Characterizing Outer Membrane Vesicles in the Methanotroph *Methylomicrobium album* BG8

by

Mariah Kristen Hermary

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences
University of Alberta

Abstract

Outer membrane vesicles (OMVs) are extracellular vesicles released from the outer leaflet of the membrane of Gram-negative bacteria. These membrane-derived vesicles are found in numerous different phyla of Gram-negative bacteria. OMV functions are as diverse as the bacteria that produce them, from exporting cellular debris to nutrient acquisition to interspecies and intraspecies communication and more. Although OMVs are incredibly common, there has yet to be a universal mechanism described for OMV production in Gram-negative bacteria.

Methanotrophs are a specialized group of microorganisms that derive their biomass and bioenergetics from single carbon sources, such as methane and methanol. Methanotrophs are major global methane cycle regulators as the only biological methane sink. In addition, methanotrophs have significant potential for converting the greenhouse gas methane into value-added industrial products.

In this study, *Methylomicrobium album* BG8 is the first methanotrophic bacterium documented to produce OMVs. Of the screened methanotrophs, *M. album* BG8 is the only bacterium to produce these extracellular structures. OMV presence has been maintained under various growth conditions. These vesicles were harvested and purified away from the bacterial biomass. The purified OMVs were sequenced to determine the protein cargo content. From the proteome, OMVs have a putative function for calcium and iron acquisition response for *M. album* BG8. The Type I secretion system is also predicted to be partially responsible for protein cargo selection in *M. album* BG8.

In addition, a mutant strain of *M. album* BG8 that does not produce OMVs regardless of the media conditions is documented. A list of potential gene candidates is generated based on the

differences in genome and transcriptome between the wild-type parental and mutant strains. These genes are hypothesized to be associated with OMV generation.

Together, the results of this research will provide necessary insight into the physiology of these unknown subcellular structures in *M. album* BG8. Future research has the potential for researchers to genetically modify other industrial methanotrophs strains to allow for OMV biogenesis. This data will improve the industrialization of this strain as OMVs can be utilized as an extraction mechanism for value-added products, advancing efforts in the carbon capture and biomass conversion field.

Preface

This thesis is an original work by Mariah Hermary. No part of this thesis has been previously published. Dr. Lisa Stein and Dr. Dominic Sauvageau were the supervisory authors who contributed in all stages of conceptualization, design, results analysis, thesis composition, and advice.

Chapter 2 will be submitted for publication as Hermary, M.K., Rodriguez Gallo M.C., Grigonyte A.M., Uhrig R.G., Sauvageau D., and Stein L.Y. "Isolation and Protein Cargo Identification of Outer Membrane Vesicles of *Methylomicrobium album* BG8". As primary author, I was responsible for the conceptualization, designing, performing experiments, bioinformatic analysis and writing of the manuscript. Maria Camila Rodriguez Gallo and Dr. R. Glen Uhrig assisted in mass spectrometry sample injection and analysis of peptide data. Dr. Aurelija Grigonyte contributed with bioinformatics analysis. Dr. Lisa Stein and Dr. Dominic Sauvageau were the supervisory authors who contributed in all stages of conceptualization, design, results analysis, manuscript composition, and advice. Arlene Oatway and Dr. Kacie Norton from the Biological Science Microscopy Unit and Haoyang Yu from nanoFAB assisted with transmission electron microscopy imagining.

Chapter 3 builds on work research from previous graduate student, Kieran McDonald who contributed the mutant *M. album* BG8 strain, genome and RNA-Seq data sets. Drs. Fabini Orata and Yusheng Tan assisted in analyzing the raw RNA and genomic sequences. Plasmids used in this chapter were engineered by Shibashis Das, who also contributed to the cloning conceptualization and design.

Acknowledgements

First and foremost, I want to thank my mentor and supervisor. Words will never describe the appreciation and respect I have for Dr. Lisa Y. Stein. Lisa took me on as a third-year undergraduate student. Since then, she has always expressed more confidence in me than I ever had in myself. Her support means the world to me and was sometimes the only reason I continued with this program. Completing graduate studies during a global pandemic wasn't easy, but it was made manageable by the support that Lisa had provided me. I am incredibly thankful to have had her mentor my research and provide unconditional support in all aspects of my life.

Likewise, thank you to my co-supervisor, Dr. Dominic Sauvageau, for his out-of-the-box approach to my science problems. Dominic's support, especially his general cheeriness and optimism, made failing experiments more bearable. My gratitude extends to the rest of my committee, particularly Dr. R. Glen Uhrig. I greatly appreciate the technical protein support you've given me, especially considering all the restrictions resulting from provincial lockdowns.

Since I started in the Stein lab as an undergraduate student, I had nothing but support from my various lab mates. The Stein and Sauvageau colleagues provided me with the technical lab skill training and supported my rambling through practice presentations, proofreading my writing, and listening to me rant about marking and whatnot. In particular, a big thank you to Shibashis Das, my biggest peer mentor and favourite lab partner. Even if I continue to research for another 30 years, I will always come to you for help and insight. Thank you to Sujani Gomes and Maria Rodriguez Gallo. You both are responsible for keeping me sane and providing the much-needed emotional support over the past two years, ily both my dudes.

Thank you to both Arlene Oatway and Dr. Kacie Norton at the Microscopy Facility for capturing my favourite part of this research, the electron micrographs. Likewise, thanks to the technical and support staff in the BioSci department. A big thanks to Dr. Anthony Cornish, who has help me in every aspect of graduate school. From scavenging equipment and reagents to allowing me the space to play instructor, Tony continues to offer advice and meaningful conversations for life both within and outside of the university to which I am grateful for.

On a personal note, I thank my mother, who sacrificed most of her life for me so I could attend university and for instilling the values of education and resilience. A thank you to my brother Kayden for constantly reminding me that there is more to life than bacteria. I especially want to thank my sister for providing nothing less than unconditional support. I know I would not have completed this degree without her. She helped me grieve my losses and celebrated all the little wins. Samantha, I will never be able to express my gratitude for being my biggest supporter throughout university.

Last but certainly not least, the biggest thanks to the start of my thesis, *Methylomicrobium album* BG8. Thanks for being the only methanotroph to make OMVs and initiating my love of wet-lab work.

Table of Contents

List of Tables

List of Supplementary Tables

List of Figures

List of Abbreviations

AMS: ammonia mineral salts

DAM: differentially abundant metabolite

EVs: extracellular vesicles

GHG: greenhouse gas

ICM: intracytoplasmic membrane

KEGG: Kyoto Encyclopedia of Genes and Genomes

LC: liquid chromatography

LP: lipopolysaccharide

LPS: lipopolysaccharides

MOB: methane oxidizing bacteria

MS/MS: tandem mass spectrometry

NMS: nitrate mineral salts

OM: outer membrane

OMVs: outer membrane vesicles

PG: peptidoglycan

PL: phospholipids

PQS: Pseudomonas quinolone signal (2-heptyl-3-hydroxy-4-quinolone)

PSM: peptide spectrum match

PTE: phosphotriesterase

RTX: repeat-in-toxin

RuMP: ribulose monophosphate

sRNA: small non-coding RNA

T1SS: Type I secretion system or Type 1 secretion system

TBDT: tonB-dependent transporters

TEM: transmission electron microscopy

1. Introduction

Approximately 60% of global methane emissions are associated with anthropogenic activities[1], predominately from agricultural practices, oil and gas production, and landfill waste. As the second most abundant greenhouse gas (GHG), methane is nearly 30 times more potent for global warming potential than carbon dioxide[2].

However, this abundant GHG is beneficial to this specialized group of microorganisms. Because of their distinctive metabolism, methanotrophs can be exploited for bioconversions of methane emissions to recycle into value-added products such as biofuels and bioplastics[3,4]. The methanotroph *Methylomicrobium album* BG8 is a candidate for biofuel industrial applications as its substrate range and growth time is preferable relative to other methanotrophs[5]. In this thesis, *M. album* BG8 has been observed to produce Outer Membrane Vesicles (OMVs) as detected by electron micrographs. As of date, there is no documentation of Gammaproteobacterial methanotrophic OMVs in the literature.

The function of OMVs are generally related to intra- and interspecies communication, adherence, transfer of virulence factors, biofilm production, and stress response[6]; however specific functions are dependent on the species and corresponding environment. Currently, there is no universal mechanism for OMV biogenesis in Gram-negative bacteria. Likewise, the current physiology models of OMV biogenesis are species-dependent.

I propose a research project to characterize the function and biogenesis mechanism of OMVs of *M. album* BG8. This research has four objectives; first, to determine which growth substrates and media conditions allow for OMV production. This objective is achieved by culturing *M. album* BG8 on various media components and screening for OMVs production. Secondly, to establish a protocol for methanotrophs to purify OMVs from the bacterial culture.

Thirdly, to determine the protein cargo of purified OMVs via mass spectrometry to elucidate the vesicle function. Lastly, to compare genomes of current lab strain *M. album* BG8 to a loss-of-function mutant strain that lacks the ability to produce OMVs. From this comparison, are a selection of candidate genes will be generated for future attempts to rescue the loss-of-function phenotype and thus provide evidence for a molecular mechanism of OMVs biogenesis.

The results of this research will provide necessary insight into the physiology of these unknown subcellular structures in *M. album* BG8. Future research has the potential for the methanotrophs researchers to genetically modify other industrial methanotrophic strains to allow for OMV biogenesis. This can improve the industrialization of this strain as OMVs can be utilized as an extraction mechanism for value-added products, advancing effort in the carbon capture and biomass conversion field.

This goal was achieved through protein identification of OMVs from *M. album* BG8. A selection of methanotrophs was first screened for the presence or absence of OMVs. Of these methanotrophs, only *M. album* BG8 displayed OMVs via transmission electron microscopy. OMV isolation and purification protocols were developed to separate the OMVs away from *M. album* BG8 bacterial cells. Purified OMVs were sequenced in liquid chromatography-tandem mass spectrometry to determine protein cargo. Of the peptides sequenced, those with statistical significance were used to create a proposed model for OMV function. In addition, a loss of function phenotype was attempted to be rescued previously identified loss of vesicle mutant strain of *M. album* BG8 but was unsuccessful. Nevertheless, potential genes have been identified to be of significance regarding OMV production.

This research provides a basic framework of OMV production, an OMV isolation procedure, prediction of OMV function, and potential genes associated with OMV biogenesis in *M. album* BG8.

2. Literature Review

2.1 Outer Membrane Vesicles (OMVs)

2.1.1 Extracellular Vesicles

Cellular production of membrane-bound vesicles is present in all three domains of life[7–10], suggesting that extracellular vesicles (EVs) are a well-documented form of secretion. EVs are frequently observed in both non-pathogenic and pathogenic microorganisms, in liquid broth and agar plates, within pure cultures[11] or in mixed community biofilms[12], under viral infection[13–15] and both *in vivo* and *in vitro*[16,17]. In addition, these extracellular structures can be manipulated under modifications of media conditions [18]. Their overwhelming presence suggests that EVs are an integral part of microbial life[16]. EVs from microbial eukaryotic organisms, such as yeast or parasites, are classified into at least two categories[8]. Exosomes are typically homogeneously shaped and are developed from multivesicular bodies. In contrast, shedding microvesicles are derived from the cell surface and are thus heterogeneously shaped.

In prokaryotes and archaea, spherical protrusions derived from the cell surface can be released and are then referred to as membrane vesicles. For example, membrane vesicles from archaeal *Sulfolobus* species are revealed to contain S-layer and membrane lipids[19]. Bacterial membrane vesicles in Gram-positive and mycobacteria also contain the distinctive thick cell wall[20]. Gram-negative bacterial membrane vesicles can have various compositions and structures[9]. *Shewanella vesiculosa* M7^T produces membrane vesicles containing both the bilayer membranes structures, coined as outer-inner membrane vesicles[21]. Appearance of vesicles can become more intricate, as found with tube-shaped membranous structures pinched off of the outer membrane of *Myxococcus xanthus*[22], as well as in a novel “beads-on-a-string” appearance from *Vibrio vulnificus*[23]. Nevertheless, the most structurally simple and best characterized

bacterial membrane vesicles are defined as outer membrane vesicles (OMVs) of Gram-negative bacteria[24].

2.1.2 Outer Membrane and OMVs of Gram-negative Bacteria

In Gram-negative bacteria, the cell envelope is contained between two membranes as illustrated in Schwechheimer and Kuehn (2015) (Fig. 1). The inner or cytoplasmic membrane is composed of a phospholipid (PL) bilayer. The outer membrane (OM) is comprised of an external primarily lipopolysaccharide (LPS) leaflet and an interior leaflet derived from PLs[25]. This asymmetry between the two leaflets is key to maintaining a barrier function[26]. Between the inner membrane and OM is the periplasmic space, which contains periplasmic proteins and the peptidoglycan (PG) layer. Envelope proteins can be soluble, integrated into either membrane (integral membrane proteins), spanning the entire envelope (transmembrane proteins) or anchored into the leaflet of either membrane via covalently attached appendages (lipoproteins)[6]. The first documented case of OMVs was in *Escherichia coli* in 1965[27]. As the name suggests, these are spherical bodies that originated from OM and thus contained components of the cellular OM but also from the periplasm and cytoplasm[9,28]. Typically between 20-300 nm in diameter[29], OMVs are pinched off from the bacterium as a separate structural entity and released into the external environment.

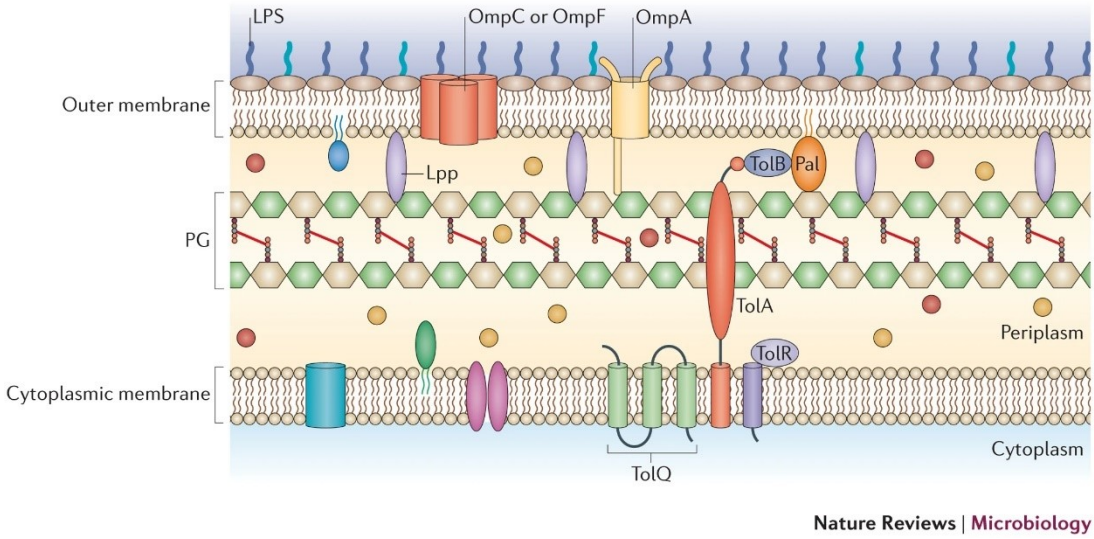


Figure 1. General overview of Gram-negative membrane. This illustration is from Schwechheimer, C., Kuehn, M. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* **13**, 605–619 (2015). https://doi.org/10.1038/nrmicro3525. Reprinted with permission of publisher.

2.1.3 Biogenesis

Originally, the production of OMVs was thought to be a direct response of the OM to the nearby external surroundings.[24,30] We now know that OMVs are not necessarily a reaction to the physical environment but rather an intentional means of secretion orchestrated by the cell[24]. In simplistic terms, OMVs are created with part of the OM that bulges or "blebs" from the PG layer and strangles itself away from the bacterium.

Currently, there is no absolute universal mechanism to describe OMV formation in Gram-negative bacteria. However, several proposed models involve a role for LPSs, PLs, and PGs in the biogenesis of OMVs (Fig. 2).

One of the earliest models suggests that OMV formation results from the decreased amounts of cross-linkage between the OM and PG layer. The three best described proteins supporting this model, which generally provide envelope stability, include outer membrane

protein A (OmpA), the Tol-Pal system, and Braun's lipoprotein, Lpp[31]. *E. coli*, *Salmonella* spp., *Vibrio cholerae* and *Acinetobacter baumanni* OmpA mutants displayed hypervesiculation phenotypes, likely due to the removal of non-covalent interactions between OmpA and PG[32–35]. The Tol-Pal complex is a five-protein system spanning the OM and the inner membrane. Although it has been proposed to have many functions, the Tol-Pal complex is best characterized in its involvement of cell division[36]. The Tol-Pal proteins localize at the site of invagination during cell division. However, Tol-Pal mutants increase bleb formation around these invagination sites[8,37]. Interestingly, these Tol-Pal-derived OMVs are distinct from OMVs generated from elsewhere on the membrane. The highly abundant OM lipoprotein Lpp acts as a molecular glue in some bacteria as approximately one-third of Lpp is covalently linked to PG, thus tethering together these two membrane components. In *E. coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhimurium, *lpp* knockouts have been documented to increase vesiculation due to decreased OM-PG cross linkage[37,38]. However, these *lpp* mutants also contained defects in the OM integrity, making it challenging to discern cell damage from OMV production. Because of these cellular leaks, it is proposed that OMV formation is either due to a temporary decrease in crosslinkage between the OM and PG or to a localized displacement of crosslinks. This model is yet to be resolved as there are no known periplasmic enzymes to liberate Lpp from its covalent bond to PG. Moreover, although the role of Lpp in OMVs was documented in *E. coli*, it is not essential for OMV formation. It has been revealed that, in *E. coli*, the overall amount of Lpp-PG crosslinks did not change in cases of increased vesiculation, suggesting a separate, Lpp-independent route for OMV formation[35]. In wild-type *E. coli* cells, relatively Lpp-free spaces in the OM, referred to as nanoterritories, allowed for envelope curvature even though the overall level of Lpp-PG links was constant[6].

The second commonly speculated model for OMV formation centers around envelope components, particularly PG, accumulating at the OM. PG fragments that are liberated in the periplasmic space by autolysin during cell growth are believed to protrude against the OM in *Porphyromonas gingivalis*[39]. This increase of turgor pressure then induces vesicle formation. As part of a stress response to temperature, *E. coli* was documented to expel misfolded proteins via OMVs [40].

The third traditional biogenesis model for OMVs revolves around altered LPS. The LPS of *P. aeruginosa* can contain different O-polysaccharides creating two forms of LPS: neutral or with anionic charge. Interestingly, LPS with anionic charge has only been detected in OMVs[41]. On the contrary, *P. gingivalis* packages both its neutral and anionic LPS types into OMVs. Here, the deacetylated lipid A that is sorted into OMVs differs from the lipid A in the OM[42]. It is hypothesized that the remodelling or compartmentalization of the OM by acetylation induces curvature of the OM, thus promoting OMV production[43].

A more contemporary take on OMV biogenesis is described as the bilayer-couple model. The faster expansion of the outer leaflet membrane in comparison to the inner leaflet stimulates the curvature of the OM. The proposal of PL accumulation forces blebbing of the OM. The VacJ/Yrb ABC transport system is responsible for the retrograde trafficking of PLs from the OM to the inner membrane. In iron-limited environments, the VacJ/Yrb transporter is down-regulated by the ferric uptake regulator creating a hypervesiculation phenotype in *H. influenzae, E. coli* and *Vibrio cholerae*[44]. Disruption of this lipid asymmetry causes accumulation of PL in the outer leaflet, creating OM expansion and subsequent OMV formation[44]. The best-studied species for the bicouple-model is *P. aeruginosa* and its signaling molecule 2-heptyl-3-hydroxy-4-quinolone. Commonly referred to as the Pseudomonas quinolone signal (PQS), this signaling molecule

mediates quorum sensing in *P. aeruginosa*[45]. Due to its hydrophobic structure, PQS has strong interactions with LPS and will intercalate into the outer leaflet causing the OM to expand rapidly[46]. However, it should be noted that this mechanism appears to be limited to *P. aeruginosa*[47].

Aside from the mechanisms described above, other niche models are used to elucidate OMV biogenesis in a subset of Gram-negative bacteria. For example, the rotation of sheathed flagella of *Vibrio fischeri* cells has been demonstrated to cause blebbing of the OM and release of OMVs[17]. The diversity of biogenesis models that has emerged suggests that investigation of OMV generation is currently multifaceted and complex.

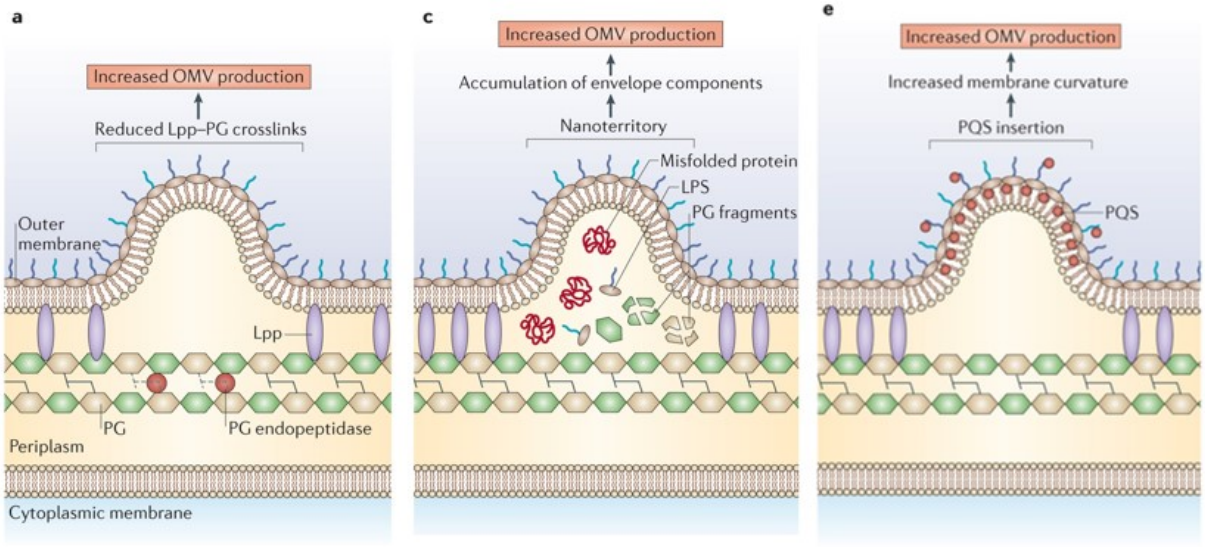


Figure 2. Schematic of frequently observed genetic-based OMV biogenesis models. This illustration is modified from Schwechheimer, C., Kuehn, M. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* **13**, 605–619 (2015). https://doi.org/10.1038/nrmicro3525. Reprinted with permission of publisher.

2.1.4 Biological cargo and functions

Although the name may suggest that OMVs contain only membrane-related components, this is misleading. OMVs can contain PL, LPS, outer membrane proteins and other cell envelope components[24]. However, periplasmic and cytoplasmic proteins, nucleic acids, ion metabolites and signaling molecules can be included during blebbing of the vesicle and thus exported away[29]. OMVs are informally referred to as the Type 0 secretion system[48]. A considerable characteristic over other secretion systems is the ability for OMVs to transport hydrophobic and insoluble biological molecules, crossing a hydrophobic lipid membrane into the aqueous environment[48]. Likewise, OMVs can carry multiple forms of cargo simultaneously. Another advantage is the concentration of cargo in a confined space. For example, cell-to-cell signaling in *P. aeruginosa* is heavily mediated by OMV release, as approximately 80% of PQS is transported in vesicles[49]. Collectively, OMVs have the unparalleled capability of transporting diverse groups of molecules efficiently in comparison to other secretion systems.

Nucleic acids detected in OMVs[50] include plasmid DNA, chromosomal DNA, viral DNA and RNA. Plasmid transfer was documented in *Neisseria gonorrhoeae,* and *Acinetobacter baumannii* with the success of transferring antibiotic resistance genes from OMVs to the cell[51,52]. *Escherichia coli* O157:H7 and other enteric species can also transport and export DNA via OMVs[5354]. This packaging of DNA into OMVs has been argued to be a mechanism of horizontal gene transfer, informally referred to as “vesiduction”[55]. This fourth proposed form of horizontal gene transfer has the advantage of physically protecting DNA from the external environment, such as exogenous nucleases or extreme conditions, enhancing the probability of successful transfer from donor to recipient[50].

mRNA, tRNA, sRNA and rRNA can also be packaged in OMVs[50]. In particular, sRNA, or small noncoding RNA, bound in OMVs are associated with inter-species communication[56,57,58]; although most of this current research is directed towards microbiome and pathogenicity studies. Notably, *Vibrio fischeri* interacts with its symbiotic squid host through OMVs containing small non-coding nucleic acids[57]. The absence of OMVs, but also the associated sRNA cargo, compromised the squid's health and inevitably led to a weakened host-microbe association[17,57].

The array of biological roles for OMVs is as diverse as the bacterial species that produce them. Because of the ability to mimic pathogenic bacterial cells, OMVs are deployed as a long-distance delivery vehicle of immunogenic molecules to trigger the host's immune system[31]. Virulence factors associated with OMVs are related to adherence, invasion, antimicrobial resistance, and modulation of host immunity[31]. The pathogenicity aspect of OMVs is of prominent interest; however, the field of OMV research has only recently begun to address potential advantages for non-pathogenic species.

For example, *V. cholerae* and *E. coli* can produce OMVs as a decoy for bacteriophage infection[59,60]. Co-incubation of T4 bacteriophage and enterotoxigenic *E. coli* OMVs resulted in nearly 90% reduction of active phage, as measured in a reduction of plaque-forming units[61]. In addition to protection against bacteriophage, OMVs contribute to the role of innate bacterial defense due to the adsorption of antimicrobial peptides. Enterotoxigenic *E. coli* demonstrated vesicle-mediated protection in the presence of polymyxin B[61]. High levels of vesiculation provided immediate protection but prevented the acquisition of resistance to polymyxin B.

The production of OMVs has been associated with stress response in both pathogenic and non-pathogenic bacterial species. Misfolded toxic products or overexpressed proteins accumulating in the periplasm of *E. coli* have been correlated with increased vesiculation[62], suggesting that OMVs act similarly to a waste disposal system. In the absence of the chaperone-protease DegP, proteinaceous waste accumulated in OMVs, whereas reduced vesiculation impaired bacterial growth. In *P. aeruginosa*, OMV production increased in the presence of a PG synthesis inhibitor[63]. Likewise, increased vesiculation was also demonstrated in the presence of physiological environmental stress such as exposure to hydrogen peroxide and polymyxin B[63]. Interestingly, stressed-induced vesicle production was shown to be independent of the alternative sigma factor for envelope stress response in *P. aeruginosa*[63].

OMVs also play roles within bacterial communities in promoting nutrient acquisition. In some *Bacteroides* species, glycosidases and proteases were preferentially packaged into OMVs[43]. *Myxococcus xanthus* OMVs are particularly efficient; they exclusively package alkaline phosphatases, liberating phosphate following lysis of their *E. coli* prey[64]. Acquisition of metals has been hypothesized as a motive for OMV production. *Moraxella catarrhalis*, *Neisseria meningitidis* and *P. gingivalis* expressed surface receptors for transferrin-binding protein B, ion transporter proteins, and haem acquisition proteins in their OMVs, respectively[6]. *Mycobacterium tuberculosis* depends on OMVs as iron donors with the ability to scavenge and deliver iron back to the iron-starved cell[65].

As mentioned before regarding quorum sensing, *P. aeruginosa* utilizes its OMVs for PQS delivery. Quorum sensing is a density-dependent signaling mechanism between bacteria, allowing for the synchronization of a whole population[66]. This regulation of expression of genes as a group is involved in virulence, competitions, stress adaptations and the formation of

biofilms. *Chromobacterium violaceum* mediates its OMVs to export violacein, a hydrophobic molecule that acts as a quorum sensing signal as well as an antimicrobial against Gram-positive competing species[67]. In *Vibrio harveyi*, CAI-1 quorum sensing molecule gets trapped in the OM because of its high lipophilicity[68]. As a result, OMVs are recognized by neighboring cells via receptors. CAI-1 is a *Vibrio*-specific singling molecules that can be utilize by different *Vibrio* species, demonstrating OMVs as a mechanism for interspecies communication[68]

Both commensal and pathogenic Gram-negative species can benefit from the production of OMVs, In fact, current OMV research emphasize the immunostimulation and immunomodulation effects of pathogenic Gram-negative bacteria. OMVs have been utilized as tools for the delivery of toxins during host infection by many pathogens including enterotoxigenic *E. coli*, the human dental pathogen *Porphyromonas gingivalis, Salmonella enterica ser.* Typhimurium and *Vibrio cholerae*[31].

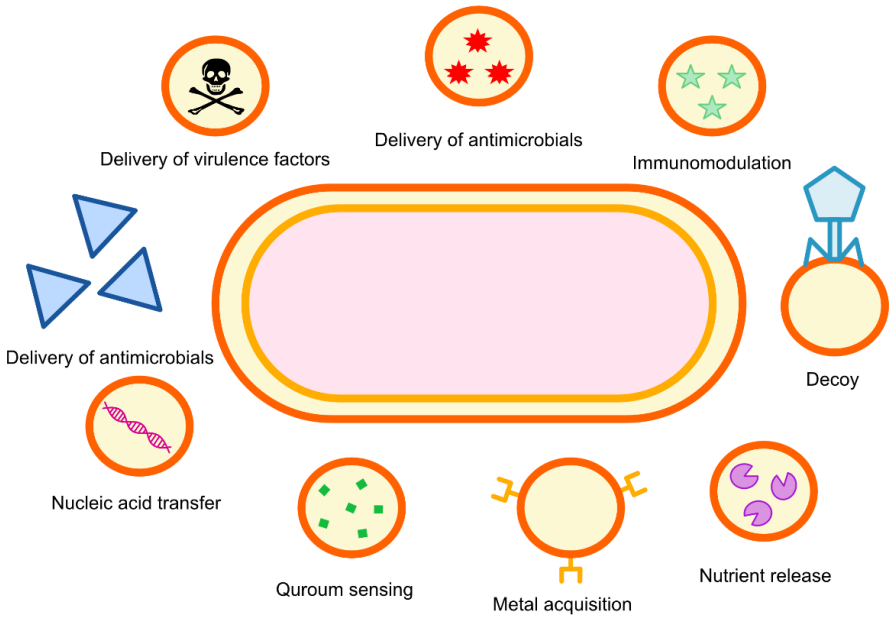


Figure 3. Schematic summary of a selection of potential OMV functions and industrial applications.

2.1.5 Biotechnology Applications

OMVs have a biotechnological appeal as a native export mechanism. The most prominent and best-studied application for OMVs is in vaccine development. OMVs have the apparent advantage of mimicking pathogenic bacterial species since the vesicle itself is decorated with microbial-associated molecular patterns but lacks the ability to infect the host. In other words, OMVs can allow for immunomodulation thus providing an alternative means of vaccine deployment. Likewise, OMVs contain natural adjuvants to stimulate both the innate and adaptive immune system[69,70]. In the past, creating a vaccine for the *N. meningitidis* serotype B was deemed impossible as its capsule had homologous structures to the human brain; thus the traditional glycoconjugate vaccine was ineffective[70,71]. The primary immunogenic OM protein PorA from *N. meningitidis* was abundant on the OMVs. OMVs derived from bioengineered strains allowed many variants of PorA to be expressed, creating efficiency amongst different strains. In 2016, the Serogroup B Meningococcal OMV-derived vaccine was the first approved by the FDA and is used in endemic areas[31]. On the other hand, OMVs are being investigated as cancer therapy to replace the use of bacterial cells in immunotherapy and cancer treatments[72]. OMVs can display more than one cancer-specific epitope on the OM but also entrap hydrophobic small molecule drugs inside the lumen of the OMV[73], while simultaneously acting as a delivery mechanism. Similarly, OMVs are also an attractive model for drug delivery. Because of their protective nature, OMVs can prolong drug activity if loaded in the lumen to slow down degradation of drugs in the bloodstream than the drug unenclosed. Similarly, OMVs display biodegradability as these vesicles can degraded itself after 48h of endocytosis within the hosts lyosome[73,74].

Another attractive avenue for OMV technology is the transport and packing of enzymes. Due to their nanostructure and low cost, OMVs can increase enzyme stability by protecting enzymes from the external environment. Phosphotriesterase (PTE) activity was maintained under various harsh storage conditions when packaged within OMVs[75]. Using a bioconjugation system, this bioremediation enzyme that decomposes organophosphates was tethered to OmpA inside the OMV. Compared to the non-packaged PTE, OMV-PTE activity was increased during long storage periods (>14 days), iterative freeze-thaw cycles, and lyophilization. With this in mind, lyophilized PTE in OMVs can be utilized as a cell-free bioremediation tool in contained environments.

Enzymatic cellulose hydrolysis can be achieved in OMVs by engineering a scaffold of three separate enzymes outside the vesicle[76]. By taking advantage of the cohesin-dockerin interaction, this scaffold allowed for subsequent protein assembly to the outside of the OMVs, bringing these enzymes close to each other. Because of this spatial advantage, the tri-enzyme scaffold on the OMVs outcompeted cellulose hydrolysis rates more than non-complexed enzymes by 23-fold.

2.2 Methanotrophs

2.2.1 Overview of Methanotrophy

Methanotrophs are microorganisms with the defined capacity to utilize single-carbon compounds as their sole carbon and energy source. Methanotrophs are a subset of a broader class of bacteria known as methylotrophs which consume compounds without carbon-carbon bonds, such as single-carbon sources like methane and methanol but also multi-carbon substrates like trimethylamine. Although methanotrophs are considered ubiquitous in the environment, they are often associated with environments with high methane levels such as rice paddies, anoxic

freshwater systems and marine waters, upland and forest soils, and sediments. Methanotrophs are often cultivated in close proximity to methane-producing archaeal methanogens.

Methanotrophs were first thought to be exclusively Gram-negative aerobic microorganisms when classified in 1970[77]. There have been hundreds of methanotrophs isolated and cultured since then, yet categorizing methanotrophs taxonomically remains a work in progress[78]. Methanotrophic bacteria, or methane-oxidizing bacteria, belong to the phyla *Proteobacteria* [79]and *Verrucomicrobia*[80]. Proteobacterial methanotrophs, that generally Methassimilate carbon from single-carbon sources, can be classified as Alpha- or Gamma – proteobacteria as discussed in detail later on. A more recently discovered clade of methanotrophs from Verrucomicrobia are autotrophic and use methane as an energy source[81]. Even beyond the paradigm of strictly bacterial life, other forms of methanotrophic lifestyles also exist. A prominent example is sulfate-reducing bacteria coupled with anaerobic methanotrophic archaea, or biologically methanogenic Archaea "operating in reverse" as they consume methane[82]. Furthermore, the candidate division NC10 functions as an oxygenic methanotroph that oxidizes methane using oxygen produced *in vivo* from nitric oxide disproportionation[83,84].

The biogeochemical significance of methanotrophs is obvious. After carbon dioxide, methane gas is the second most abundant greenhouse gas (GHG). However, the global warming potential of methane, based on the ability of a GHG to trap heat, is nearly 30 times that of carbon dioxide[2]. A comprehensive assessment on climate change from the Intergovernmental Panel on Climate Change has noted that the concentration of methane has risen by nearly 150% since 1750[85]. Biogenic methane sources are prominent due to the activity of methanogens, and comprise anaerobic environments such as natural wetlands and rice paddies, oxygen-poor

freshwater reservoirs, digestive systems of ruminants and termites, and organic waste deposits (such as manure, sewage and landfills)[86].

Anthropogenic methane sources of interest predominantly concern the industrialization of biogenic sources through agriculture practice, landfill expansion, but also through the exploitation of coal, oil and natural gas industries[86]. For example, it has been estimated that upstream oil and gas activity accounts for 70% of Alberta's methane emissions[87]. Methanotrophs are the only biological sink that will oxidize this GHG from the environment. Both aerobic and anaerobic methanotrophs play a significant role in controlling atmospheric concentrations[88].

2.2.2 Aerobic Proteobacterial Methanotrophs

Aerobic methanotrophs oxidize methane to methanol in the presence of oxygen. The reaction is catalyzed by methane monooxygenase (MMO), which exists in two structurally and biochemically distinct forms. The particulate form of methane monooxygenase (pMMO) is a copper-containing enzyme integrated within the intracytoplasmic membrane (ICM), whereas the soluble form (sMMO) is a cytoplasmic non-heme iron enzyme complex[81]. Methanotrophic bacteria contain at least one form of MMO. However, some species can produce both pMMO and sMMO, such as *Methylococcus capsulatus* Bath, *Methylomicrobium buryatense* 5G and *Methylosinus trichosporium* OB3b[89]. The expression of these enzyme forms is controlled by the canonical "copper switch", in which copper-limited conditions simulate expression of sMMO over pMMO[89]. As the availability of copper increases, by default as the copper-to-biomass ratio increases, the expression of sMMO will decrease as pMMO expression increases[89,90].

A vast number of methanotrophs can produce peptidic natural products that chelate copper. These copper-binding peptides are dubbed chalkophores, analogous to iron-binding

siderophores. The largest and best-understood groups of these metal-biding peptides are referred to as methanobactins.[91] *Methylosinus trichosporium* OB3b is the first methanotroph known to utilize methanobactins and are produced primarily at low copper levels to scavenge for copper[92], thus reiterating the importance of copper for methanotrophs.

Once methane is oxidized by either form of MMO, methanol will be further oxidized by the periplasmic methanol dehydrogenase MxaFI into formaldehyde. Historically, this calcium-dependent MxaFI was thought to be the primary form of methanol dehydrogenase. However, a novel methanol dehydrogenase possessing a lanthanide- (or rare earth metal) associated active site was identified. XoxF has been documented in a number of strains[89]. Formaldehyde will be converted to formate through either the tetrahydrofolate or tetrahydromethanopterin pathway. The last oxidation step is to convert formate into carbon dioxide using formate dehydrogenase, and this enzyme is present in all methanotrophs[93].

Importantly, the pathway intermediate, formaldehyde, is used as the starting molecule for the branch point of two distinct carbon assimilation pathways, depending on the group of Proteobacteria (Fig. 3). Generally, proteobacterial methanotrophs are divided into two main categories based primarily on their carbon assimilation pathway and also from some structural components. Classically, Type I Gammaproteobacteria utilize the ribulose monophosphate (RuMP) pathway for carbon assimilation[79]. Gammaproteobacterial methanotrophs have fatty acid chain lengths of 16 carbons. The ICM of gammaproteobacterial methanotrophs contains an unusually high amount of lipids per cell compared to other bacterial groups. This highly structured internal membrane is essential to methanotrophs to house the pMMO enzymes. The gammaproteobacterial methanotroph ICM consists of specific C16 lipids and are arranged in discs throughout the cell[81]. The second group of Type II Alphaproteobacteria utilizes the serine

pathway for formaldehyde assimilation[79]. In contrast to Gammaproteobacteria, the ICM of alphaproteobacterial methanotrophs exists in paired layers around the cell's periphery, consisting of C18 fatty acid lipids[81].

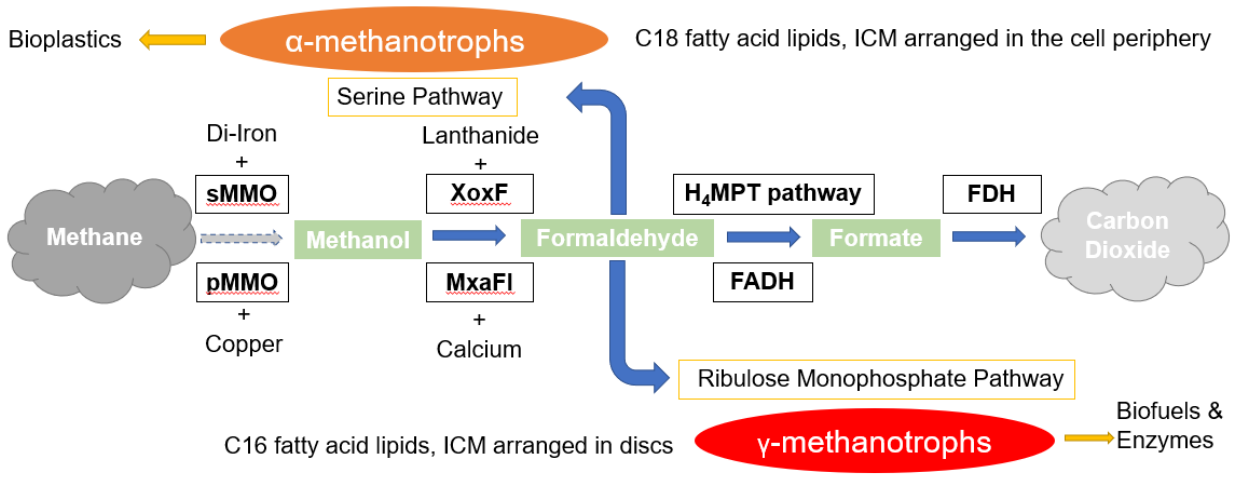


Figure 4. Summary of aerobic methane oxidation pathways and highlighted differences between Alpha- and Gammaproteobacterial methanotrophs.

2.2.3 Industrial Applications

One of the environmental applications of methanotrophy is to use both the pMMO and sMMO enzymes for bioremediation of hydrocarbon pollutants. It was demonstrated that pMMO could oxidize alkanes up to C5 and sMMO can oxidize alkanes up to C8 and ethers, cyclic alkane, and aromatic hydrocarbons[94]. Both forms of MMO can attack chlorinated ethenes, commonly founds at hazardous waste sites[95]. However, one of the potential limitations of this application is that the pollutants cannot serve as a carbon or energy source for methanotrophs and are thus co-metabolized with methane as the growth substrate[95].

Methanotrophs are sensitive to copper because of its necessity for pMMO but also it regulation of gene expression and protein synthesis[89,96,97]. As a result, methanotrophs can respond strongly various available copper concentrations[96], but their ability to sequester metals is

not exclusive to copper. Methanotrophs have been shown to bind and detoxify other metals, including toxic forms of chromium and selenium[98,99]. However, more research is needed to understand how these metals are taken up and transformed [95]. Similarly, detoxification of metals is dependent on the availability of methane. The methanobactin produced by *M. capsulatus* Bath has been shown to bind and detoxify mercury, even in the presence of copper[100]. Methylmercury uptake has been seen in methanotrophs, including those without the methanobactin gene cluster[101]. Methanobactin polypeptide precursor gene has been identified as *mbnA*, as well as several gene involved in the heterocyclic rings associated with this polypeptide, *mbnBCN*[102]. In addition, the uptake of methanobactin is through a Ton-B dependent transporter, encoded by *mbnT.* However, this transporter is also seen in methanotrophs which lack the genes associated with methanobactin production, suggesting non-methanobactin producing methanotrophs can "steal" MB from others[102]. Moreover, although methanobactins have the capacity to bind to other metals, the predominant function is to collect copper to support pMMO. Sequestering copper via methanobactins may prevent other microorganisms from utilizing this metal. Copper is required for other microorganisms in trace amounts and needed for respiration by some groups, like denitrifying bacteria[103]. When co-incubated with the presence of methanobactin or active cultures of *M. trichosporium* OB3b, denitrifying bacteria produced substantial amounts of nitrous oxide, indicating that the copper-dependent nitrous oxide reductase was inhibited[104]. Under a medical application lens, methanobactins may be useful for treating copper-associated diseases. For example, methanobactins are an appealing therapeutic agent for controlling copper homeostasis in Wilson's disease patients, a disease caused by copper accumulation in vital organs such as the liver[105]. Methanobactins have been demonstrated to prevent copper buildup but also removal of pre-existing copper, leading to liver repair[106]. This is a significant finding as

methanobactin may reduce the need for liver-transplants if severe liver damage may potentially be reversed[106]. Copper increases the severity of Alzheimer's disease by leading to large plaque formation in brain tissue. Excess copper increases the production of misfolded proteins but prevents the export of these proteins from the brain tissue[107,108].

Although much more research is needed for the application of MMO in bioremediation and methanobactin applications, the predominant commercial application of methanotrophs is methanotrophy to valorize the inexpensive substrate methane into value-added products, such as protein for feedstock, bioplastic precursors, liquid biofuel precursors and osmoprotectants[95,109].

Methanotrophs have been used to generate single-cell protein sources for monogastric species, including pigs, chickens, mink, fox and certain fish species[110]. Based on the criteria of amino acids composition, digestibility, and animal performance of using *M. capsulatus* Bath as bacterial biomass, methanotrophs have been considered a promising and inexpensive protein source for animal health[110]. Another current commercial application of methanotrophs is the production of bioplastics. In particular, alpha-proteobacterial methanotrophs, which utilize the serine cycle for carbon assimilation, are targeted to produce polyhydroxyalkanoates, like poly-3-hydroxybutyrate (PHB)[111]. This bacterial storage polymer is seen as a potential substitute for traditional petroleum-based plastics[110]. PHB has many beneficial properties such as biodegradability, biocompatibility and thermostability. It has been demonstrated that alphaproteobacterial methanotrophs can accumulate PHB to more than 50% of the dry cell biomass[109]. Although PHB production by methanotrophs has been of interest for decades, the cheap cost of the substrate makes this venture more desirable due to the potential of lower production costs[110]. As a result, several companies are pursuing industrial scale-up of methanotroph-produced PHB.

The ICM of methanotrophs allows for high lipid content, upwards of 20% of the biomass[112]. As such, methanotrophic lipids could be used for production of biofuels. However, these lipids are polar PLs mainly composed of phosphatidylglycerol, phosphatidylcholine and phosphatidylethanolamine. Unfortunately, the high heteroatom content of N and P make downstream processing more difficult than nonpolar lipid equivalents[110,112,113]. Nevertheless, the idea of utilizing methane substrate is of interest as it avoids the food versus fuel dilemma of traditional agriculture sources and susbstrates[114]. Intriguingly, methanotrophic lipids are of interest for human health supplements to reduce the ratio of low- to high-density lipoprotein plasma cholesterol levels[115].

A group of secondary metabolites referred to as isoprenoids, also known as terpenoids or terpenes, are the most prominent family of natural products[116]. With more than 40 000 members identified, isoprenoids have been used in various applications such as cosmetics and fragrances, food additives, and medicinal purposes[117]. Due to this extensive structural and functional diversity, there has been increasing interest in exploring these biological molecules as next-generation biofuels. Their low hygroscopic properties, high energy density and suitable fluidity temperature allow them to be candidates for the precursor of diesel and gasoline alternatives[118]. All isoprenoids are synthesized from a five-carbon precursor and its isomer, which can be generated in methanotrophs using two pathways: the mevalonate pathway and the methylerythritol 4-phosphate pathway[116]. As more genetic tools become available in methanotrophs, engineering these pathways to compete with other bacterial systems is deemed a potentially promising venture.

Some halophilic methanotrophs will naturally produce osmotic stabilizers or compatible solutes to tolerate their highly saline environments. Various *Methylotuvimicrobium* species will

produce ectoine as an osmotic stabilizer[112,119]. Ectoine is released into the surrounding medium when the bacterium is exposed to low salt concentrations; thus, bacterial cells could be reused multiple times for the production of product[114]. With the ability to stabilize proteins and nucleic acids under osmotic stress, these compounds have been used in the cosmetic industry as a moisturizer[120].

2.2.4 *Methylomicrobium album* BG8 as an Industrial Microbe

Methylomicrobium album BG8, a genetically well-identified gammaproteobacterial methanotroph and has many physiological studies aimed to explore its behaviour[5,121–1235]. *M. album* BG8 was isolated decades ago[77] and has undergone numerous changes in nomenclature resulting in various names in the literature, including *Methylobacter albus, Methylomonas albus*, and *Methylomonas alba*[124].

Regarding the flexibility of carbon source, *M. album* BG8 has a potential industrial advantage. Although traditionally methanotrophs have been cultivated using methane gas, many studies have explored the use of alternative substrates such as methanol[5,125–127]. Methanol is desirable as it can be inexpensively synthesized from carbon dioxide or methane[128] and is easier to use than methane in continuous-culture systems since it is a liquid. Likewise, methanol avoids gas-liquid mass transfer issues associated with low solubility gases such as methane[129].

Tays et al. described the culturing impact on the optimization of growth of methanotrophs, comparing *M. album* BG8 growth to a selection of other methanotrophs when given either methane or methanol as the sole substrate. *M. album* BG8 had the highest optical density (OD) when cultured on methanol alone compared to the other Gamma- and alphaproteobacterial methanotrophs[5]. Surprisingly, *M. album* BG8 preferred methanol over

methane as a carbon source which is generally rare since most strains are more sensitive to methanol toxicity. In addition to the solvent properties of methanol disrupting membrane integrity[130], accumulation of methanol subsequently leads to buildup of formaldehyde in methantrophs[123]. When grown on methanol *M album* BG8 indicated an upregulating of glutathione-dependent formaldehyde detoxification[123]. Likewise, *M album* BG8 manages methanol toxicity by modifying its sugar catabolism pathways flux, upregulating translation machinery, alternating PL production to adapt the membrane to solvent stress[123] *M. album* BG8 was also shown as the only strain not to excrete measurable formate into its media. This provides evidence that *M. album* BG8 can fully oxidize its single-carbon substrate, either methane or methanol, into carbon dioxide without experiencing toxicity.

In terms of metabolite production, *M. album* BG8 has been demonstrated to increase metabolite production when cultured with methane over methanol.[123] Out of 104 differentially abundant metabolites (DAMs), 90 DAMs were more abundant in methane than methanol. An increase of these methane-specific DAMs includes amino acids, co-factors, and carbohydrates. Likewise, this study observed a difference between nitrate over ammonium as nitrogen sources. Metabolic differences were 2-fold larger when grown with ammonium rather than with nitrate. In contrast, nitrate-grown cultures saw an increased expression of genes associated with inorganic ion transport and metabolism. Interestingly, the nitrogen source response to the number of DAMs was influenced primarily by the use of methanol over methane rather than by the change in nitrogen source.

This flexibility of metabolism shown for *M. album* BG8 is advantageous in the industrial sense in terms of substrate cost and fast growth rate. Its ability to be cultivated on methanol allows for an industrial advantage for ease of liquid substrate and as a potential deterrent against

contamination because many bacterial species experience methanol toxicity. Likewise, the ability to change metabolite pools based on the combination of carbon and nitrogen sources can lead to enhanced production of several valuable products by a single strain. However, an interesting physiological phenomenon described in this thesis can increase the value of *M. album* BG8 as an industrial microbe. In this thesis, OMVs are documented to be produced by *M. album* BG8 under a variety of growth conditions and under all growth phases. This natural export system allows for potential advantages for industrialization of this methanotroph.

2.3 Industrial Applications of OMVs

2.3.1 Potential Applications of *M. album* BG8 OMVs

As mentioned above, the literature is experiencing an expansion of OMV descriptions from commensal bacterial species and their utilization in biotechnological applications. Regarding *M. album* BG8, the information about OMVs will be beneficial to its industrialization. This native secretion system has the potential to be genetically engineered to aid in the exportation of value-added products such as enzymes or isoprenoids. On the other hand, OMVs are an energetically intensive process, directing many cellular resources towards their production. The need to understand OMV physiology is necessary to scale up production of *M. album* BG8 biomass and products. Elucidation of the biogenesis mechanism of OMVs would allow for potential redirecting of these cellular resources, thus *M. album* BG8's growth rate and biomass yield could be more tightly manipulated. The use of OMVs can be directed towards a natural extraction process for collecting value-added products without the need for the harsh solvent treatments currently used for breaking open cells to harvest intracellular products. With the increased knowledge of *M. album* BG8 OMVs and with a more explicit biogenesis hypothesis, perhaps OMVs can be engineered into other methanotrophs to add more value to

currently industrialized strains. On the flip side, perhaps when *M. album* BG8 genetic tools are more advanced, we can create strains for production of other value-added products and utilize OMVs for directed export. When we have more insight into OMV generation, the collection of OMVs at an industrial scale can allow for more efficient use and reuse of cellular membrane and cargo materials.

To conclude, this thesis documents the presence of OMVs in the Gammaproteobacterial methanotroph *Methylomicrobium album* BG8. This research aims to provide a basic framework of characterizing these vesicles, including a purification procedure, assaying OMV presence within different growth substrates, identifying OMVs protein cargo, and showing the uniqueness of the OMVs and related genes to *M. album* BG8 among methanotrophic Proteobacteria. This thesis also attempts to identify OMV production genes through the retrieval of the loss-of-OMV phenotype in a naturally mutated *M. album* BG8 strain. Overall, this thesis provides a starting point for more in-depth hypotheses and research proposals to examine the physiology of OMVs from methanotrophs. Understanding this fundamental physiology of *M. album* BG8 is needed to further develop it as an industrialized strain.

3. Isolation and Protein Cargo Identification of Outer Membrane Vesicles of *Methylomicrobium album* BG8

3.1 Abstract

Outer Membrane Vesicles (OMVs) are spherical bodies derived from the outer membrane of Gram-negative bacteria. Fully encapsulated by membrane, these extracellular structures transport biological cargo, such as proteins, from within the bacterium out into the external environment. Although there are multiple studies regarding the characteristics of OMVs released by pathogenic bacteria, understanding the role of OMVs released from commensal species is less well understood. This study describes the first OMVs from a methanotrophic bacterium, *Methylomicrobium album* BG8, and proteome contents of these vesicles. The size and morphology of these OMVs were similar to other well-characterized OMVs of other Gram-negative bacteria. Proteomic data revealed an abundance of membrane-located proteins, of which many support the putative function of metal acquisitions for the OMVs. The presence of Type I secretion system (T1SS) terminal tag, TolC and RTX-related proteins suggests the potential selection mechanism for protein cargo is mediated partially through the T1SS.

3.2 Introduction

The secretion system of extracellular vesicles is found in all three domains of life. In Gram-negative bacteria, this active transport of cargo from the interior to the exterior of cells is referred to as Outer Membrane Vesicles or OMVs. OMVs are derived from the blebbing of both the outer lipopolysaccharide (LPS) leaflet and the inner phospholipid (PL) leaflet originating from the outer membrane (OM). These vesicles can range from approximately 20-300 nm[29] and contain a similar composition to the OM from which it was originated from. The production of

OMVs is considered common within Gram-negative phyla and in both pathogenic and non-pathogenic species.

Current scientific literature emphasizes the functionality of OMVs in pathogenicity, but OMVs do not provide advantages to only pathogenic bacteria[6]. Specific functionality of OMVs varies depending on the bacterium: they can play physiological roles in stress responses [62,131], disposal of proteinaceous waste[62], nutrient acquisition[44,65,132], intra-species[68,133] and inter-species[17,68,133] interactions, and biofilm production[133]. OMVs are the only secretion system to maintain the capacity of translocating hydrophobic molecules[48,134]. Likewise, no universal genetic mechanism has been described for this energetically expensive secretion mechanism[6].

To date there has been no documentation of OMVs in methanotrophic bacteria. Also known as methane-oxidizing bacteria, methanotrophs oxidize single-carbon molecules such as methane and methanol to be utilized for biomass and energy. Aerobic methanotrophs are primarily defined into two categories, Gammaproteobacteria and Alphaproteobacteria, depending on their assimilation of formaldehyde via the RuMP or serine pathway, respectively[80]. These non-pathogenic bacteria are of particular industrial interest for their capacity to convert the greenhouse gas methane into valuable molecules for production of bioplastics and biofuels. *Methylomicrobium album* BG8, a Gammaproteobacterium, is of interest for industrialization purposes due to its well-defined genome[124], generation of industrial precursors of such as isoprenoids and other metabolites, and ability to grow rapidly to high biomass on various carbon and nitrogen sources [5,124].

The present study demonstrates *M. album* BG8 as the first methanotroph documented to produce OMVs. Of the eight methanotrophic isolates screened, *M. album* BG8 was the only

strain that displayed the presence of OMVs constitutively and under a variety of media formulations. Here, we introduce a purification protocol for OMVs from *M. album* BG8 in addition to proteomic analysis of their protein cargo utilizing LC-MS/MS. Gaining insight on the morphology, protein cargo and potential cargo selection mechanism for OMVs of *M. album* BG8 expands our understanding of its Type I secretion system as a promising tool in its production of valuable industrial products.

3.3 Materials and Methods

3.3.1 Culturing conditions and media

Eight methanotrophs species were cultivated; *Methylocystis* sp. WRRC1, *Methylocystis* sp. Rockwell, *Methylosinus trichosporium* OB3b, *Methylomonas denitrificans* FJG1, *Methylicorpusculum oleiharenae* XLMV4T, *Methylotuvimicrobium buryatense* 5GB1, *Methylococcus capsulatus* Bath and *Methylomicrobium album* BG8. Majority of these methanotroph strains were cultured in 250-mL Wheaton bottles fitted with screw cap lids inlaid with a butyl rubber stopper. The methanotrophs were cultured in either 100 mL of standard nitrate minerals salts (NMS) media or ammonia mineral salts (AMS) media, as described by Tays et al. 2018[5]. Per bottle, 1.5 mL of sterile phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4), pH of 6.8, was added following sterilization of the base media. *Methylotuvimicrobium buryatense* 5GB1 was cultured in 100 mL of modified NMS media (NMS2), as reported by Puri et al. 2015[135]. To make NMS2 media, 4 mL of sterile carbonate buffer (94.5 g/L $NaHCO_3$, 13.2 g/L Na_2CO_3), pH 8.65, and 1 mL of sterile phosphate buffer (25.1 g/L Na_2HPO_4, 17.1 g/L KH_2PO_4), pH 6.8, were added following sterilization of the base NMS medium. 50 mL of gas headspace was removed from each bottle via sterile syringe after which 60 mL of sterilized methane was injected with a syringe fitted with a 0.22-µm filter.

Modification of NMS and AMS media to the appropriate *M. album* BG8 experiments are as follows: the addition of 100 µL of pure high performance liquid chromatography (HPLC) grade methanol instead of methane, and NMS medium containing 50x standard trace element solution. In addition, copper-free media prepared in acid-washed Wheaton bottles lacked $CuSO_4$ and all media components except the trace-elements solution were treated with Chelex 100 (BioRad) to remove all traces of copper. For all cultures, the inoculum contained 1 mL (1%) of a previously grown culture that had been passaged once under identical conditions for each of the experimental conditions. All strains were incubated at 30°C, with the exception of *M. capsulatus* Bath incubated at 37°C, whilst shaken at 150 rpm until early stationary phase as determined by growth curves. Growth curves were established by measuring the optical density (OD) at 540 nm for 500 µL of extracted samples using sterile techniques, as described by Tays et al. 2018[5].

3.3.2 Micrograph imaging

Cells were removed from cultures during stationary phase and concentrated by centrifuging 1 to 2 mL of culture for 5 min at 8,000 rpm (~6,010 x *g*) (Eppendorf Centrifuge 5424), then discarding the supernatant. Pelleted cells were rinsed with phosphate buffer. Cell samples were placed on a 300-nm copper grid for 4 to 6 min prior to staining with 1% phosphotungistic acid. Micrographs were captured using a Philips/FEI (Morgagni) Transmission Electron Microscope with Gatan Camera. Figure 7A was imaged with a JEOL JEM-ARM200CF S/TEM electron microscope at an accelerating voltage of 200 kV. The HAADF-STEM images were collected with the JEOL HAADF detector using the following experimental conditions: probe size 6C, condenser lens aperture 30 µm, scan speed 32 µs per pixel, and camera length 8 cm. The average diameter of OMVs starting from the outer ring of the closed vesicles were measured using ImageJ (n=50). Micrographs contain wide ranges of scales to highlight

membrane morphology and adjacent surrounding area. TEM imaging was complete with the assistance of Arlene Oatway, Dr. Kacie Norton from Microscopy Facility (U of A) and Dr. Haoyang Yu from nanoFAB (U of A).

3.3.3 OMV purification

Three technical replicates were used for OMV purification and mass spectrometry, with each replicate containing 1 L total of *M. album* BG8 culture grown in standard NMS medium with methane. The purification method used is illustrated in Figure 6. For each replicate, the cultures were filtered using a 0.45-µm PES stericup (Millipore) to separate biomass from filtrate containing the OMVs. The filtrate was passed through a 100-kDa MW cutoff protein concentrator (GE Vivaspin) and retentate was collected by rinsing the filter with 1 mL phosphate buffer pH 6.8. Retentate aliquots were spotted on NMS agar and incubated with methane to confirm the absence of viable *M. album* BG8 cells.

The crude retentate was mixed with OptiPrep (iodixanol) (60%) and placed at the bottom of a sterile centrifuge tube. A discontinuous density gradient was created by layering 2 mL of diluted OptiPrep (40, 35, 30, 25, 20% using OptiPrep Diluent) as prepared per manufacturer's instructions. Samples were centrifuged for 12 h at 53,000 rpm (~288,350 x *g*) at 4°C in a Ti70 Fixed rotor ultracentrifuge (Beckman Optima L-90K Ultracentrifuge).

After ultracentrifugation, 1 mL fractions were removed by pipetting from the top of the density gradient, with a total of 18 fractions per replicate. Each fraction was measured for refractive index (Reichert AR200 Automatic Digital Refractometer) to ensure that the density gradient was maintained. Per fraction, 50 µL was denatured using 50 µL of 2x Laemmli Sample Buffer (Bio Rad) and incubation on a heat block (Baxter Canlab H2025-1 Dry Block Heater) at

95°C for 5 min. The prepared samples were separated on an 8% acrylamide gel and stained with colloidal Coomassie to determine protein presence. Protein-containing fractions were again denatured and concentrated using a protein centrifuge concentrator (Amicon) to a final volume of 50 μL. Concentrated protein samples were partially run through a precast 4-20% gradient gel (Bio Rad) until no residual sample was present in the wells of the gel to remove the Optiprep solvent. Protein samples were excised from the acrylamide gel and submitted for LC-MS/MS.

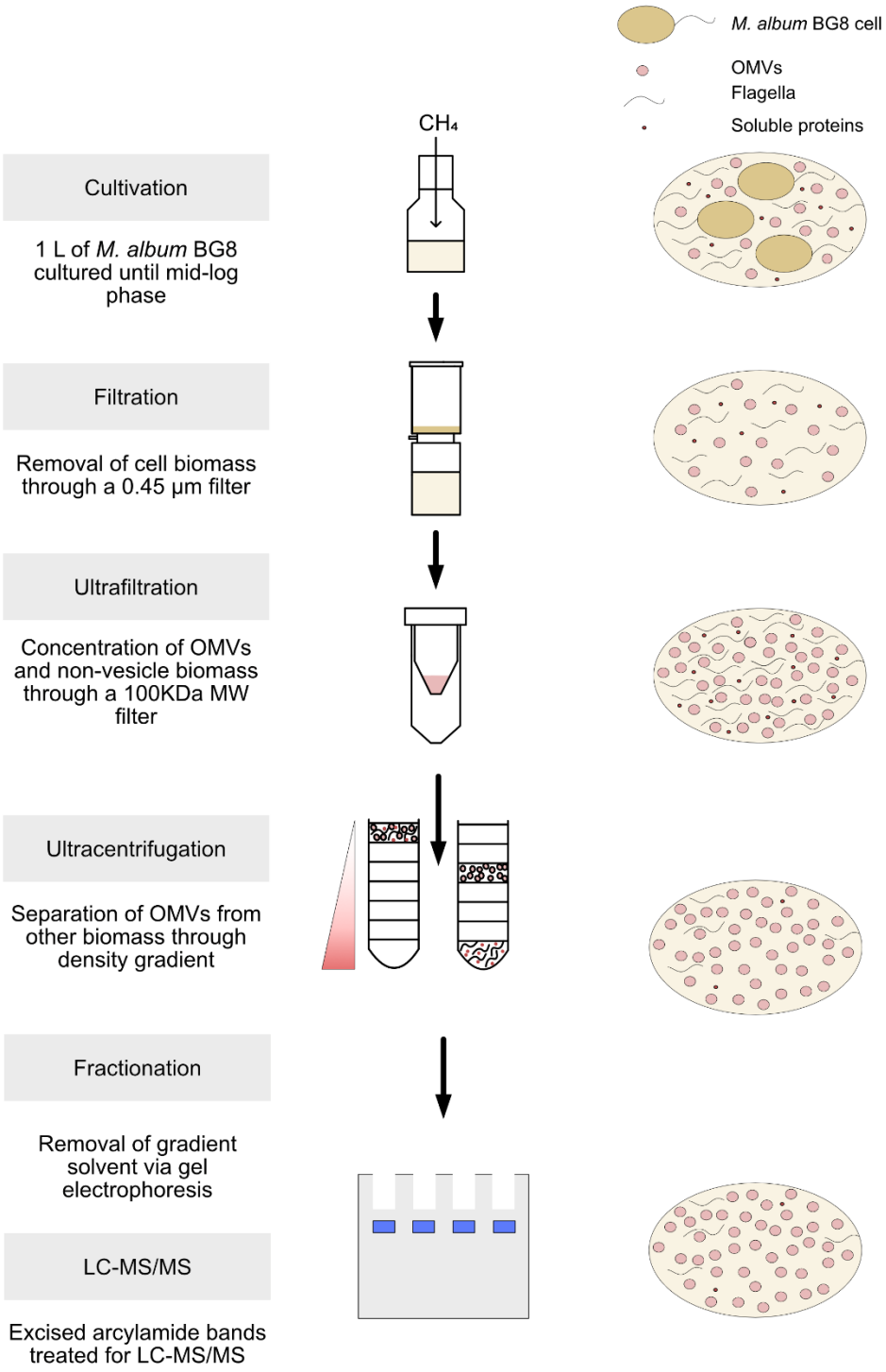


Figure 5. OMVs isolation and purification protocol for *M. album* BG8. This figure was adapted from Klimentová, J., & Stulík, J. (2015) Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological research*, 170, 1-9.

3.3.4 In-Gel Digest of OMV Peptides

All peptide digestion and LC-MS/MS preparation as well as mass spectrometry analysis was completed by Maria Camila Rodriguez Gallo and Dr. R. Glen Uhrig in association with the Alberta Proteomics and Mass Spectrometry Facility (U of A).

The gel bands corresponding to vesicles were cut into small pieces and destained with 50% 50 mM ammonium bicarbonate / 50% ACN at 37°C for 10 min, repeated 4 times. Gel pieces were then washed with 100 mM ammonium bicarbonate at 37°C for 10 min. Gel pieces were dehydrated by incubating in 100 % ACN at room temperature for 10 min, repeated twice. The gel piece was dried completely in a SpeedVac Concentrator 5301 (Eppendorf, Germany). Reducing cysteines was carried out with a 10 mM dithiothreitol solution in 100 mM ammonium bicarbonate for 45 min at 37°C. Reduction solution was replaced with 55 mM solution of iodoacetamide in 100 mM ammonium bicarbonate buffer and incubated in darkness for 1 h at 37°C to alkylate cysteine residues. The alkylation solutions were replaced by 50 mM ammonium bicarbonate buffer for 10 min. Gel pieces were washed in 100% ACN and dried by SpeedVac concentration. Gel pieces were rehydrated by adding 6 ng/µL trypsin (Promega Sequencing Grade - V5113) in 50 mM ammonium bicarbonate. Peptides were digested for 16 h at 37 °C shaking at 150 rpm. Tryptic peptides were retained and in-gel digested peptides were further extracted by adding 1% formic acid, 2% acetonitrile in 100 mM ammonium bicarbonate and incubated for 1 h at 37°C. This was followed by a second 1 h 37°C extraction using a 1:1 1 % formic acid in 50 mM ammonium bicarbonate and 100 % acetonitrile extraction buffer. Digested peptide fractions were pooled and dried down in a SpeedVac.

Isolated peptides were then dried, re-suspended and desalted using ZipTip C18 pipette tips (ZTC18S960; Millipore), as previously described (https://doi.org/10.1111/tpj.14315). All

peptides were then dried and re-suspended in 3% (v/v) ACN / 0.1% (v/v) FA immediately prior to MS analysis.

3.3.5 Mass spectrometry data analysis

Re-suspended tryptic peptides were analyzed using nano flow HPLC (Easy-nLC 1000, Thermo Scientific) with an EASY-Spray capillary HPLC column (PepMap RSLC C18, 75um x 25cm, 100Å, 2µm, Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). Dissolved samples were injected using an Easy-nLC 1000 system (Thermo Scientific). The column was equilibrated with 100% solvent A (0.1% formic acid (FA), 4% ACN in water). Peptides were eluted using the following gradient of solvent B (0.1% FA in 80% ACN): 0–18% B, 0–73 min; 18–30% B, 73-101 min; 30–46% B, 101–120 min; 46–100% B, 120-123, min at a flow rate of 0.35 µl min−1 at 50°C. High-accuracy mass spectra were acquired in data-dependent acquisition mode. All precursor signals were recorded in a mass range of 300–1,700 m/z and a resolution of 35,000 at 200 m/z. The maximum accumulation time for a target value of 1×10^6 was set to 120 ms. Up to 12 data-dependent MS/MS were recorded using quadrupole isolation with a window of 2 Da and higher-energy collisional dissociation fragmentation with 26% fragmentation energy. A target value of 5×10^4 was set for MS/MS using a maximum injection time of 250 ms and a resolution of 17,500 at 200 m/z. Precursor signals were selected for fragmentation with charge states from +2 to +7 and a signal intensity of at least 1×10^4. All precursor signals selected for MS/MS were dynamically excluded for 30 s.

Data was processed using MaxQuant 1.6.14.0 (http://www.maxquant.org/; https://doi.org/10.1038/nbt.1511), using *Methylomicrobium album* BG8 (proteomic ID: UP000005090) database (https://www.uniprot.org/) with the reverse decoy option selected. Search parameters included a peptide, protein and PSM false discovery rate (FDR) of 1%, a

precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.01 Da and a total of 2 missed cleavages. Peptides were searched with the following considerations: carbamidomethyl cysteine as a fixed modification, as cysteines are reduced during digestion; oxidized methionine; and phosphorylated serine, threonine and tyrosine as variable modifications as these amino acids are often phosphorylated.

3.3.6 Identifying similar protein sequences of OMV-related proteins in other methanotrophs using BLASTp

To determine if proteins identified in the OMV proteome where unique to *M. album* BG8, each OMV-related protein sequence was compared to the other Gammaproteobacterial non-vesicle producing methanotrophs to detect similarity using BLASTp from NCBI (https://blast.ncbi.nlm.nih.gov/). Due to the prominence of T1SS proteins in Tables 2 and 3, proteins found in *M. album* BG8 genome (GenBank: CM001475.1) associated with TISS were also analyzed. For the sequences with multiple ranges of hits, only the sequence with the highest percentage were selected. Non-vesicle producing strains are listed as follows, with reference taxonomy ID: *Methylomonas denitrificans* FJG1 (taxon:416); *Methylicorpusculum oleiharenae* XLMV4T (taxon:1338687); *Methylotuvimicrobium buryatense* 5GB1 (taxon:1338687); *Methylococcus capsulatus* Bath (taxon:414). A positive control was determined by aligning the 16S sequence of *M. album* BG8 to a known OMV-producing bacterial species outside of the Methylococcaceae family. Bioinformatics analysis was completed with the help of Dr. Aurelija Grigonyte.

3.4 Results

3.4.1 *M. album* BG8 produces OMVs in all tested media formulations

Transmission electron microscopy of *M. album* BG8 grown in standard NMS medium conditions displayed bilayer membrane vesicles (Fig. 7). The vesicle diameters ranged from 49.5 to 75.2 nm with an average diameter of 60.9 ± 5.7 (n=50) (Table 1). OMVs were observed when *M. album* BG8 was grown in media containing methanol instead of methane as a carbon source. Both NMS and AMS media devoid of copper showed production of OMVs, as seen in Fig. 7b. When cultured with excess trace elements, OMVs were present in *M. album* BG8 cultures even after 72 h of growth (Fig. 7c). Thus, changes in carbon source, nitrogen source, and metal availability did not alter the ability of *M. album* BG8 to produce OMVs.

Table 1. Diameter of *M. album* BG8 OMVs per media condition tested.

Media Condition	Average (nm)	Range (nm)
CH_4 NMS	60.9 ± 5.7	49.5 – 75.2
CH_4 AMS	64.5 ± 6.5	49.2 – 78.7
CH_3OH NMS	64.8 ± 8.1	49.7 – 84.0
CH_3OH AMS	66.9 ± 4.8	54.4 – 77.5
CH_4 x50 trace element	61.0 ± 6.8	53.1 – 78.0
CH_4 vitamin stock	67.0 ± 6.7	49.4 – 82.0

n=50

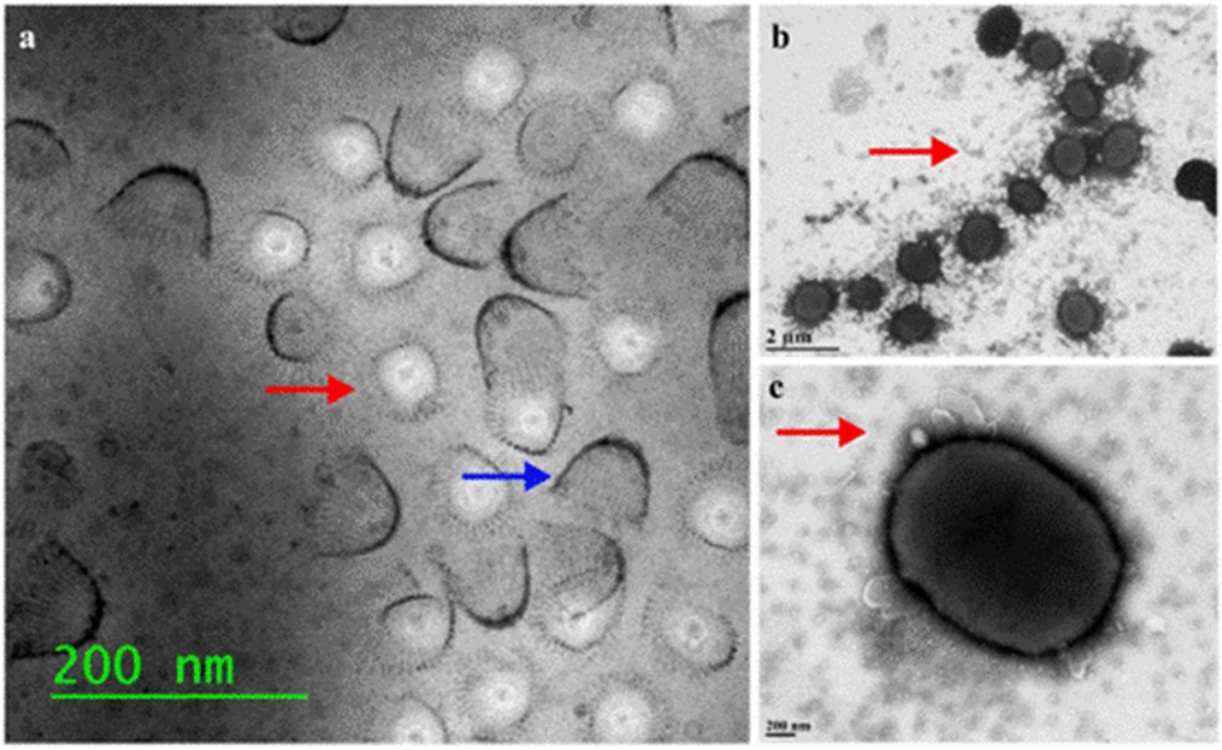


Figure 6. Transmission electron micrographs of *M. album* BG8 with OMVs (as depicted with arrows) when cultured with methane in various media conditions. (a) NMS media (b) AMS media without copper (c) NMS media with 50x trace elements solution

3.4.2 Only *M. album* BG8 produced OMVs in a screen of 8 methanotrophic isolates in standard media

Seven other strains of methanotrophic bacteria were not able to produce OMVs under the conditions tested. Fig. 8 presents the three Alphaproteobacteria and an additional four Gammaproteobacteria that were screened for OMVs production. Three Alphaproteobacterial strains, *Methylocystis* sp. WRRC1, *Methylocystis* sp. Rockwell and *Methylosinus trichosporium* OB3b, and four Gammaproteobacterial strains, *Methylomonas denitrificans* FJG1, *Methylicorpusculum oleiharenae* XLMV4^{T}, and *Methylotuvimicrobium buryatense* 5GB1 were grown as described in the Materials and Methods section and none of the cultures showed OMV production. *M. album* BG8 was seen to produce OMVs immediately after inoculation, and at every 12 h internal, for 48 h total.

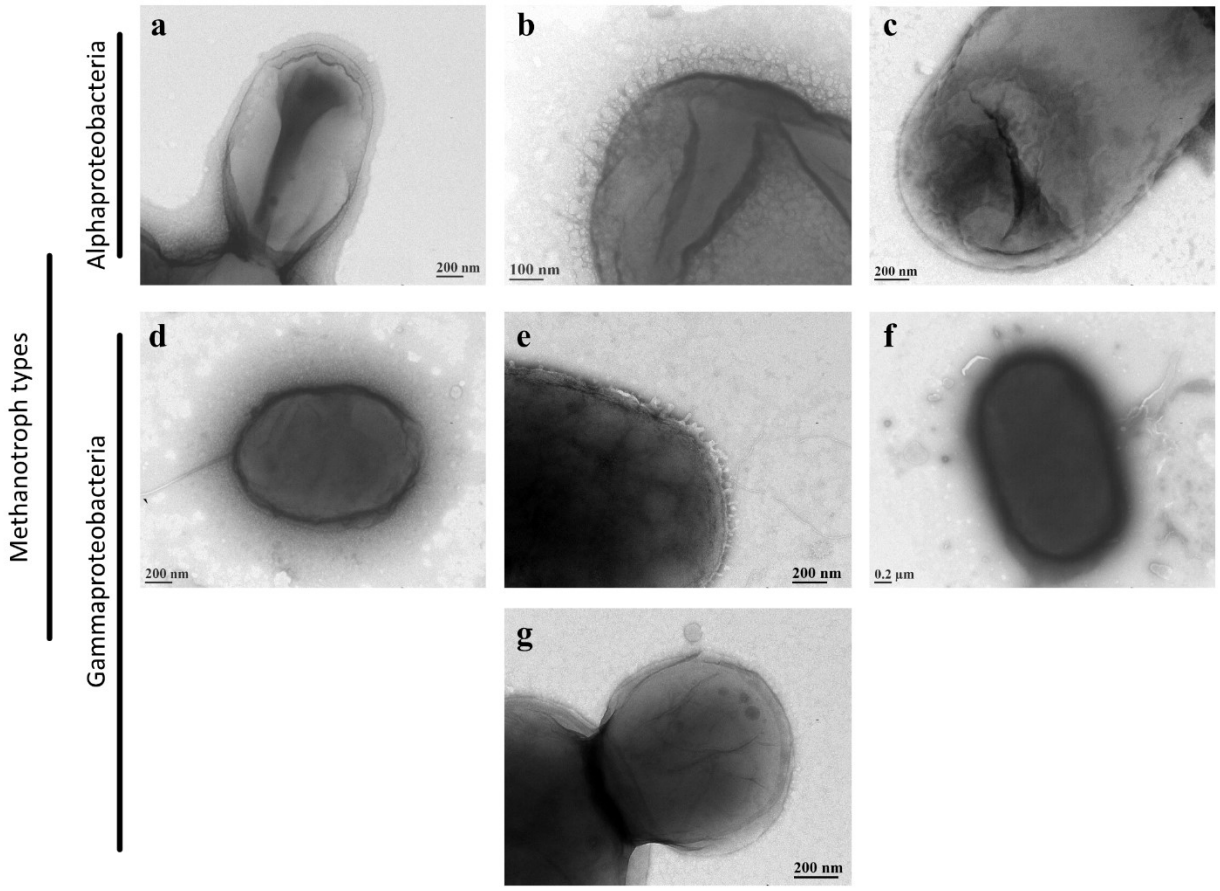


Figure 7. Transmission electron micrographs of selected Alphaproteobacterial and Gammaproteobacterial methanotrophs grown cultivated using methane. (a) *Methylocystis* sp. WRRC1 (b) *Methylocystis* sp. Rockwell (c) *Methylosinus trichosporium* OB3b (d) *Methylomonas denitrificans* FJG1 (e) *Methylicorpusculum oleiharenae* XLMV4^{T} (f) *Methylotuvimicrobium buryatense* 5GB1 (g) *Methylococcus capsulatus* Bath

3.4.3 OMV proteome using LC-MS/MS

Of the purified OMVs samples analyzed by LC-MS/MS, a total of 310 peptides were identified (Supplemental Table 1). 14 proteins had high statistical significance in triplicate samples with a ranking PSM >4, peptides >5 and score>10 (Table 2). The highest-ranking proteins included flagellin, a prepilin-type N-terminal cleavage/methylation domain-containing protein, a metallo-protease-like uncharacterized protein, Type I secretion C-terminal target

domain protein and flagella hook protein FlgE. The presence of Type I secretion C-terminal target domain protein was consistent with earlier trials of protein identification using mass spectrometry (data not shown). The proteins were matched against the Cluster of Orthologous Genes (COG) category for function annotation. Of these 14 proteins, 4 proteins were involved in secondary metabolites biosynthesis, transport and catabolism.

Table 2. Significant peptide hits from LC-MS/MS detection of purified OMVs (n=3).

Accession	UniProt Description	Mean PSM	Mean peptide	Mean score	COG Category
H8GMU5	Flagellin	331.7	22.3	1316.7	Cell motility
H8GHU9	Prepilin-type N-terminal cleavage/methylation domain-containing protein	64.3	4.0	231.4	Intracellular trafficking, secretion, and vesicular transport
H8GNA1	Uncharacterized protein (Metallo-protease PrtC*)	30.3	15.7	106.7	Secondary metabolites biosynthesis, transport and catabolism
H8GIF1	Type I secretion C-terminal target domain protein	24.5+	12.5+	71.9+	Secondary metabolites biosynthesis, transport and catabolism
H8GNF7	Flagellar hook protein FlgE	16.0	8.7	51.7	Cell motility
H8GGQ6	Uncharacterized protein	16.0	4.7	52.5	-
H8GIA0	ATP synthase epsilon chain	9.7	1.0	22.4	Lipid transport and metabolism
H8GJI6	LPS-assembly protein LptD	16.7	2.7	43.8	Cell wall/membrane/envelope biogenesis
H8GJ49	Outer membrane cobalamin receptor protein	8.0	6.3	22.5	Coenzyme transport and metabolism
H8GGW0	TonB-dependent siderophore receptor	7.0	6.3	20.2	Inorganic ion transport and metabolism
H8GKK7	Ca2+-binding protein, RTX toxin	5.7	5.7	18.5	Secondary metabolites biosynthesis, transport and catabolism
H8GKR0	Uncharacterized protein (Outer membrane protein*)	5.0	2.0	17.9	Cell wall/membrane/envelope biogenesis
H8GFV3	Uncharacterized protein	7.3	6.3	23.2	Secondary metabolites biosynthesis, transport and catabolism
H8GPP0	Uncharacterized protein	7.0	1.0	20.0	Inorganic ion transport and metabolism

*HHpred protein annotation (>95% probability)
+ replicates n=2

In addition to these 14 proteins, Table 3 represents a selection of additional proteins of interest. Notable transporter/secretion peptides include TolB from the Tol-Pal system, a TolC-like OM protein, a cation diffusion facilitator protein family protein and a general secretion pathway protein D. The presence of OmpA-like protein was expected as this protein is characteristic of other bacterial OMVs.

3.4.4 Some similarity of OMV -related and Type I Secretion-related proteins and targeted cargo of *M. album* BG8 to other Gammaproteobacterial methanotrophs.

Of the methanotroph screened, only two proteins were unique to *M. album* BG8; prepilin-type N-terminal cleavage/methylation domain-containing protein and uncharacterized protein (H8GFV3). ATP synthase epsilon chain had the highest similarity amongst the other Gammaproteobactieral methantrophs with ≥97% query coverage and 84% identity in *M. denitrificans* FJG1, *M. oleiharenae* XLMV4^{T} and *M. buryatense* 5GB1 and 67% identity in *M. capsulatus* Bath (Table 4). *M. buryatense* 5GB1 had the overall highest similarity to *M. album* BG8, particularity with Type I secretion membrane fusion protein, HlyD family and Type I secretion system ABC transporter, PrtD family with query coverage of 100% and 97% as well as 70% and 80% identity, respectively. Also, Type I secretion membrane fusion protein, HlyD family did not have significant similarity to *M. oleiharenae* XLMV4^{T} and *M. capsulatus* Bath. As mentioned above, *P. aeruginosa* is well-documented to produce OMVs nut did not display high %identity. Regarding the T1SS-related proteins, *P. aeruginosa* query coverage ranged from 88 to 97% but only 34 to 59% in identity.

Table 3. Additional protein hits of interest from LC-MS/MS detection of purified OMVs.

Accession	UniProt Accession	Mean PSM	Mean Peptide	Mean Score	COG Category
H8GMU7	Flagellar hook-associated protein 2	5.00	5.00	13.49	Cell motility
H8GL31	Cell division coordinator CpoB	5.00	5.00	11.65	Cell cycle control, cell division, chromosome partitioning
H8GNA4	Uncharacterized protein	4.67	2.00	11.77	-
H8GL29	Tol-Pal system protein TolB	4.67	4.33	14.22	Intracellular trafficking, secretion, and vesicular transport
H8GRD0	Transaldolase	4.33	4.00	10.21	Nucleotide transport and metabolism
H8GN21	Uncharacterized protein	4.00	1.00	8.92	Function unknown
H8GNM8	Hemolysin-coregulated protein (Uncharacterized)	3.67	3.00	10.30	Function unknown
H8GM80	Uncharacterized protein	3.33	1.00	7.05	-
H8GG32	Adenosylhomocysteinase	3.33	1.33	4.80	Coenzyme transport and metabolism
H8GFV2	Type I secretion outer membrane protein, TolC family	3.00	3.00	8.09	Cell wall/membrane/envelope biogenesis; Intracellular trafficking, secretion, and vesicular transport
H8GK80	Outer membrane protein/peptidoglycan-associated (Lipo)protein	3.00	2.33	8.32	Cell wall/membrane/envelope biogenesis
H8GI44	PQQ-dependent dehydrogenase, methanol/ethanol family	3.00	3.00	7.40	Carbohydrate transport and metabolism
H8GQ61	Uncharacterized protein	3.00	2.67	7.27	Function unknown
H8GNG3	Flagellar hook-associated protein 1	2.67	2.67	8.48	Cell motility
H8GPY3	Outer membrane protein/peptidoglycan-associated (Lipo)protein	2.67	2.33	7.49	Cell wall/membrane/envelope biogenesis
H8GGQ9	DNA-binding transcriptional regulator NtrC	2.67	1.00	6.79	Signal transduction mechanisms
H8GGW4	Uncharacterized protein	2.67	1.00	6.78	Function unknown
H8GNG4	Flagellar hook-associated protein 3	2.33	2.33	6.55	Cell motility
H8GI88	Probable cytosol aminopeptidase	2.33	2.00	6.21	Amino acid transport and metabolism
H8GPH9	Uncharacterized protein	1.67	1.67	6.06	Function unknown
H8GI24	Uncharacterized protein	4.00[a]	1.00	10.57	-
H8GI56	Uncharacterized protein	2.50[a]	2.50	6.29	-
H8GH59	PAS domain S-box/diguanylate cyclase (GGDEF) domain-containing protein	3.50[a]	1.00	9.16	Signal transduction mechanisms
H8GK04	Cation diffusion facilitator family transporter	2.50	1.00[b]	6.19	Inorganic ion transport and metabolism
H8GHK8	Gly-zipper_OmpA domain-containing protein	3.50	2.00	12.03[c]	Function unknown
H8GIF2	VCBS repeat-containing protein	2.67	2.00	11.46[c]	Secondary metabolites biosynthesis, transport and catabolism

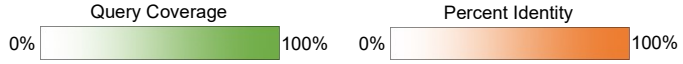
H8GPX1	Citrate lyase beta subunit	2.33	1.00	8.01[c]	Carbohydrate transport and metabolism
H8GIF8		3.00	2.50	7.94[c]	Cell motility; Intracellular trafficking, secretion, and vesicular transport

[*] HHpred protein annotation (>95%)
[a] Absent in replicate A
[b] Absent in replicate B
[c] Absent in replicate C

Table 4. Protein sequence comparison of OMV- and T1SS- related proteins of *M. album* BG8 to Gammaproteobacterial non-OMV producing methanotrophs *M. denitrificans* FJG1, *M. oleiharenae* XLMV4^{T}, *M. buryatense* 5GB1 and *M. capsulatus* Bath.

Methylomicrobium album BG8 (taxon:686340)				*Methylomonas denitrificans* FJG1 (taxon:416)				*Methylicorpusculum oleiharenae* XLMV4^{T} (taxon:1338687)				*Methylotuvimicrobium buryatense* 5GB1 (taxon:1338687				*Methylococcus capsulatus* Bath (taxon:414)			
GeneBank ID	Accession	Description	Length (aa)	Reference Sequence	Query Cov	E value	% Identity	Reference Sequence	Query Cov	E value	% Identity	Reference Sequence	Query Cov	E value	% Identity	Reference Sequence	Query Cov	E value	% Identity
EIC29550.1	H8GNF7	Flagellar hook protein FlgE	431	WP_036275189	100%	1.0E-144	52%	WP_159659411.1	100%	9.0E-137	51%	WP_017842460.1	100%	1.0E-135	50%	-	-	-	-
EIC29497.1	H8GMU5	Flagellin	761	WP_036276946.1	31%	1.0E-44	61%	WP_231089167.1	13%	4.0E-24	51%	QCW83869.1	33%	6.0E-48	67%	-	-	-	-
EIC28933.1	H8GHU9	Prepilin-type N-terminal cleavage/methylation domain-containing protein	158	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EIC28015.1	H8GKK7	Ca2+-binding protein, RTX toxin	1044	AMK76855.1	23%	1.0E-08	29%	WP_159658860.1	27%	3.0E-10	39%	WP_017842713.1	89%	0.0E+00	47%	-	-	-	-
EIC31463.1	H8GIF1	Type 1 secretion C-terminal target domain protein	1392	AMK76855.1	7%	3.0E-12	55%	WP_159658860.1	17%	1.0E-14	55%	WP_175421712.1	14%	3.0E-12	40%	-	-	-	-
EIC29232.1	H8GKR0	Uncharacterized protein	299	WP_036278985.1	91%	4.0E-68	42%	WP_159657453.1	86%	2.0E-70	47%	WP_017840936.1	87%	2.0E-80	46%	WP_010960992.1	87%	2.0E-39	31%
EIC28502.1	H8GPP0	Uncharacterized protein	118	WP_036273854.1	92%	4.0E-57	76%	WP_159658499.1	92%	8.0E-46	62%	-	-			-	-		
EIC31556.1	H8GJ49	Outer membrane cobalamin receptor protein	691	WP_052142199.1	22%	7.0E-07	25%	WP_159657167.1	26%	3.0E-13	27%	WP_017840490.1	21%	5.0E-08	24%	-	-	-	-
EIC30073.1	H8GGW0	TonB-dependent siderophore receptor	787	AMK76721.1	94%	2.0E-65	30%	WP_159658092.1	100%	0.0E+00	73%	WP_017841134.1	92%	3.0E-63	28%	AAU92033.1	22%	2.0E-14	29%
EIC31412.1	H8GIA0	ATP synthase epsilon chain	140	WP_033156953.1	100%	1.0E-49	80%	WP_159657005.1	100%	2.0E-53	84%	WP_026130213.1	100%	1.0E-53	84%	WP_010959386.1	97%	5.0E-35	67%
EIC30346.1	H8GJI6	LPS-assembly protein LptD	1003	WP_062329806.1	95%	0.0E+00	43%	WP_159657794.1	96%	0.0E+00	45%	WP_017842102.1	96%	0.0E+00	47%	WP_228370330.1	85%	0.0E+00	36%
EIC28704.1	H8GFV3	Uncharacterized protein	4036	-	-	-	-	-	-	-	-	WP_017842715.1	43%	1.0E-36	35%	-	-	-	-
EIC28703.1	H8GFV2	Type I secretion outer membrane protein, TolC family	464	WP_036273387.1	89%	1.0E-47	30%	WP_231089141.1	88%	2.0E-41	28%	WP_138767192.1	94%	0.0E+00	63%	WP_010960559.1	94%	3.0E-32	28%
EIC28702.1	H8GFV1	Type I secretion membrane fusion protein, HlyD family*	440	WP_062329159.1	99%	3.0E-38	28%	-	-	-	-	WP_017842711.1	100%	0.0E+00	70%	-	-	-	-
EIC28701.1	H8GFV0	Type I secretion system ABC transporter, PrtD family*	570	WP_062329155.1	92%	1.0E-61	29%	WP_231089186.1	83%	6.0E-55	29%	WP_017842710.1	97%	0.0E+00	80%	WP_010960270.1	42%	1.0E-35	33%
EIC30282.1	H8GIU2	Type I secretion system ATPase, LssB family*	724	WP_062329155.1	96%	0.0E+00	73%	WP_231089186.1	87%	2.0E-86	32%	WP_017841836.1	96%	0.0E+00	63%	WP_010960270.1	42%	3.0E-49	39%
EIC29875.1	H8GR48	Type I secretion outer membrane protein, TolC family*	469	WP_036273387.1	92%	1.0E-151	53%	WP_231089141.1	92%	2.0E-180	60%	WP_017840392.1	94%	0.0E+00	63%	WP_010960559.1	91%	3.0E-98	44%

*Not explicitly present in OMV proteome



3.5 Discussion

3.5.1 *M. album* BG8 is the only methanotroph to produce OMVs in this study

Although the presence of OMVs is common amongst Gram-negative bacterial species, here we present the first identified OMV produced by a methanotrophic bacterium. The morphology of these extracellular vesicles is similar to other OMVs from Gammaproteobacteria. OMVs are spherical in shape with a diameter ranging from approximately 40 to 80 nm with an average diameter of approximately 65 nm based on TEM assessment. The cup-shaped morphology as depicted with the blue arrow in Fig. 7a is likely due to TEM sample preparation[10], is standard for bacterial OMV preparations. The biological samples require drying on the copper grid, which could potentially lead to osmotic shock and may rupture the OMVs. However, the origin of this cup-shaped morphology is remains unclear.

Amongst the other methanotrophs, TEM imagine highlighted artifact are not believed to be OMVs. White patches surrounding the cell are consequence of the phosphotungistic acid negative stain, as seen in Fig. 8d. *M. oleiharenae* XLMV4^T has membrane protrusions consistent with other studies[136] but did not present any OMVs in the sample (Fig. 8e). *M. buryatense* 5GB1 displayed some spherical particles but were deem too large to be OMVs any were only present around select cells (Fig. 8f). Lastly, *M. capsulatus* Bath contains a capsule[77] surrounding its membrane (Fig. 8g).

OMV isolation and purification can differ depending on the physical attributes of the cell and the OMVs themselves[137]. Generally, OMVs are isolated by first removing the cellular biomass by either pelleting the cells or filtration of the cells after cultivation. The remaining filtrate contains the OMVs but also non-vesicle materials such as flagella, pili and extracellular

secreted proteins. Frequently, this remaining material will require concentration since the total amount of OMVs is relatively low. To remove the non-vesicle components, purification by either density gradient ultracentrifugation or gel filtration is necessary.

For *M. album* BG8, an OMV isolation and purification protocol was established. Filtration was preferred over centrifugation to remove cellular biomass, as we found pelleting large volumes of *M. album* BG8 biomass inefficient and time-consuming Instead, we found that using a 0.45-µm PES sterile filter was optimal. Both PVDF and PES filters were used for filtration of viruses and OMVs due to the low adsorption of the membrane material thus allowing for good recovery[138]. However, we found that PES clogged less frequently than the PVDF filters even with a larger pore size. Following the concentration of filtrate, the retentate was separated by ultracentrifugation using Optiprep iodixanol density gradient, a common solvent for OMV purification. Iodixanol gradients maintain several advantages over sucrose gradients. Iodixanol maintains a lower viscosity, is metabolically inert, and display lower osmolality at similar density allowing of iso-osmatic conditions which have been shown to better preserve integrity of other EVs[139]. However, the viscosity of the Optiprep solution was incompatible with subsequent downstream processes and LC MS/MS. As a result, the fractions containing the OMVs were lysed with Lamelli buffer and electrophoresed on a precast acrylamide gel to remove the iodixanol solvent. Because of the limited quantities of OMVs, gel purification was preferred over washing of OMVs. Once the sample had been incorporated into the gel, all of the bands were excised and processed with LC-MS/MS.

3.5.2 Proposed speculative model for OMV function in *M. album* BG8

Several metal-related proteins were identified among those associated with *M. album* BG8 OMVs (Fig. 9). As a result, we predict these OMVs likely play a role in metal acquisition in *M. album* BG8. The presence of metals is significant to methanotrophs. Aerobic methanotrophs are known for their characteristic single-carbon assimilation into four distinct steps. Methane is converted into methanol via methane monooxygenase, and subsequently becomes oxidized into carbon dioxide as a final production of the remaining three reactions[79,140]. Of the initial oxidation step of methane, many methanotrophs utilize the membrane-bound, copper-containing particulate form of methane monooxygenase (pMMO). Unsurprisingly, our lab found that limiting the availability of copper decreased the average cell diameter of *M. album* BG8 (unpublished data). Unexpectedly, this did not affect the presence of OMVs (Fig. 7b), suggesting that OMVs are significant even when the bacterium itself is under pressure to maintain its biomass as copper is an essential cofactor for pMMO[96]

Iron accumulation is not a unique vesicle function as the production and regulation of *Haemophilus influenzae* OMVs respond to the availability of iron[44]. Limitation of iron down-regulated the VacJ/ABC transport system, increasing vesiculation. Biogenesis of *H. influenzae* OMV is due to the lipoprotein VacJ trafficking phospholipids from the outer membrane to Yrb ABC transporter within the inner membrane. This model proposes that under the control of the ferric uptake receptor (Fur) repressor, lipid asymmetry of the outer and inner leaflet of the outer membrane induces a curvature that blebs into a vesicle. *Escherichia coli* and *Vibrio cholerae* also showed increased OMV production under iron-limiting conditions [44]. Surprisingly, even with 50x iron (trace elements) availability, *M. album* BG8 still produced OMVs despite iron

being non-limiting. This result suggests a lack of regulatory control for OMV production in this strain if OMVs are related to iron-acquisition Within our data, we note that both OM cobalamin receptor protein and the TonB-dependent siderophore receptor are structures associated with Ton-B dependent transporters, TBDT (Table 2). TBDTs are bacterial OM proteins that bind and transport iron complexes. Although the binding affinity is high for siderophores, TBDTs also transport vitamin B12 (cobalamin), nickel complexes and carbohydrates across the outer membrane[141]. In *E. coli*, TBDTs were found to be regulated by the Fur repressor in order to maintain iron homeostasis[141].

A recent study demonstrated that *Beijerinckiaceae* bacterium RH AL1 produced OMVs[132]. As a methylotroph, with the ability to reduce carbon substrates with no carbon-carbon bond as their sole source of carbon[142], its OMVs were proposed to be involved in extracellular lanthanide (rare earth metals) accumulation. A high concentration of lanthanides in the medium generated lanthanide crystals around which the strain RH AL1 cells formed. In the limited lanthanide conditions, these large crystalline structures disappeared. In its place, OMVs containing small lanthanide crystals were seen with strain RH AL1 cells maintaining contact with the OMVs.

As a subset group of methylotrophs, methanotrophs conceivably could accumulate iron within their OMVs in a similar nature. Of the media conditions tested, we saw OMVs presence in high concentration of Whittenbury trace element solution[77] (Table 1). This solution contains trace amounts of metals, including iron (II), zinc, manganese, cobalt, nickel, disodium and boric acid. However, we did not quantitate the OMVs in this media condition. A more extensive

investigation, including quantifying OMVs under various metal concentrations, particularly iron, would be needed to validate this proposal.

The most abundant proteins seen in the proteome are related to flagellar transport (Table 2 & 3). Flagellin and other flagella-related proteins are generally common contamination of purified OMV proteomes[143]. Interestingly, a commonly used *E. coli* K-12 strain has been demonstrated to package flagellin monomer subunit inside the lumen of its OMVs[144]. Likewise, a recent study demonstrated that wild type strains of *E. coli* will preferentially package major structural monomers of fimbriae and flagella into their OMVs[145], with proposed function of host immunoevasion and immunostimulation regarding pathogenicity. However, autotrophic *M. album* BG8, like all methanotrophs, are non-pathogenic to other organisms; therefore, potential directed flagellin packaging functionality would be unclear.

Lastly, as for the cargo selection, we propose the involvement of the Type I secretion system (T1SS). This secretion system comprises three parts: an ABC transporter located within the inner membrane; a membrane fusion protein that spans the periplasmic space; and an outer membrane porin protein. Together these components create an ATP-dependent translocation mechanism widespread in Gram-negative bacteria. Often, the outer membrane porin component is TolC. We see the presence of Type I secretion outer membrane protein of the TolC family (Table 3) as well as substrate, Ca^{2+}-binding protein, RTX toxin (Table 2). The substrates of the T1SS typically are defined by the presence of several blocks of nonapeptide-binding consensus sequence located at the C-terminal[146]. Here we propose that Type I secretion C-terminal target domain protein (Table 2) is connected to the selection of protein cargo in *M. album* BG8 OMVs.

The variety of RTX (repeat-in-toxin) proteins is vast and expressed broadly within Gram-negative bacteria, yet with the commonality that the secretion of these toxins always involves the T1SS. The secretion of RTX proteins is not entirely exclusive to OMVs, but in some instances, it can be. In *E. coli,* α-hemolysin RTX toxins are tightly associated with its OMVs. α-hemolysin secretion is dependent on the domain located on the C-terminal[147]. This C-terminal tag was essential for secretion but insufficient for localization to OMVs. For *V. cholerae* in stationary phase, all RTX toxin activity is associated with the OM, possibly coupled with TolC and/or inserted into OMVs[148].

The largest studied group of RTX proteins, like α-hemolysin, are generally found in pore-forming, pathogenic bacterial strains. However, there are groups of RTX proteases to bind zinc ions and contain a C-terminal calcium-binding RTX domain. Considering the predicted metallo-protease PrtC hypothetical protein (Table 1), zinc availability is another research area of interest going forward for *M. album* BG8 OMVs.

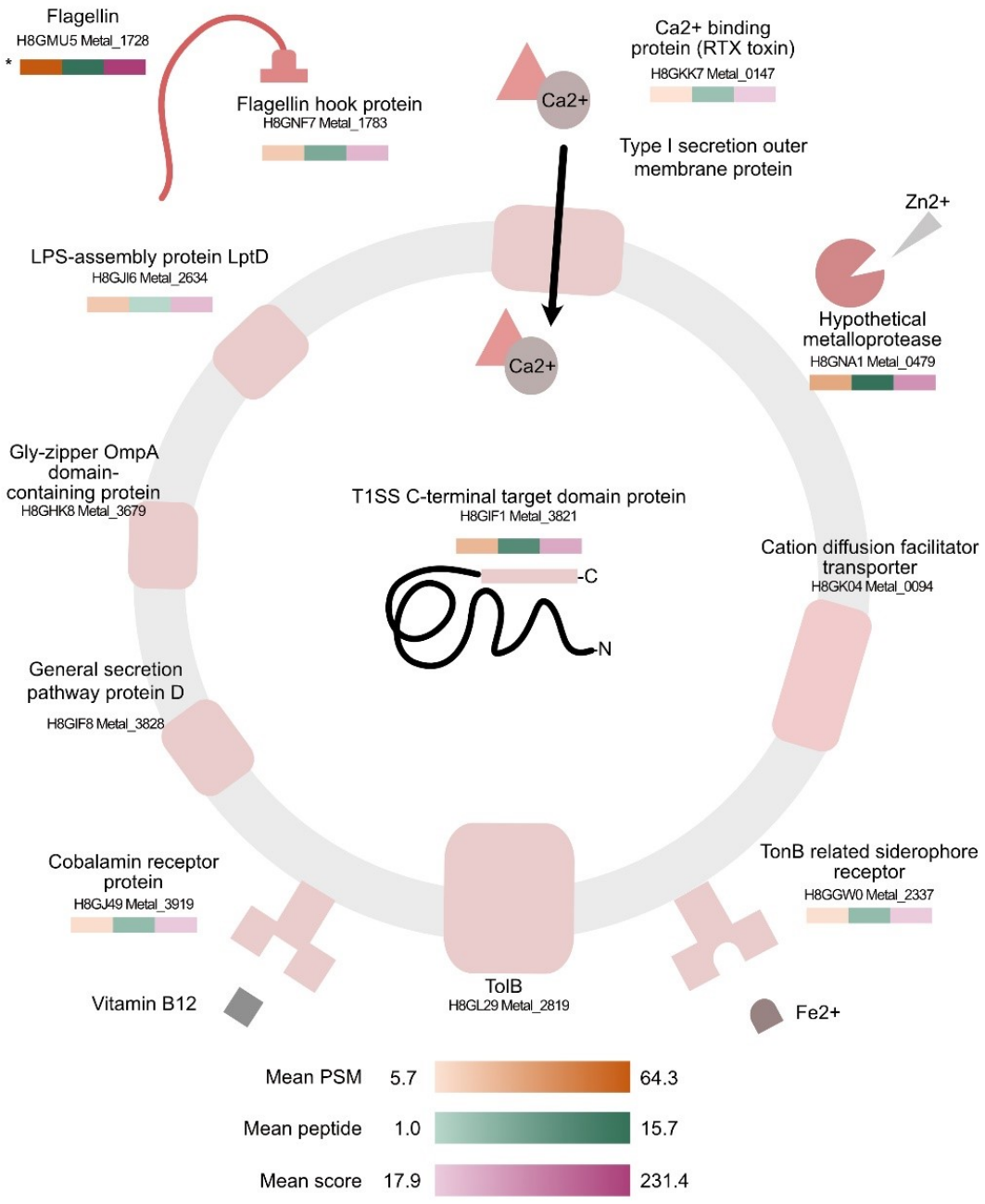


Figure 8. Proposed functional model for OMV of *M. album* BG8 based on LC-MS/MS proteome hits. Statistically significant peptides are displayed with mean PSM, peptide and score. *Flagellin heatmap adjusted to the remaining peptides.

The constant laboratory passaging of *M. album* BG8 pure cultures may have led to the need to scavenge for metal resources. The initial isolation of *M. album* BG8 was derived from freshwater and soil samples[77], likely containing more free ions than the traditional NMS minimal medium. OMVs were observed even in cultures of our oldest available stock culture of *M. album* BG8, dating back to 2013. It would be interesting to see if older stock cultures grown or other lab-adapted strains of *M. album* BG8 contain these methanotrophic OMVs.

None of the other seven additional Gamma- nor Alphaproteobacterial methanotrophs screened were seen to produce OMVs. Protein alignment analysis of Gammaproteobacterial strains indicated there were some similarity comparing sequences of the OMV-specific proteins but also the T1SS. With this is mind, *M. buryatense* 5GB1 may have the potential to produce OMVs in other media conditions aside from standard methane NMS2 media formulation. Aside from the prepilin-type N-terminal cleavage/methylation domain-containing protein, the uncharacterized protein (H8GFV3) is unique to *M. album* BG8 (Table 4). This protein is 4036 amino acids long and contains a hemolysin-type calcium-binding region conserved site (IPR018511) and a CalX-like domain superfamily (IPR038081) when searching against Interpro (https://www.ebi.ac.uk/)[149]. This uncharacterized protein is suggested to be involved with the T1SS and may be a contributing factor to OMV biogenesis in *M. album* BG8.

3.5.3 The impacts of OMVs on the industrialization of *M. album* BG8

Although the current biotechnological application of OMVs focuses on vaccine development, cancer therapy and drug delivery[72], we can still take advantage of non-pathogenic bacterial OMVs. These OMVs can serve as a fully biodegradable delivery vehicle to transport products of interest from within the cell to the external environment. Wild-type OMVs naturally

shared similar characteristics of the OM of the bacterial cell of orgins[28,150]. If the desired products of interest were membrane-bound or periplasmic-bound, then engineered export should intuitively include OMVs[151]. Likewise, engineering the movement of the desired protein to the outer membrane through a two-hybrid system has been shown in *E. coli*.[152] Packaging of bioremediation enzymes inside of OMVs has been shown to maintain function and integrity under various stressful storage conditions[75]. Compared to free-phosphotriesterase, the OMV-bound phosphotriesterase demonstrated enzymatic activity when subjected to extreme conditions such as elevated temperature, iterative freeze-thaw cycles and lyophilization.

With the proposed function of metal acquisition, native *M. album* BG8 OMVs have the potential to be mobilized for remediation of polluted environments. Moreover, with more in-depth understanding of OMVs function and biogenesis, we could potentially engineer the movement of methanotroph-related peptides or metabolites into a native secretion system. For example, *M. capsulatus* Bath and *M. trichosporium* OB3b have been highlighted for methanobactin production[91]. As a type of chalkophore, methanobactins are copper-binding peptides that are analogous to iron-binding siderophores[91]. However, these methanobactins have yet to be identified in *M. album* BG8. Interestingly, *M. album* BG8 can "steal" methanobactin from other species whilst producing a novel chalkophores[153]. Further research will have to look at the tags of peptides, like chalkophore, to the OM to see if we can choose the cargo packaged in OMVs. Siderophores have been studied for applications including within bioremediation, agriculture and medicine[154]. However, the physical interaction between *M. album* BG8 and its OMVs after blebbing needs to be resolved.

Biotechnological application of methanotrophs is an emerging field with the rise of methane emissions now higher than ever[155]. Industrialization of methanotrophs have been especially appealing due to the bioconversion of this greenhouse gas but also the industrial pollutant methanol as a low-cost feedstock into products of interest[114]. Some value-added products in methanotrophs include: metabolites such as lactic and succinic acids; precursors to biofuels like isoprenoids, isobutanol, lipids; and precursors to plastic and polymer products, like polyhydroxybutyrate and 1,4-butanediol[114,156]. Metabolomic studies on other OMVs from bacterial species have yet to be conducted. Irrespective of the type of product, exportation through a naive secretion system is a worthwhile venture for efficiently harvesting such material.

Regardless of the use of OMVs as a delivery vehicle, the research of *M. album* BG8 OMVs is still essential to bacterial physiology. Despite its ubiquitous presence, the OMV biogenesis mechanism is still unclear. Although there are species-specific hypotheses of OMV formation, there is no universal mechanism as of yet. Nevertheless, with the discovery of OMVs from more and more bacterial species, especially non-pathogenic bacterial stains, we are in a better context to gain insight on this abundant yet unsolved secretion system. We know that OMV production, regardless of the bacterium, is an energetically expensive form of secretion. Therefore, if we want to optimize *M. album* BG8 as an industrial strain, we need to understand the energetics of OMV generation.

3.5.4 Conclusions

OMVs were identified in *M. album* BG8. To characterize these vesicles, an OMV isolation and purification protocol was performed for the first time for methanotrophic bacteria. The protein cargo of the purified OMVs was characterized by LC-MS/MS. Proteomic analysis

revealed an abundance of membrane proteins, many of which functionally related to secretion and transportation of substrates across the membrane. The presence of metal-related transporters and other proteins suggest a role for *M. album* BG8 OMVs in metal acquisition, specifically iron acquisition. In addition, proteins related to the Type I secretion system was noted. This may suggest involvement of the T1SS in protein cargo selection into the OMVs. This preliminary research provides a protocol for future studies of methanotrophic OMV harvesting, as well as a baseline OMV proteome for *M. album* BG8 when cultured at standard media conditions. The proteome also serves as a list for potential indicator gene products that are involved in OMV biosynthesis. As the use of methanotrophic bacteria increase in biotechnological applications, understanding the physiology of *M. album* BG8 and its extracellular components is critical. Not only are OMVs an attractive scale up export/harvesting mechanism for value-added products, but its fundamental that we gain insight on how and why OMVs generated to optimize *M. album* BG8 cultivation in the future.

3.6 Acknowledgments

Maria Camila Rodriguez Gallo and Dr. R. Glen Uhrig assisted in mass spectrometry sample injection and analysis of peptide data. Dr. Aurelija Grigonyte contributed with bioinformatics analysis. Drs. Lisa Stein and Dominic Sauvageau were the supervisory authors who contributed in all stages of conceptualization, design, results analysis, manuscript composition, and advice. Thank you to Arlene Oatway and Dr. Kacie Norton from the Biological Science Microscopy Unit and Haoyang Yu from nanoFAB with assisting in transmission electron microscopy imaging.

4. Attempt to Rescue Loss-of-Function Outer Membrane Vesicles Production of Mutant *Methylomicrobium album* BG8

4.1 Abstract

Methylomicrobium album BG8 produces OMVs in various media formulations, yet there is one exception. A mutant strain was generated from previous work in the Sauvageau lab, through sequential adaptation passaging. This acid-adapted *M. album* BG8 strain acquired multiple mutations that allowed growth as low as pH 3.8 and maintained a loss-of-function phenotype regarding OMV production. A genomic and transcriptomic comparison between the OMV-absent mutant and the wild-type was performed. Compared to the wild-type *M. album* BG8 strain, the acid-adapted mutant displayed a down regulation of flagella- and T1SS-related gene products. Genomic comparison revealed mutations within T1SS-related genes, particularly the gene encoding the outer membrane protein TolC. Selected gene candidates were identified and transformed into the acid-adapted *M. album* BG8 in an attempt to recover OMV production. Neither introduction and expression of *tolC* nor *groL* independently rescued OMV production. The omics data collectively suggest that the T1SS plays a role in OMV biogenesis in *M. album* BG8 and should be further investigated.

4.2 Introduction

Previous work by Kieran McDonald, a former MSc student in the Sauvageau laboratory, evolved a strain of *M. album* BG8 that was adapted to acidic media formulations. This project successfully adapted the parental *M. album* BG8 with nitrate as the nitrogen source from pH 6.8 to pH 3.95 in methanol and pH 3.8 in methane. These acid-adapted strains of *M. album* BG8 maintained the ability to grow normally at the neutral pH 6.8 condition. Both mutant strains were

passaged from the lowest pH to neutral pH and back to the lowest pH, without demonstrating any significant growth changes. Since growth performance at neutral pH was not altered, this passaging suggested independent adaption rather than mutation accumulation or antagonistic pleiotrophys[157].

An interesting artifact from this adaptation was the loss of OMVs in both of the acid-adapted strains. Regardless of the pH, the acid-adapted strains of *M. album* BG8 were unable to produce OMVs. The acid-adapted mutant strains were analyzed for genomic changes and global gene expression for comparison to the wildtype to determine the genetic elements involved in OMV production in *M. album* BG8.

4.3 Materials and Methods

4.3.1 Transcriptome Analysis

Kieran McDonald performed the RNA extraction. To summarize, acid-adapted and wildtype *M. album* BG8 were cultured in 250 ml Wheaton bottles containing NMS medium at pH 3.80 and pH 6.8, respectively, as described in McDonald K. 2019 in biological triplicates. Both cultures were harvested in early stationary phase and processed through the MasterPure RNA purification kit (Epicentre). RNA-Seq was performed by the Centre d'Expertise et de Services at Génome Québec (Montreal, Québec, Canada). Raw reads were assembled by Dr. Yusheng Tan using Geneious RNASeq assembler to align reads to genome files, Geneious read count program to calculate aligned read counts, and Geneious DESeq2 wrapped to calculate DE. From the whole transcriptome, a subset of data was used in the present analysis in the context of genes potentially involved in OMV biogenesis and function.

4.3.2 Genome Analysis

Kieran McDonald also performed the genomic DNA extraction. To summarize, acid-adapted and wild-type *M. album* BG8 were harvested at late stationary phase. The genomic DNA was extracted using GeneJET Genomic DNA Purification kit (ThermoFisher Scientific) following the manufacturer's protocol. The samples were further processed through Zymo DNA clean and concentrator kit (Zymo Research). Sequencing was performed by the University of Washington PacBio (Seattle, Washington, USA), and Kieran McDonald and Dr. Fabini Orata completed the initial genomic analysis.

4.3.3 Construction and conjugation of pQSDP$_{MMO}$ and pQSDP$_{GAP}$ into acid-adapted *M. album* BG8

The promoter sequences for methane monooxygenase (P_{MMO}) and glyceraldehyde 3-phosphate dehydrogenase (P_{GAP}) were derived from *M. album* BG8 genome and cloned into an IncQ BHR pQCH plasmid by Shibashis Das. These constructs contain a gentamycin resistance marker referred to as pQSDP$_{MMO}$ and pQSDP$_{GAP}$ and maintained in *E. coli* DH10β.

Sequences for TolC, GroL and OmpL were amplified by PCR from *M. album* BG8 genome using primers compatible with Gibson Assembly (Supplemental Table 3). The TolC and GroL inserts were assembled into pQSDP$_{MMO}$ and OmpL-His was assembled into pQSDP$_{GAP}$ via Gibson assembly, then transformed into *E. coli* DH10β. These plasmids were purified using Qiagen Miniprep and transformed into *E. coli* S17λ as the donor strain for conjugation. Acid-adapted *M. album* BG8 was cultured using 250ml Wheaton bottles containing NMS media, pH 6.8 with methane, as described in Chapter 2. The acid-adapted *M. album* BG8 biomass was collected and co-cultured with *E. coli* S17λ on NMS + mating plates (NMS agar with 15% v/v

nutrient broth) in an anaerobic jar supplied with methane gas for 48 h at 30°C. After conjugation, this biomass and plated on NMS agar plates with gentamycin (15 ug/ml). Transformants were incubated in serum vials containing 7 mL NMS liquid media containing methanol until growth was observed. Incubations lasted 5-7 days and passaged 3 separate times to remove *E. coli* S17λ donor strain. Transformed acid-adapted *M. album* BG8 was imaged for OMV production via TEM, as described in Chapter 2.

4.4 Results

4.4.1. Acid-adapted *M. album* BG8 downregulates flagella-associated genes

The lack of OMVs was documented by TEM imaging. Neither the methane nor methanol acid-adapted strain produces vesicles at low pH or neutral pH (Fig. 10). The acid-adapted strain still maintains the production of pili (Fig. 10b) and flagella (image not shown).

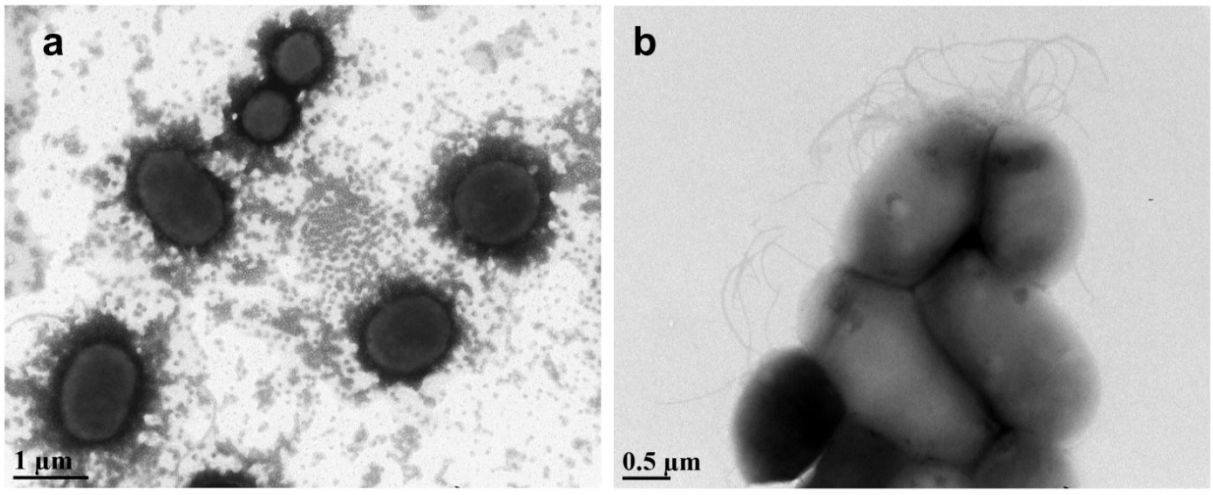


Figure 9. Transmission electron micrographs *M. album* BG8 cultivated with methane NMS pH 6.8; a) parental wild-type *M. album* BG8 with OMVs present b) acid-adapted *M. album BG8* with the absence of OMVs

Based on the RNASeq transcriptome of OMV-related proteins described in Chapter 2, 9 genes were significantly down-regulated (differential expression Log2 Ratio ≤ -1) in the acid-adapted strain compared to the parental (Table 5). Of these, 5 genes associated with flagella showed significant decrease, the largest being the flagellar hook protein. The remaining down-regulated genes included prepilin-type N-terminal cleavage/methylation domain-containing protein, an OmpA domain-containing protein, as well as the two prominent T1SS proteins from Chapter 2; Ca^{2+}-binding RTX protein and the T1SS C-terminal target domain protein.

In contrast, only 2 genes were significantly up-regulated 9 (Differential Expression Log2 Ratio ≥ 1) in the acid-adapted mutant. The PAS domain S-box/diguanylate cyclase (GGDEF) domain-containing protein and uncharacterized protein (H8GFV3) maintained a differential expression Log2 ratio of 1.15 and 2.62, respectively.

Table 5. RNASeq transcriptome of *M. album* BG8 cultivated at pH 3.8 over pH 6.8 of OMV-related proteins.

Locus tag	Accession	Description	Minimum	Maximum	Differential Expression Log2 Ratio
Metal_1783	H8GNF7	Flagellar hook protein	1603279	1604574	-2.51
Metal_1790	H8GNG4	Flagellar hook-associated protein 3	1596181	1597107	-2.38
Metal_1789	H8GNG3	Flagellar hook-associated protein 1	1597117	1598772	-2.26
Metal_1728	H8GMU5	Flagellin	1656779	1659064	-2.10
Metal_1116	H8GHU9	Prepilin-type N-terminal cleavage/methylation domain-containing protein	2353691	2354167	-1.98
Metal_3679	H8GHK8	Gly-zipper_OmpA domain-containing protein	3963376	3963771	-1.25
Metal_0147	H8GKK7	Ca2+-binding protein, RTX toxin	3459470	3462604	-1.13
Metal_3821	H8GIF1	Type I secretion C-terminal target domain protein	3810217	3814395	-1.08
Metal_1730	H8GMU7	Flagellar hook-associated protein 2	1654589	1655965	-1.00
Metal_0094	H8GK04	Cation diffusion facilitator family transporter	3514636	3515601	-0.66

Metal_2282	H8GGQ9	DNA-binding transcriptional regulator NtrC	1024428	1025837	-0.45
Metal_2515	H8GI88	Probable cytosol aminopeptidase	730051	731538	-0.41
Metal_2469	H8GI44	PQQ-dependent dehydrogenase, methanol/ethanol family	782421	784232	-0.35
Metal_2345	H8GGW4	Uncharacterized protein	925357	925647	-0.33
Metal_1419	H8GK80	Outer membrane protein/peptidoglycan-associated (Lipo)protein	2014298	2015179	-0.27
Metal_3106	H8GNM8	Hemolysin-coregulated protein (Uncharacterized)	98295	98789	-0.26
Metal_2207	H8GG32	Adenosylhomocysteinase	1106756	1108045	-0.25
Metal_1986	H8GPX1	Citrate lyase beta subunit	1359341	1360291	-0.20
Metal_1447	H8GKR0	Uncharacterized protein	1983015	1983914	-0.15
Metal_0659	H8GPP0	Uncharacterized protein	2834359	2834715	-0.12
Metal_3255	H8GPH9	Uncharacterized protein	4390812	4391090	0.03
Metal_0482	H8GNA4	Uncharacterized protein	3055180	3055491	0.05
Metal_2821	H8GL31	Cell division coordinator CpoB	403812	404663	0.06
Metal_1666	H8GM80	Uncharacterized protein	1716673	1720563	0.08
Metal_3919	H8GJ49	Outer membrane cobalamin receptor protein	3687178	3689253	0.09
Metal_2337	H8GGW0	TonB-dependent siderophore receptor	929577	931940	0.12
Metal_3768	H8GIA0	ATP synthase epsilon chain	3868004	3868426	0.15
Metal_2446	H8GI24	Uncharacterized protein	809820	812723	0.17
Metal_2819	H8GL29	Tol-Pal system protein TolB	405468	406745	0.18
Metal_3055	H8GN21	Uncharacterized protein	164573	166417	0.19
Metal_0879	H8GFV2	Type I secretion outer membrane protein, TolC family	2617731	2619125	0.21
Metal_3460	H8GRD0	Transaldolase	4200221	4201228	0.22
Metal_3338	H8GQ61	Uncharacterized protein	4318245	4319984	0.25
Metal_1998	H8GPY3	Outer membrane protein/peptidoglycan-associated (Lipo)protein	1344830	1346023	0.35
Metal_2481	H8GI56	Uncharacterized protein	769328	770185	0.39
Metal_3828	H8GIF8	General secretion pathway protein D	3792584	3794851	0.55
Metal_2634	H8GJI6	LPS-assembly protein LptD	619290	622301	0.67
Metal_1031	H8GH59	PAS domain S-box/diguanylate cyclase (GGDEF) domain-containing protein	2454790	2456739	1.15
Metal_0880	H8GFV3	Uncharacterized protein	2604290	2616400	2.61
Metal_2279*	H8GGQ6	Uncharacterized protein	-	-	-
Metal_0479*	H8GNA1	Uncharacterized protein	-	-	-
Metal_3822*	H8GIF2	VCBS repeat-containing protein	-	-	-

* Absent in RNASeq dataset

Bolded proteins are statistically significant from Table 2 in Chapter 2

4.4.2 T1SS associated genes mutated in the acid-adapted *M. album* BG8 genome

Table 6 displays 19 mutations disrupting 16 coding sequences. There were an additional 25 mutations within non-coding regions, repeat regions and hypothetical proteins, which were not included in this analysis. Of these 19 mutations, 6 different transposase sequences were disrupted.

Table 7 displays SNPs and point mutations documented between the acid-adapted strain to the wildtype genome. Mutations listed here were seen in both the methane and methanol acid-adapted strains. Likewise, only mutations in coding regions were analyzed. However, genes potentially associated with OMVs or outer membrane are displayed, resulting in 27 SNPs or point mutations in 14 genes. Eleven of these SNPs resulted in silent mutations. Three different transposase sequences contained point mutations. Notably, the Type I secretion membrane fusion protein, HlyD family, had a frameshift mutation.

Based on the genes listed in Table 6, a few were selected to be transformed into the acid-adapted *M. album* BG8 strain in an attempt to rescue its loss-of-function phenotype. TolC outer membrane protein and GroL chaperonin (also referred to as GroEL) were independently expressed under a strong consecutively promotor P_{MMO}. The porin OmpL with a His-tag was expressed under a weaker promoter, P_{GAP}. Only pQSDP$_{MMO}$TolC and pQSDP$_{GAP}$OmpL-His were successfully conjugated into the acid-adapted *M. album* BG8 strain. TEM imaging of these two transformants displayed no OMV presence (Fig. 11). Notably, the pQSDP$_{GAP}$OmpL-His transformant displayed bulging of the membrane.

Table 6. Mutations identified in methane acid-adapted to wild-type *M. album* BG8 genome.

Locus Tag	IMG Reference Gene	Function	Start Position	Length (bp)	Type of Mutation
Metal_0315	2508546663	WD40 repeat-containing protein	326462	254	Insertion
Metal_0463	2508546811	Serine/threonine protein kinase	517080	51	Deletion
Metal_0464	2508546812	Transposase IS5 family	517237	957	Deletion
Metal_0570	2508546918	Helicase family protein with metal-binding cysteine cluster	666904	181	Insertion
Metal_1578	2508547926	Transposase	1782881	351	Deletion
Metal_1579	2508547927	Transposase	1783306	1,635	Deletion
Metal_1926	2508548273	Carbamoylphosphate synthase large subunit	2173171	1,185	Insertion
Metal_2279	2508548626	Porin, OmpL	2571781	30	Insertion
	2508548626	Porin, OmpL	2571890	12	Deletion
Metal_2305	2508548652	Non-ribosomal peptide synthase/amino acid adenylation enzyme	2628177	12,974	Insertion
	2508548652	Non-ribosomal peptide synthase/amino acid adenylation enzyme	2629940	1,763	Deletion
Metal_2569	2508548916	Type I secretion protein TolC	2914335	8	Insertion
Metal_2675	2508549022	Transposase IS5 family	3016314	957	Deletion
Metal_2953	2508549300	Hybrid non-ribosomal peptide synthetase/type I polyketide synthase	3325215	9	Repeat Deletion
Metal_3584	2508549931	Chaperonin GroL	3989868	9	Repeat Expansion
Metal_3611	2508549958	Transposase IS5 family	4014110	957	Deletion
Metal_3822	2508550169	VCBS repeat-containing protein	4246250	568	Deletion
	2508550169	VCBS repeat-containing protein	4246928	1,126	Insertion
Metal_3848	2508550195	Transposase IS5 family	4281503	957	Deletion

Table 7. SNPs and point mutations of interest from *M. album* BG8 adapted when grown at pH 3.85 compared to *M. album* BG8 reference genome.

Locus tag	Position in Ref	Change	IMG Reference Gene	Amino Acid Change	Function
Metal_0915	1,030,576	.-G	2508547263	Ala-Gly +	Drug resistance transporter, EmrB/QacA subfamily
Metal_1247	1,392,148	C-T	2508547595	Asp-Asp	Transposase
Metal_1247	1,392,950	C-A	2508547595	Pro-Gln	Transposase
Metal_1247	1,392,998	T-C	2508547595	Val-Ala	Transposase
Metal_1247	1,393,073	C-A	2508547595	Pro-His	Transposase
Metal_1464	1,641,433	A-G	2508547812	Asp-Gly	DNA-binding protein H-NS
Metal_1726	1,942,714	A-G	2508548073	Asn-Ser	Flagellar basal body-associated protein
Metal_1909	2,153,431	A-G	2508548256	Leu-Leu	DNA/RNA helicase, superfamily II, SNF2 family
Metal_2019	2,277,970	G-A	2508548366	Asp-Asn	Succinate dehydrogenase, hydrophobic membrane anchor protein
Metal_2101	2,359,509	T-G	2508548448	Val-Gly	Putative permease
Metal_2196	2,477,863	G-.	2508548543	Arg-Arg +	Transposase IS5 family
Metal_2196	2,477,913	G-.	2508548543	Gly-Ala +	Transposase IS5 family
Metal_2323	2,647,577	A-G	2508548670	Glu-Gly	Cytochrome c
Metal_2567	2,913,121	A-.	2508548914	Asn-Thr +	Type I secretion membrane fusion protein, HlyD family
Metal_2678	3,019,029	A-C	2508549025	Glu-Ala	Tfp pilus assembly protein PilN
Metal_3143	3,535,118	C-T	2508549490	His-Tyr	Transposase
Metal_3143	3,535,239	A-G	2508549490	Val-Val	Transposase
Metal_3143	3,535,245	T-C	2508549490	Ala-Ala	Transposase
Metal_3143	3,535,314	G-A	2508549490	Gln-Gln	Transposase
Metal_3143	3,535,326	A-G	2508549490	Ala-Ala	Transposase
Metal_3143	3,535,601	T-C	2508549490	Pro-Pro	Transposase
Metal_3143	3,535,619	A-G	2508549490	Pro-Pro	Transposase
Metal_3143	3,535,658	T-C	2508549490	Phe-Phe	Transposase
Metal_3143	3,535,737	C-G	2508549490	Thr-Arg	Transposase
Metal_3143	3,535,751	T-C	2508549490	His-His	Transposase
Metal_3171	3,563,785	G-A	2508549518	Ser-Asn	Phosphatidylserine decarboxylase precursor
Metal_3777	4,195,163	T-C	2508550124	Val-Ala	Outer membrane protein

+ frameshift mutation

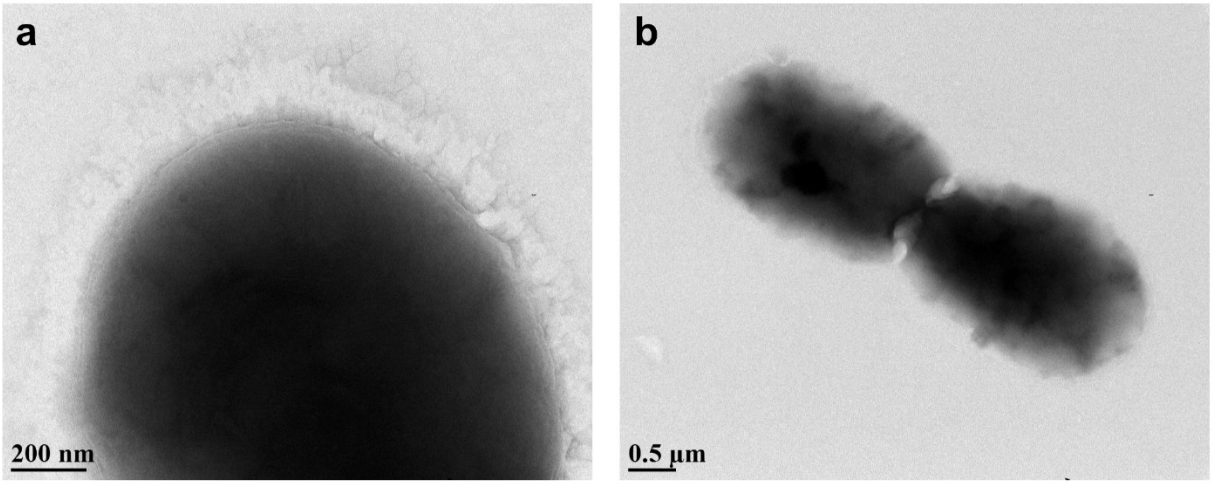


Figure 10. TEM of transformed acid-adapted *M. album* BG8; a) pQSDP$_{MMO}$-TolC, b) pQSDP$_{GAP}$-OmpL-His.

4.5 Discussion

4.5.1 Flagella-related proteins down-regulated and Ca^{2+}-binding proteins up-regulated in acid-adapted *M. album* BG8

As mentioned in the previous chapter, recent evidence describes flagellin involvement in OMVs related to pathogenicity of *E. coli*. Table 5 shows a general downregulation of flagella-related proteins in the acid-adapted strain. However, TEM imaging suggests that flagella are still evident. Similar to flagellin, pilin-related peptides are known to co-purify with OMV prepartions[31]. This decrease in flagella-related gene expression may result in a change in the proton motive force since the difference between the internal cellular pH to the external environment pH is increased.

The genes encoding Gly-zipper_OmpA domain-containing protein was slightly down-regulated in the acid-adapted strain. OmpA is a signature OMV-associated protein, so the

absence of OMVs correlates with less OmpA production. Interestingly, in *V. cholera* and S. Typhimurium, mutants lacking *ompA* are shown to increase in vesiculation[34,158]

The CalX beta motif is present in domains of Calx sodium-calcium exchangers, which are used to transport calcium from the cell and calcium-binding and regulation[159]. This result further suggests a role in calcium binding and/or involvement of the TISS in wildtype OMVs.

4.5.2 T1SS involvement in OMV production *M. album* BG8

Ca^{2+}-binding protein, RTX toxin and Type I secretion C-terminal target domain protein were down-regulated in the acid-adapted stain. These T1SS gene differences provide additional evidence that the T1SS could be significant to OMV cargo selection, supporting the proposed model (Fig. 9).

One well-studied use of the T1SS is the export of the pore-forming hemolysin RTX toxin, HlyE. The gene encoding the membrane fusion protein for this tri-protein complex, HlyD, is mutated in the acid-adapted *M. album* BG8 strain. In *Salmonella enterica* ser. Typhi, the global regulator H-NS is hypothesized to regulate the expression of other genes involved in vesiculation[33]. In contrast, *ompA* is involved in HlyE secretion, and *hns* is reported to repress *hlyE* expression.

In *E. coli* and *Salmonella enterica* serovar Choleraesuis, evidence suggests that TolC plays a role in low pH adaptation as it is part of the glutamate decarboxylase system[160,161].

The most abundant family of genes showing mutation in the acid-adapted strain is transposases. However, the relationship between OMVs and transposase is not explicitly documented, with the exception of *Acinetobacter baumannii* OMVs. The OMVs of this pathogen

exports transposases to the host cell nucleus during infection[162]. Transposases are used to modify transcription regulation of the host cell via methylation of CpG island. Once again, as *M. album* BG8 is not a known pathogen, it is unclear whether there is potential use of transporting transposases.

4.5.3 TolC and OmpL independently are not sufficient for OMV production in the acid-adapted *M. album* BG8 strain

As mentioned above, the T1SS system is speculated to be involved in OMV production. TolC is often the outer membrane protein in this three-protein secretion system. As such, TolC is an ideal candidate to attempt to rescue OMV loss of function in the acid-adapted strain.

The soil nematode symbiont, *Xenorhabdus nematophila,* associates the molecular chaperonin GroL with its OMVs. In addition to being a molecular chaperonin responsible for the correct folding of nascent proteins in the cell, GroL contains insecticidal activity with chitin-binding properties[163]. Unfortunately, GroL was unable to be transformed into the acid-adapted *M. album* BG8 strain. However, it still makes for an interesting gene candidate. Like many methanotrophs, *M. album* BG8 was isolated from freshwater and soils and may benefit from a chitin inhibition activity.

Porin proteins produce transmembrane diffusion channels that allow for diffusion of small hydrophobic molecules, such as sugars, amino acids and vitamins. OmpC and OmpF are porins that are major component of OMVs in *E. coli.* These two porins have been found to transport β-lactam antibiotics into OMVs where β-lactamase is held[164]. As a result, OmpL was

considered to be a potential gene product that associated with *M. album* BG8 OMVs, especially because the substrate for OmpL has yet to be elucidated[165]

Methane monooxygenase is consecutively expressed. Because of this, a weaker promoter was used for the second round of OMV-restoring gene candidates to as a concern to address lethal protein overexpression. With this in mind, OmpL was expressed under the weaker promotor derived from the glyceraldehyde 3-phosphate dehydrogenase gene. In addition, the OmpL construct was generated with a His-tag for further protein quantification. Regardless of the promotor, neither TolC under P_{MMO} or OmpL under P_{GAP} rescued the loss-of-function mutation. Both transformants displayed distorted and bulging membranes (Fig. 11), suggesting that the membrane was mutated. The recombinant OmpL-His protein has yet to be quantified.

From a screen of 15 000 random insertion mutants, 9 genes in *S. Typhi* were identified to increase HlyE secretion and were involved in OMV biogenesis, which includes *ompA* and *hns*. Each individual gene was implicated in a different OMV biogenesis stage and played a role in OMV cargo selection. Thus, multiple genes potentially determine the function and properties like the diameter of OMVs[33,166]. As for any species of Gram-negative bacteria, it is unlikely that a single gene is responsible for OMV production in *M. album* BG8. Genetic models for well-documented OMVs systems consist of orchestrating multiple gene products. Further investigation of the above gene candidates is warranted to determine how they interact to form OMVs in *M. album* BG8 such as creating a genome wide library for CRISPR knockout

4.6 Acknowledgments

This chapter builds on the research of Kieran McDonald, of which generated the acid-adapted mutant *M. album* BG8 strains and completed both the genome and RNA extraction. Thank you to Dr. Fabini Orata, Cerrise Weiblen, Dr. Aurelija Grigonyte, and Yusheng Tan for clarifying the genomic and transcriptomic analysis. Additional thanks to Shibashis Das for help with cloning.

5. Conclusion

5.1 Summary and Conclusions

OMVs are widespread structures released by many Gram-negative bacteria. Yet the functions and production of these ubiquitous structures are as distinctive as the species that generate them. OMV research emphasizes the role of these vesicles in pathogenicity and medical purposes. However, pathogens encompass a small range of species that produce these elegant extracellular structures.

This thesis is a reference framework for the characterization of OMVs in *M. album* BG8. As part of a non-pathogenic group of bacteria, characterizing obligate methanotrophic OMVs is needed to expand the understanding of how and why OMVs are generated. In chapter 3, OMVs were documented in only one of eight methanotrophs. *M. album* BG8 produces OMVs in many iterations of media formulations. We proposed the first OMV purification protocol specific to methanotrophs. Proteome results suggest that metal scavenging, particularly calcium and iron, is a functional trait of OMVs of *M. album* BG8. In addition, the presence of the proteins related to the Type I secretion system suggests its involvement in protein cargo selection directed into the vesicles.

To support the proposed model derived from the OMV proteome, chapter 4 compares the transcriptome and genome of an acid-adapted *M. album* BG8 strain lacking the ability to generate OMVs. This acid-adapted strain showed changes in the regulation of, as well as mutations/SNPs of, the T1SS and cargo related genes. Taken together, these omics based approaches provide insight into the genes potentially associated with biogenesis, cargo-selection, and function of *M. album* BG8 OMVs.

5.2 Future Directions

Because vesiculation is often a response to non-ideal growth conditions[113,167], this might suggest that there are non-ideal aspects of the current culturing environment for *M. album* BG8. Investigation of other forms of methanotrophic media may increase or decrease vesiculation. Likewise, a crucial next step would be to quantify OMVs using flow cytometry[168]. In addition, to validate the functional metal acquisition proposed model, metal-based assay experiments are required, particularly for calcium and iron.

This thesis only examined the protein cargo of *M. album* BG8 OMVs but did not investigate the presence of other biological cargo such as nucleic acids, lipopolysaccharides, peptidoglycan, and phospholipids. Fluorescent staining of nucleic acids was limited by the magnification range of fluorescent microscopy equipment at the time of screening. Detecting the presence and potentially quantifying nucleic acids would provide more information on possible function of methanotroph OMVs.

To further investigate OMV biogenesis, lipidomics might be an interesting route[137]. The lipid composition of OMVs in *E .coli* changes when released from the cells in exponential phase versus stationary phase[169,170]. The flexibility of the fatty acid structures is thought to influence OMV biogenesis as these fatty acids affect the fluidity and rigidity of the lipid membrane. *M. album* BG8 adapts its membrane in the presence of methanol[123]. Identifying if the lipid membrane is altered throughout the cell lifecycle would provide insight into vesicle production.

Regarding the rescue of the loss-of-function mutation in the acid-adapted *M. album* BG8 strain, cloning and expressing multiple gene candidates simultaneously may be a better approach.

Likewise, a high throughput random mutagenesis screen of the wildtype strain would highlight the specific genes involved in OMV production. Granted, establishing an OMV screen that is not dependent on electron microscopy might be required before any high throughput screening methods are employed due to the time and effort required.

Unified References

1. Hogan, K., Hoffman, J. & Thompson, A. Methane on the greenhouse agenda. *Nature* **354,** 181–182 (1991). https://doi.org/10.1038/354181a0

2. Jackson, R.B., Solomon, E.I., Canadell, J.G. *et al.* Methane removal and atmospheric restoration. *Nat. Sustain.* **2,** 436–438 (2019). https://doi.org/10.1038/s41893-019-0299-x

3. Fei, Q., Puri, A.W., Smith, H. *et al.* Enhanced biological fixation of methane for microbial lipid production by recombinant *Methylomicrobium buryatense*. *Biotechnol Biofuels* **11,** 129 (2018). https://doi.org/10.1186/s13068-018-1128-6.

4. Khosravi-Darani, K., Mokhtari, ZB., Amai, T. *et al.* Microbial production of poly(hydroxybutyrate) from C_1 carbon sources. *Appl. Microbiol. Biotechnol.* **97,** 1407–1424 (2013). https://doi.org/10.1007/s00253-012-4649-0

5. Tays, C., Guarnieri, M. T., Sauvageau, D. & Stein, L. Y. Combined Effects of Carbon and Nitrogen Source to Optimize Growth of Proteobacterial Methanotrophs. *Front. Microbiol.* **9**, 2239 (2018). https://doi.org/10.3389/fmicb.2018.02239

6. Schwechheimer, C., Kuehn, M. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat. Rev. Microbiol.* **13,** 605–619 (2015). https://doi.org/10.1038/nrmicro3525

7. Schatz, D. & Vardi, A. Extracellular vesicles — new players in cell–cell communication in aquatic environments. *Curr. Opin. Microbiol.* **43**, 148–154 (2018). https://doi.org/10.1016/j.mib.2018.01.014

8. Deatherage, B. L., Lara, J. C., Bergsbaken, T., Barrett, S. L. R., Lara, S., & Cookson, B. T. Biogenesis of bacterial membrane vesicles. *Mol. Microbiol.* **72**, 1395-1407 (2009). doi:10.1111/j.1365-2958.2009.06731.x

9. Toyofuku, M., Nomura, N. & Eberl, L. Types and origins of bacterial membrane vesicles. *Nat. Rev. Microbiol.* **17,** 13–24 (2019). https://doi.org/10.1038/s41579-018-0112-2

10. Gill, S., Catchpole, R. & Forterre, P. Extracellular membrane vesicles in the three domains of life and beyond. *FEMS Microbiol. Rev.* **43**, 273–303 (2019). https://doi.org/10.1093/femsre/fuy042

11. Choi, D. S., Kim, D. K., Choi, S. J., Lee, J., Choi, J. P., Rho, S., *et al.* Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. *Proteomics* **11**, 3424–3429 (2011). https://doi.org/10.1002/pmic.201000212

12. Beveridge, T. J., Makin, S. A., Kadurugamuwa, J. L. & Li, Z. Interactions between

biofilms and the environment. *FEMS Microbiol. Rev.* **20**, 291–303 (1997). https://doi.org/10.1111/j.1574-6976.1997.tb00315.x

13. Turnbull, L., Toyofuku, M., Hynen, A. *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **7,** 11220 (2016). https://doi.org/10.1038/ncomms11220

14. Toyofuku, M., Cárcamo-Oyarce, G., Yamamoto, T. *et al.* Prophage-triggered membrane vesicle formation through peptidoglycan damage in *Bacillus subtilis*. *Nat. Commun.* **8,** 481 (2017). https://doi.org/10.1038/s41467-017-00492-w

15. Martins, S. de T. & Alves, L. R. Extracellular Vesicles in Viral Infections: Two Sides of the Same Coin? *Front. Cell. Infect. Microbiol.* **10**, 737 (2020). https://doi.org/10.3389/fcimb.2020.593170

16. Deatherage, B. L. & Cookson, B. T. Membrane vesicle release in bacteria, eukaryotes, and archaea: A conserved yet underappreciated aspect of microbial life. *Infect. Immun.* **80**, 1948–1957 (2012). https://doi.org/10.1128/IAI.06014-11

17. Aschtgen, M. S., Lynch, J. B., Koch, E., Schwartzman, J., McFall-Ngai, M., & Ruby, E. Rotation of Vibrio fischeri flagella produces outer membrane vesicles that induce host development. *J. Bacteriol.*, **198**, 2156-2165 (2016). doi:10.1128/JB.00101-16

18. Orench-Rivera, N. & Kuehn, M. J. Environmentally controlled bacterial vesicle-mediated export. *Cell. Microbiol.* **18**, 1525–1536 (2016). https://doi.org/10.1111/cmi.12676

19. Ellen, A.F., Albers, SV., Huibers, W. *et al.* Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species reveals the presence of endosome sorting complex components. *Extremophiles* **13,** 67 (2009). https://doi.org/10.1007/s00792-008-0199-x

20. Brown, L., Wolf, J. M., Prados-Rosales, R. & Casadevall, A. Through the wall: Extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat. Rev. Microbiol.* **13**, 620–630 (2015).

21. Perez-Cruz, C., Carrion, O., Delgado, L., Martinez, G., Lopez-Iglesias, C., & Mercade, E. New Type of Outer Membrane Vesicle Produced by the Gram-Negative Bacterium *Shewanella vesiculosa* $M7^T$: implications for DNA Content. *Appl. Environ. Microbiol.*, **79**, 1874-81 (2013). doi:10.1128/AEM.03657-12

22. Remis, J. P., Wei, D., Gorur, A., Zemla, M., Haraga, J., Allen, S., *et al.* Bacterial social networks: Structure and composition of *Myxococcus xanthus* outer membrane vesicle chains. *Environ. Microbiol.* **16**, 598–610 (2014). https://doi.org/10.1111/1462-2920.12187

23. Hampton, C. M., Guerrero-Ferreira, R. C., Storms, R. E., Taylor, J. V., Yi, H., Gulig, P. A., & Wright, E. R. The opportunistic pathogen *Vibrio vulnificus* produces outer membrane vesicles in a spatially distinct manner related to capsular polysaccharide. *Front. Microbiol.* **8**, (2017). https://doi.org/10.3389/fmicb.2017.02177

24. Avila-Calderón, E. D., Ruiz-Palma, M. D. S., Aguilera-Arreola, M. G., Velázquez-Guadarrama, N., Ruiz, E. A., Gomez-Lunar, Z., *et al.* Outer Membrane Vesicles of Gram-Negative Bacteria: An Outlook on Biogenesis. *Front. Microbiol.* **12**, (2021). https://doi.org/10.3389/fmicb.2021.557902

25. Silhavy, T. J., Kahne, D. & Walker, S. The Bacterial Cell Envelope. *Cold Spring Harb. Perspect. Biol.* **2**, (2010). doi: 10.1101/cshperspect.a000414

26. Hughes, G.W., Hall, S.C.L., Laxton, C.S. *et al.* Evidence for phospholipid export from the bacterial inner membrane by the Mla ABC transport system. *Nat. Microbiol.* **4,** 1692–1705 (2019). https://doi.org/10.1038/s41564-019-0481-y

27. Bishop, D. G. & Work, E. An Extracellular Glycolipid Produced by *Escherichia coli* Grown under Lysine-Limiting Conditions. *Biochem. J* **96**, 567 (1965). https://doi.org/10.1042/bj0960567

28. Beveridge, T. J. Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. *J. Bacteriol.* **181**, (1999). https://doi.org/10.1128/JB.181.16.4725-4733.1999

29. Jan, A. T. Outer Membrane Vesicles (OMVs) of gram-negative bacteria: A perspective update. *Front. Microbiol.* **8**, (2017). https://doi.org/10.3389/fmicb.2017.01053

30. Koike, M., Iida, K. & Matsuo, T. Electron microscopic studies on mode of action of polymyxin. *J. Bacteriol.* **97**, 448–452 (1969). https://doi.org/10.1128/jb.97.1.448-452.1969

31. Sartorio, M. G., Pardue, E. J., Feldman, M. F. & Haurat, M. F. Annual Review of Microbiology Bacterial Outer Membrane Vesicles: From Discovery to Applications. *Annu. Rev. Microbiol*. **75**, 609-630 (2021). doi:10.1146/annurev-micro-052821

32. Moon, D.C., Choi, C.H., Lee, J.H. *et al. Acinetobacter baumannii* outer membrane protein a modulates the biogenesis of outer membrane vesicles. *J. Microbiol.* **50,** 155–160 (2012). https://doi.org/10.1007/s12275-012-1589-433.

33. Nevermann, J. *et al.* Identification of genes involved in biogenesis of Outer Membrane Vesicles (OMVs) in Salmonella enterica Serovar Typhi. *Front. Microbiol.* **10**, (2019). https://doi.org/10.3389/fmicb.2019.00104

34. Valeru, S. P., Shanan, S., Alossimi, H., Saeed, A., Sandström, G., & Abd, H. Lack of

outer membrane protein a enhances the release of outer membrane vesicles and survival of *Vibrio cholerae* and suppresses viability of *Acanthamoeba castellanii. Int. J. Microbiol.* **2014**, (2014). https://doi.org/10.1155/2014/610190

35. Schwechheimer, C., Kulp, A. & Kuehn, M.J. Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiol* **14,** 324 (2014). https://doi.org/10.1186/s12866-014-0324-1

36. Gerding, M. A., Ogata, Y., Pecora, N. D., Niki, H. & De Boer, P. A. J. The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in *E. coli*. *Mol. Microbiol.* **63**, 1008 (2007). https://doi.org/10.1111/j.1365-2958.2006.05571.x

37. Bernadac, A., Gavioli, M., Lazzaroni, J. C., Raina, S. & Lloubès, R. *Escherichia coli* tol-pal mutants form outer membrane vesicles. *J. Bacteriol.* **180**, 4872–4878 (1998). https://doi.org/10.1128/JB.180.18.4872-4878.1998

38. Wessel, A. K., Liew, J., Kwon, T., Marcotte, E. M. & Whiteley, M. Role of *Pseudomonas aeruginosa* Peptidoglycan-Associated Outer Membrane Proteins in Vesicle Formation. *J. bacteriol*, **195**, 213-219 (2013). doi:10.1128/JB.01253-12

39. Hayashi, J., Hamada, N. & Kuramitsu, H. K. The autolysin of *Porphyromonas gingivalis* is involved in outer membrane vesicle release. *FEMS Microbiol. Lett.* **216**, 217–222 (2002). https://doi.org/10.1111/j.1574-6968.2002.tb11438.x

40. Schwechheimer, C. & Kuehn, M. J. Synthetic Effect between Envelope Stress and Lack of Outer Membrane Vesicle Production in *Escherichia coli*. *J. bacteriol.* **195**, (2013). doi:10.1128/JB.02192-12

41. Kadurugamuwa, J. L. & Beveridge, T. J. Virulence Factors Are Released from Pseudomonas aeruginosa in Association with Membrane Vesicles during Normal Growth and Exposure to Gentamicin: a Novel Mechanism of Enzyme Secretion. *J. Bacteriol.* **177**, 3998–4008 (1995). https://doi.org/10.1128/jb.177.14.3998-4008.1995

42. Haurat, M. F., Aduse-Opoku, J., Rangarajan, M., Dorobantu, L., Gray, M. R., Curtis, M. A., & Feldman, M. F. Selective sorting of cargo proteins into bacterial membrane vesicles. *J. Biol. Chem.* **286**, 1269–1276 (2011). https://doi.org/10.1074/jbc.M110.185744

43. Elhenawy, W., Debelyy, M. O. & Feldman, M. F. Preferential Packing of Acidic Glycosidases and Proteases into Bacteroides Outer Membrane Vesicles. *MBio* **5**, (2014). https://doi.org/10.1128/mBio.00909-

44. Roier, S., Zingl, F., Cakar, F. *et al.* A novel mechanism for the biogenesis of outer

membrane vesicles in Gram-negative bacteria. *Nat Commun* **7,** 10515 (2016). https://doi.org/10.1038/ncomms10515

45. Lin, J., Cheng, J., Wang, Y. & Shen, X. The Pseudomonas Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore. *Front. Cell. Infect. Microbiol.* **8**, 230 (2018). https://doi.org/10.3389/fcimb.2018.00230

46. Schertzer, J. W. & Whiteley, M. A bilayer-couple model of bacterial outer membrane vesicle biogenesis. *MBio* **3**, 1–7 (2012). https://doi.org/10.1128/mBio.00297-11

47. Horspool, A.M., Schertzer, J.W. Reciprocal cross-species induction of outer membrane vesicle biogenesis via secreted factors. *Sci. Rep.* **8,** 9873 (2018). https://doi.org/10.1038/s41598-018-28042-4

48. Guerrero-Mandujano, A., Hernández-Cortez, C., Ibarra, J. A. & Castro-Escarpulli, G. The outer membrane vesicles: Secretion system type zero. *Traffic* **18**, 425–432 (2017). https://doi.org/10.1111/tra.12488

49. Mashburn-Warren, L. M. & Whiteley, M. Special delivery: vesicle trafficking in prokaryotes. *Mol. Microbiol.* **61**, 839–846 (2006). https://doi.org/10.1111/j.1365-2958.2006.05272.x

50. Domingues, S. & Nielsen, K. M. Membrane vesicles and horizontal gene transfer in prokaryotes. *Curr. Opin. Microbiol.* **38**, 16–21 (2017). https://doi.org/10.1016/j.mib.2017.03.012

51. Dorward, D. W., Garon, C. F. & Judd, R. C. Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae. *J. Bacteriol.* **171**, 2499–2505 (1989). https://doi.org/10.1128/jb.171.5.2499-2505.1989

52. Rumbo, C., Fernández-Moreira, E., Merino, M., Poza, M., Mendez, J. A., Soares *et al.* Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: A new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **55**, 3084–3090 (2011). https://doi.org/10.1128/AAC.00929-10

53. Kolling, G. L. & Matthews, K. R. Export of virulence genes and Shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **65**, 1843–1848 (1999). https://doi.org/10.1128/AEM.65.5.1843-1848.19

54. Yaron, S., Kolling, G. L., Simon, L. & Matthews, K. R. Vesicle-mediated transfer of virulence genes from Escherichia coli O157:H7 to other enteric bacteria. *Appl. Environ. Microbiol.* **66**, 4414–4420 (2000). https://doi.org/10.1128/AEM.66.10.4414-4420.2000

55. Soler, N. & Forterre, P. Vesiduction: the fourth way of HGT. *Environ. Microbiol.* **22**, 2457–2460 (2020). https://doi.org/10.1111/1462-2920.15056

56. Koeppen, K., Hampton, T. H., Jarek, M., Scharfe, M., Gerber, S. A., *et al.* A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. *PLoS Pathog.* **12**, 1–22 (2016). https://doi.org/10.1371/journal.ppat.1005672

57. Moriano-Gutierrez, S., Bongrand, C., Essock-Burns, T., Wu, L., McFall-Ngai, M. J., & Ruby, E. G. The noncoding small RNA SsrA is released by *Vibrio fischeri* and modulates critical host responses. *PLOS Biol.* **18**, e3000934 (2020). https://doi.org/10.1371/journal.pbio.3000934

58. Ahmadi Badi, S., Bruno, S. P., Moshiri, A., Tarashi, S., Siadat, S. D., & Masotti, A. Small RNAs in Outer Membrane Vesicles and Their Function in Host-Microbe Interactions. *Front. Microbiol.* **0**, 1209 (2020). https://doi.org/10.3389/fmicb.2020.01209

59. Chutkan, H., Macdonald, I., Manning, A. & Kuehn, M. J. Chapter 16 Quantitative and Qualitative Preparations of Bacterial Outer Membrane Vesicles. *Methods Mol. Biol.* **966**, 259-272 (2013). doi: 10.1007/978-1-62703-245-2_16.

60. Langlete, P., Krabberød, A. K., & Winther-Larsen, H. C. *Vibrio cholerae* Outer Membrane Vesicles Inhibit Bacteriophage Infection. *Front. Microbiol.* **10**, 2708 (2018). doi:10.1128/JB.00792-17

61. Manning, A. J. & Kuehn, M. J. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiol.* **11**, (2011). doi: 10.1186/1471-2180-11-258.

62. McBroom, A. J. & Kuehn, M. J. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol. Microbiol.* **63**, 545–558 (2007). https://doi.org/10.1111/j.1365-2958.2006.05522.x

63. MacDonald, I. A. & Kuehna, M. J. Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa. *J. Bacteriol.* **195**, 2971–2981 (2013). https://doi.org/10.1128/JB.02267-12

64. Evans, A. G., Davey, H. M., Cookson, A., Currinn, H., Cooke-Fox, G., Stanczyk, P. J., & Whitworth, D. E. Predatory activity of *Myxococcus xanthus* outer-membrane vesicles and properties of their hydrolase cargo. *Microbiology*, **158**, 2742-2752. doi:10.1099/mic.0.060343-0

65. Prados-Rosales, R., Weinrick, B. C., Piqué, D. G., Jacobs Jr, W. R., Casadevall, A., & Rodriguez, G. M. Role for *Mycobacterium tuberculosis* membrane vesicles in iron acquisition. *J. Bacteriol.* **196**, 1250–1256 (2014). https://doi.org/10.1128/JB.01090-13

66. Pena, R. T., Blasco, L., Ambroa, A., González-Pedrajo, B., Fernández-García, L., *et al.* Relationship between quorum sensing and secretion systems. *Front. Microbiol.* **10**, 1100 (2019). https://doi.org/10.3389/fmicb.2019.01100

67. Batista, J. H. *et al.* Interplay between two quorum sensing-regulated pathways, violacein biosynthesis and VacJ/Yrb, dictates outer membrane vesicle biogenesis in *Chromobacterium violaceum*. *Environ. Microbiol.* **22**, 2432–2442 (2020). doi: 10.1111/1462-2920.15033.

68. Brameyer, S., Plener, L., Müller, A., Klingl, A., Wanner, G., & Jung, K. Brameyer, S. Outer membrane vesicles facilitate trafficking of the hydrophobic signaling molecule CAI-1 between *Vibrio harveyi* cells. *J. Bacteriol.* **200**, (2018). https://doi.org/10.1128/JB.00740-17

69. Ellis, T. N., Leiman, S. A. & Kuehn, M. J. Naturally produced outer membrane vesicles from Pseudomonas aeruginosa elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect. Immun.* **78**, 3822–3831 (2010). doi: 10.1128/IAI.00433-10.

70. van der Pol, L., Stork, M. & van der Ley, P. Outer membrane vesicles as platform vaccine technology. *Biotechnol. J.* **10**, 1689–1706 (2015). https://doi.org/10.1002/biot.201400395

71. Holst, J., Oster, P., Arnold, R., Tatley, M., Næss, L., Aaberge, *et al.* Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): lessons from past programs and implications for the future. *Hum. Vaccin. Immunother.* **9**, 1241–1253 (2013). https://doi.org/10.4161/hv.24129

72. Huang, Y., Nieh, M. P., Chen, W. & Lei, Y. Outer membrane vesicles (OMVs) enabled bio-applications: A critical review. *Biotechnol. Bioeng.* **119**, 34–47 (2022). doi: 10.1002/bit.27965

73. Zhang, Y., Fang, Z., Li, R., Huang, X. & Liu, Q. Design of Outer Membrane Vesicles as Cancer Vaccines: A New Toolkit for Cancer Therapy. *Cancers (Basel).* **11**, (2019). https://doi.org/10.1002/bit.27965

74. Li, R. & Liu, Q. Engineered Bacterial Outer Membrane Vesicles as Multifunctional Delivery Platforms. *Front. Mater.* **7**, 202 (2020). https://doi.org/10.3389/fmats.2020.00202

75. Alves, N., Turner, K., Medintz, I. *et al.* Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. *Sci Rep* **6,** 24866 (2016). https://doi.org/10.1038/srep24866

76. Park, M., Sun, Q., Liu, F., DeLisa, M. P. & Chen, W. Positional Assembly of Enzymes on Bacterial Outer Membrane Vesicles for Cascade Reactions. *PLoS One* **9**, e97103 (2014). https://doi.org/10.1002/bit.27965

77. Whittenbury, R. & Wilkinson, J. F. Enrichment, Isolation and Some Properties of Methane-utilizing Bacteria. *J. Gen. Microbiol.* **61**, 205–218 (1970). https://doi.org/10.1099/00221287-61-2-205

78. Orata, F. D., Meier-Kolthoff, J. P., Sauvageau, D. & Stein, L. Y. Phylogenomic analysis of the gammaproteobacterial methanotrophs (order methylococcales) calls for the reclassification of members at the genus and species levels. *Front. Microbiol.* **9**, (2018). https://doi.org/10.3389/fmicb.2018.03162

79. Hanson, R. S. & Hanson, T. E. Methanotrophic bacteria. *Microbiol. Rev.* **60**, 439–71 (1996). doi: 10.1128/mr.60.2.439-471.1996.

80. Khmelenina V.N., Colin Murrell J., Smith T.J., Trotsenko Y.A. Physiology and Biochemistry of the Aerobic Methanotrophs. In: Rojo F. (eds) Aerobic Utilization of Hydrocarbons, Oils and Lipids. *Handbook of Hydrocarbon and Lipid Microbiology.* (2018) Springer, Cham. https://doi.org/10.1007/978-3-319-39782-5_4-1

81. Kalyuzhnaya M.G., Gomez O.A., Murrell J.C. The Methane-Oxidizing Bacteria (Methanotrophs). In: McGenity T. (eds) Taxonomy, Genomics and Ecophysiology of Hydrocarbon-Degrading Microbes. *Handbook of Hydrocarbon and Lipid Microbiology*. (2019) Springer, Cham. https://doi.org/10.1007/978-3-319-60053-6_10-1

82. Strous, M. & Jetten, M. S. M. Anaerobic oxidation of methane and ammonium. *Annu. Rev. Microbiol.* **58**, 99–117 (2004). https://doi.org/10.1146/annurev.micro.58.030603.123605

83. Ettwig, K., Butler, M., Le Paslier, D. *et al.* Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464,** 543–548 (2010). https://doi.org/10.1038/nature08883

84. He, Z., Cai, C., Wang, J. *et al.* A novel denitrifying methanotroph of the NC10 phylum and its microcolony. *Sci Rep* **6,** 32241 (2016). https://doi.org/10.1038/srep32241

85. IPCC (2014) Climate Change 2014: synthesis report. Contribution of working groups I. II and III to the Fifth report of the intergovernmental panel of climate change, Geneva

86. Kirschke, S., Bousquet, P., Ciais, P. *et al.* Three decades of global methane sources and sinks. *Nature Geosci* **6,** 813–823 (2013). https://doi.org/10.1038/ngeo1955

87. Schiffner, D., Kecinski, M. & Mohapatra, S. An updated look at petroleum well leaks, ineffective policies and the social cost of methane in Canada's largest oil-producing

province. *Climatic Change* **164,** 60 (2021). https://doi.org/10.1007/s10584-021-03044-w

88. Conrad, R. The global methane cycle: Recent advances in understanding the microbial processes involved. *Environ. Microbiol. Rep.* **1**, 285–292 (2009). https://doi.org/10.1111/j.1758-2229.2009.00038.x

89. Semrau, J. D., DiSpirito, A. A., Gu, W., & Yoon, S. Metals and Methanotrophy. *Applied and environmental microbiology*, **84**, (2018). https://doi.org/10.1128/AEM.02289-17

90. Stanley, S.H., Prior, S.D., Leak, D.J. *et al.* Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: Studies in batch and continuous cultures. *Biotechnol Lett* **5,** 487–492 (1983). https://doi.org/10.1007/BF00132233

91. Kenney, G. E. & Rosenzweig, A. C. Chalkophores. *Annu. Rev. Biochem.* **87**,645-676 (2018). doi:10.1146/annurev-biochem-062917-012300

92. DiSpirito, A. A., Zahn, J. A., Graham, D. W., Kim, H. J., Larive, C. K., *et al.* Copper-binding compounds from Methylosinus trichosporium OB3b. *J. Bacteriol.* **180**, 3606–3613 (1998). https://doi.org/10.1128/JB.180.14.3606-3613.1998

93. Trotsenko, Y. A. & Murrell, J. C. Metabolic Aspects of Aerobic Obligate Methanotrophy. *Adv. Appl. Microbiol.* **63**, 183–229 (2008). https://doi.org/10.1016/S0065-2164(07)00005-6

94. Burrows, K. J., Cornish, A., Scott, D. & Higgins, I. J. Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b. *J. Gen. Microbiol.* **130**, 3327–3333 (1984). https://doi.org/10.1099/00221287-130-12-3327

95. Semrau, J. D. & DiSpirito, A. A. Methanotrophy - Environmental, Industrial and Medical Applications. *Curr. Issues Mol. Biol.* **33**, 1–22 (2019). https://doi.org/10.21775/cimb.033.001

96. Semrau, J. D., Dispirito, A. A. & Yoon, S. Methanotrophs and copper. *FEMS Microbiol. Rev.* **34**, 496–531 (2010). https://doi.org/10.1111/j.1574-6976.2010.00212.x

97. Murrell, J. C., McDonald, I. R. & Gilbert, B. Regulation of expression of methane monooxygenases by copper ions. *Trends Microbiol.* **8**, 221–225 (2000). https://doi.org/10.1016/S0966-842X(00)01739-X

98. Eswayah, A.S., Smith, T.J., Scheinost, A.C. *et al.* Microbial transformations of selenite by methane-oxidizing bacteria. *Appl. Microbiol. Biotechnol.* **101,** 6713–6724 (2017). https://doi.org/10.1007/s00253-017-8380-8

99. Al Hasin, A., Gurman, S. J., Murphy, L. M., Perry, A., Smith, T. J., & Gardiner, P. H. Remediation of chromium (VI) by a methane-oxidizing bacterium. *Environ. Sci. Technol.* **44**, 400–405 (2010). https://doi.org/10.1021/es901723c

100. Vorobev, A., Jagadevan, S., Baral, B. S., DiSpirito, A. A., Freemeier, B. C., Bergman, B. H. Detoxification of mercury by methanobactin from *Methylosinus trichosporium* OB3b. *Appl. Environ. Microbiol.* **79**, 5918–5926 (2013). https://doi.org/10.1128/AEM.01673-13

101. Lu, X., Gu, W., Zhao, L., Farhan Ul Haque, M., DiSpirito, A. A., Semrau, J. D., & Gu, B. Methylmercury uptake and degradation by methanotrophs. *Sci. Adv.* **3**, (2017). DOI: 10.1126/sciadv.1700041

102. Kang-Yun, C.S., Liang, X., Dershwitz, P. *et al.* Evidence for methanobactin "Theft" and novel chalkophore production in methanotrophs: impact on methanotrophic-mediated methylmercury degradation. *ISME J* **16,** 211–220 (2022). https://doi.org/10.1038/s41396-021-01062-1

103. Brown, K., Tegoni, M., Prudêncio, M. *et al.* A novel type of catalytic copper cluster in nitrous oxide reductase. *Nat. Struct. Mol. Biol.* **7,** 191–195 (2000). https://doi.org/10.1038/73288.

104. Chang, J., Gu, W., Park, D. *et al.* Methanobactin from *Methylosinus trichosporium* OB3b inhibits N_2O reduction in denitrifiers. *ISME J* **12,** 2086–2089 (2018). https://doi.org/10.1038/s41396-017-0022-8

105. Summer, K. H., Lichtmannegger, J., Bandow, N., Choi, D. W., DiSpirito, A. A., & Michalke, B. The biogenic methanobactin is an effective chelator for copper in a rat model for Wilson disease. *J. Trace Elem. Med. Biol.* **25**, 36–41 (2011). https://doi.org/10.1016/j.jtemb.2010.12.002

106. Semrau, J. D., Dispirito, A. A., Obulisamy, P. K. & Kang-Yun, C. S. Methanobactin from methanotrophs: genetics, structure, function and potential applications. *FEMS Microbiol. Lett.* **367**, 45 (2020). https://doi.org/10.1093/femsle/fnaa045

107. Singh, I., Sagare, A. P., Coma, M., Perlmutter, D., Gelein, R., Bell, R. D., *et al.* Low levels of copper disrupt brain amyloid-β homeostasis by altering its production and clearance. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 14771–14776 (2013). https://doi.org/10.1073/pnas.1302212110

108. Gamez, P. & Caballero, A. B. Copper in Alzheimer's disease: Implications in amyloid aggregation and neurotoxicity. *AIP Adv.* **5**, (2015). https://doi.org/10.1063/1.4921314

109. Kalyuzhnaya, M. G., Puri, A. W. & Lidstrom, M. E. Metabolic engineering in methanotrophic bacteria. *Metab. Eng.* **29**, 142–152 (2015). https://doi.org/10.1016/j.ymben.2015.03.010

110. Strong, P. J., Xie, S. & Clarke, W. P. Methane as a Resource: Can the Methanotrophs Add Value? **49**, 4001–4018 (2015). https://doi.org/10.1021/es504242n

111. Pieja, A.J., Rostkowski, K.H. & Criddle, C.S. Distribution and Selection of Poly-3-Hydroxybutyrate Production Capacity in Methanotrophic Proteobacteria. *Microb. Ecol.* **62,** 564–573 (2011). https://doi.org/10.1007/s00248-011-9873-0

112. Khmelenina, V.N., Rozova, O.N., But, S.Y. *et al.* Biosynthesis of secondary metabolites in methanotrophs: Biochemical and genetic aspects (Review). *Appl Biochem Microbiol* **51,** 150–158 (2015). https://doi.org/10.1134/S0003683815020088

113. Kulp, A. & Kuehn, M. J. Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annu. Rev. Microbiol.* **64**, 163–184 (2010). https://doi.org/10.1146/annurev.micro.091208.073413

114. Strong, P. J., Kalyuzhnaya, M., Silverman, J. & Clarke, W. P. A methanotroph-based biorefinery: Potential scenarios for generating multiple products from a single fermentation. *Bioresour. Technol.* **215**, 314–323 (2016). https://doi.org/10.1016/j.biortech.2016.04.099

115. Müller, H., Hellgren, L. I., Olsen, E. & Skrede, A. Lipids rich in phosphatidylethanolamine from natural gas-utilizing bacteria reduce plasma cholesterol and classes of phospholipids: A comparison with soybean oil. *Lipids.* **39**, 833–841 (2004). https://doi.org/10.1007/s11745-004-1304-5

116. Li, Y. & Wang, G. Strategies of isoprenoids production in engineered bacteria. *J. Appl. Microbiol.* **121**, 932–940 (2016). https://doi.org/10.1111/jam.13237

117. Jeon, Y. C., Nguyen, A. D. & Lee, E. Y. Bioproduction of isoprenoids and other secondary metabolites using methanotrophic bacteria as an alternative microbial cell factory option: Current stage and future aspects. *Catalysts* **9**, (2019). https://doi.org/10.3390/catal9110883

118. Phulara, S. C., Chaturvedi, P. & Gupta, P. Isoprenoid-based biofuels: Homologous expression and heterologous expression in prokaryotes. *Appl. Environ. Microbiol.* **82**, 5730–5740 (2016). https://doi.org/10.1128/AEM.01192-16

119. Awasthi, D., Tang, Y. H., Amer, B., Baidoo, E. E., Gin, J., Chen, Y., *et al.* Adaptive evolution of *Methylotuvimicrobium alcaliphilum* to grow in the presence of rhamnolipids improves fatty acid and rhamnolipid production from CH_4. *J. Ind. Microbiol.*

Biotechnol., **49**, (2022). https://doi.org/10.1093/jimb/kuac002

120. Pastor, J. M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L. N., *et al.* Ectoines in cell stress protection: Uses and biotechnological production. *Biotechnol. Adv.* **28**, 782–801 (2010). https://doi.org/10.1016/j.biotechadv.2010.06.005

121. Cáceres, M., Gentina, J.C. & Aroca, G. Oxidation of methane by *Methylomicrobium album* and *Methylocystis* sp. in the presence of H_2S and NH_3 . *Biotechnol Lett* **36,** 69–74 (2014). https://doi.org/10.1007/s10529-013-1339-7

122. Wang, J., Mendelsohn, R., Dinar, A., Huang, J., Rozelle, S., & Zhang, L. Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria. *FEMS Microbiol. Lett.* **322**, 82–89 (2011). https://doi.org/10.1111/j.1574-6968.2011.02340.x

123. Sugden, S., Lazic, M., Sauvageau, D. & Stein, L. Y. Transcriptomic and Metabolomic Responses to Carbon and Nitrogen Sources in Methylomicrobium album BG8. *Appl. Environ. Microbiol.* **87**, (2021). https://doi.org/10.1128/AEM.00385-21

124. Kits, K. D., Kalyuzhnaya, M. G., Klotz, M. G., Jetten, M. S., Op den Camp, H. J., Vuilleumier, *et al.* Genome Sequence of the Obligate Gammaproteobacterial Methanotroph *Methylomicrobium album* Strain BG8. *Genome Announc.* **1**, (2013). https://doi.org/10.1128/genomeA.00170-13

125. Nguyen, A. D., Park, J. Y., Hwang, I. Y., Hamilton, R., Kalyuzhnaya, M. G., Kim, D., & Lee, E. Y. Genome-scale evaluation of core one-carbon metabolism in gammaproteobacterial methanotrophs grown on methane and methanol. *Metab. Eng.* **57**, 1–12 (2020). https://doi.org/10.1016/j.ymben.2019.10.004

126. Lazic, M., Sugden, S., Sauvageau, D. & Stein, L. Y. Metabolome profiles of the alphaproteobacterial methanotroph *Methylocystis* sp. Rockwell in response to carbon and nitrogen source. *FEMS Microbiol. Lett.* **368**, 1–8 (2021). https://doi.org/10.1093/femsle/fnaa219

127. Fu, Y., He, L., Reeve, J., Beck, D. A. C. & Lidstrom, M. E. Core metabolism shifts during growth on methanol versus methane in the methanotroph *Methylomicrobium buryatense* 5GB1. *MBio* **10**, (2019). https://doi.org/10.1128/mBio.00406-19

128. Schrader, J., Schilling, M., Holtmann, D., Sell, D., Villela Filho, M., Marx, A., & Vorholt, J. A. Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. *Trends Biotechnol.* **27**, 107–115 (2009). https://doi.org/10.1016/j.tibtech.2008.10.009

129. E. Bjorck, C., D. Dobson, P. & Pandhal, J. Biotechnological conversion of methane to

methanol: evaluation of progress and potential. *AIMS Bioeng.* **5**, 1–38 (2018). doi: 10.3934/bioeng.2018.1.1

130. Torres, S., Pandey, A. & Castro, G. R. Organic solvent adaptation of Gram positive bacteria: Applications and biotechnological potentials. *Biotechnol. Adv.* **29**, 442–452 (2011). https://doi.org/10.1016/j.biotechadv.2011.04.002

131. Baumgarten, T. *et al.* Membrane Vesicle Formation as a Multiple-Stress Response Mechanism Enhances *Pseudomonas putida* DOT-T1E Cell Surface Hydrophobicity and Biofilm Formation. *Appl. Environ. Microbiol.* **78**, 6217 (2012). https://doi.org/10.1128/AEM.01525-12

132. Wegner, C. E., Westermann, M., Steiniger, F., Gorniak, L., Budhraja, R., Adrian, L., & Küsel, K. Extracellular and Intracellular Lanthanide Accumulation in the Methylotrophic Beijerinckiaceae Bacterium RH AL1 . *Appl. Environ. Microbiol.* **87**, (2021). ttps://doi.org/10.1128/AEM.03144-20

133. Remis, J. P., Wei, D., Gorur, A., Zemla, M., Haraga, J., Allen, S., *et al.* Bacterial social networks: structure and composition of *Myxococcus xanthus* outer membrane vesicle chains. *Environ. Microbiol.* **16**, 598–610 (2014). https://doi.org/10.1111/1462-2920.12187

134. Hearn, E., Patel, D., Lepore, B. *et al.* Transmembrane passage of hydrophobic compounds through a protein channel wall. *Nature* **458,** 367–370 (2009). https://doi.org/10.1038/nature07678

135. Puri, A. W., Owen, S., Chu, F., Chavkin, T., Beck, D. A., Kalyuzhnaya, M. G., & Lidstrom, M. E. Genetic tools for the industrially promising methanotroph *Methylomicrobium buryatense*. *Appl. Environ. Microbiol.* **81**, 1775–1781 (2015). https://doi.org/10.1128/AEM.03795-14

136. Saidi-Mehrabad, A., Kits, D.K., Kim, J.J., Tamas, I., Schumann, P., Khadka, R., Strilets, T., Smirnova, A.V., Rijpstra, W.I.C., Damsté, J.S.S. and Dunfield, P.F. *Methylicorpusculum oleiharenae* gen. Nov., sp. nov., an aerobic methanotroph isolated from an oil sands tailings pond. *Int. J. Syst. Evol. Microbiol.* **70**, 2499–2508 (2020). https://doi.org/10.1099/ijsem.0.004064

137. Klimentová, J. & Stulík, J. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological Research* **170**, 1–9 (2015). https://doi.org/10.1016/j.micres.2014.09.006

138. Mocé-Llivina, L., Jofre, J. & Muniesa, M. Comparison of polyvinylidene fluoride and polyether sulfone membranes in filtering viral suspensions. *J. Virol. Methods* **109**, 99–101 (2003). https://doi.org/10.1016/S0166-0934(03)00046-6

139. Li, K., Wong, D. K., Hong, K. Y. & Raffai, R. L. Cushioned–Density Gradient Ultracentrifugation (C–DGUC): a Refined and High Performance Method for the Isolation, Characterization & Use of Exosomes. *Methods Mol. Biol.* **1740**, 69 (2018). doi: 10.1007/978-1-4939-7652-2_7.

140. Vorholt, J.A. Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. *Arch. Microbiol.* **178,** 239–249 (2002). https://doi.org/10.1007/s00203-002-0450-2

141. Noinaj, N., Guillier, M., Barnard, T. J. & Buchanan, S. K. TonB-dependent transporters: regulation, structure, and function. *Annu. Rev. Microbiol.* **64**, 43 (2010). https://doi.org/10.1146/annurev.micro.112408.134247

142. Chistoserdova, L., Kalyuzhnaya, M. G. & Lidstrom, M. E. The Expanding World of Methylotrophic Metabolism. **63**, 477-499 (2009) https://doi.org/10.1146/annurev.micro.091208.073600

143. Bauman, S. J. & Kuehn, M. J. Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response. *Microbes Infect.* **8**, 2400-2408 (2006). https://doi.org/10.1016/j.micinf.2006.05.001

144. Manabe, T., Kato, M., Ueno, T. & Kawasaki, K. Flagella proteins contribute to the production of outer membrane vesicles from *Escherichia coli* W3110. *Biochem. Biophys. Res. Commun.* **441**, 151–156 (2013). https://doi.org/10.1016/j.bbrc.2013.10.022

145. Blackburn, S. A., Shepherd, M. & Robinson, G. K. Reciprocal Packaging of the Main Structural Proteins of Type 1 Fimbriae and Flagella in the Outer Membrane Vesicles of "Wild Type" *Escherichia coli* Strains. *Front. Microbiol.* **12**, 209 (2021). https://doi.org/10.3389/fmicb.2021.557455

146. Spitz, O., Erenburg, I. N., Beer, T., Kanonenberg, K., Holland, I. B., & Schmitt, L. Type I Secretion Systems—One Mechanism for All? *Microbiol. Spectr.* **7**, (2019). https://doi.org/10.1128/microbiolspec.PSIB-0003-2018

147. Balsalobre, C. *et al.* Release of the type I secreted α-haemolysin via outer membrane vesicles from *Escherichia coli*. *Mol. Microbiol.* **59**, 99–112 (2006). https://doi.org/10.1111/j.1365-2958.2005.04938.x

148. Boardman, B. K., Meehan, B. M. & Satchell, K. J. F. Growth phase regulation of Vibrio cholerae RTX toxin export. *J. Bacteriol.* **189**, 1827–1835 (2007). https://doi.org/10.1128/JB.01766-06

149. Blum, M., Chang, H. Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., *et*

al. The InterPro protein families and domains database: 20 years on. *Nucleic Acids Res.* **49**, D344–D354 (2021). https://doi.org/10.1093/nar/gkaa977

150. Lee, E. Y., Bang, J. Y., Park, G. W., Choi, D. S., Kang, J. S., Kim, H. J., *et al.* Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli. Proteomics* **7**, 3143–3153 (2007). https://doi.org/10.1002/pmic.200700196

151. Baker, J. L., Chen, L., Rosenthal, J. A., Putnam, D. & DeLisa, M. P. Microbial biosynthesis of designer outer membrane vesicles. *Current Opinion in Biotechnology* **29**, 76–84 (2014). https://doi.org/10.1016/j.copbio.2014.02.018

152. Alves, N., Turner, K., Medintz, I. *et al.* Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. *Sci. Rep.* **6,** 24866 (2016). https://doi.org/10.1038/srep24866

153. Kang-Yun, C.S., Liang, X., Dershwitz, P. *et al.* Evidence for methanobactin "Theft" and novel chalkophore production in methanotrophs: impact on methanotrophic-mediated methylmercury degradation. *ISME J* **16,** 211–220 (2022). https://doi.org/10.1038/s41396-021-01062-1

154. Saha, M., Sarkar, S., Sarkar, B. *et al.* Microbial siderophores and their potential applications: a review. *Environ Sci Pollut Res* **23,** 3984–3999 (2016). https://doi.org/10.1007/s11356-015-4294-0

155. IPCC, 2021: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, In press, doi:10.1017/9781009157896

156. Lee, O. K., Hur, D. H., Nguyen, D. T. N. & Lee, E. Y. Metabolic engineering of methanotrophs and its application to production of chemicals and biofuels from methane. *Biofuels, Bioprod. Biorefining* **10**, 848–863 (2016). https://doi.org/10.1002/bbb.1678

157. Elena, S., Lenski, R. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* **4,** 457–469 (2003). https://doi.org/10.1038/nrg1088

158. Deatherage, B. L., Lara, J. C., Bergsbaken, T., Barrett, S. L. R., Lara, S., & Cookson, B. T. Biogenesis of bacterial membrane vesicles. *Mol. Microbiol.* **72**, 1395–1407 (2009). https://doi.org/10.1111/j.1365-2958.2009.06731.x

159. Schwarz, E. M. & Benzer, S. Calx, a Na-Ca exchanger gene of *Drosophila melanogaster*. *PNAS.* **94**, 10249–10254 (1997). https://doi.org/10.1073/pnas.94.19.10249

160. Deininger, K. N., Horikawa, A., Kitko, R. D., Tatsumi, R., Rosner, J. L., Wachi, M., & Slonczewski, J. L. A requirement of tolc and MDR efflux pumps for acid adaptation and GadAB induction in *Escherichia coli*. *PLoS One* **6**, (2011). https://doi.org/10.1371/journal.pone.0018960

161. Lee, J. J., Wu, Y. C., Kuo, C. J., Hsuan, S. L. & Chen, T. H. TolC is important for bacterial survival and oxidative stress response in *Salmonella enterica* serovar Choleraesuis in an acidic environment. *Vet. Microbiol.* **193**, 42–48 (2016). https://doi.org/10.1016/j.vetmic.2016.08.006

162. Moon, D. C., Choi, C. H., Lee, S. M., Lee, J. H., Kim, S. I., Kim, D. S., & Lee, J. C. Nuclear Translocation of *Acinetobacter baumannii* Transposase Induces DNA Methylation of CpG Regions in the Promoters of E-cadherin Gene. *PLoS One,* **7**, (2012) https://doi.org/10.1371/journal.pone.0038974

163. Joshi, M. C., Sharma, A., Kant, S., Birah, A., Gupta, G. P., Khan, S. R. An insecticidal GroEL protein with chitin binding activity from *Xenorhabdus nematophila*. *J. Biol. Chem.* **283**, 28287–28296 (2008). https://doi.org/10.1074/jbc.M804416200

164. Kim, S. W., Lee, J. S., Park, S. B., Lee, A. R., Jung, J. W., Chun, J. H. The Importance of Porins and β-Lactamase in Outer Membrane Vesicles on the Hydrolysis of β-Lactam Antibiotics. *Int. J. Mol. Sci.* **21**, 2822 (2020). https://doi.org/10.3390/ijms21082822

165. Sardesai, A. A., Genevaux, P., Schwager, F., Ang, D. & Georgopoulos, C. The OmpL porin does not modulate redox potential in the periplasmic space of *Escherichia coli. EMBO J.* **22**, 1461–1466 (2003). https://doi.org/10.1093/emboj/cdg152

166. Yoon, H. Bacterial outer membrane vesicles as a delivery system for virulence regulation. *J. Microbiol. Biotechnol.* **26**, 1343–1347 (2016). https://doi.org/10.4014/jmb.1604.04080

167. Kalyuzhnaya, M. G., Puri, A. W. & Lidstrom, M. E. Metabolic engineering in methanotrophic bacteria. *Metab. Eng.* **29**, 142–152 (2015). https://doi.org/10.1016/j.ymben.2015.03.010

168. Wieser, A., Storz, E., Liegl, G., Peter, A., Pritsch, M., Shock, J., *et al.* Efficient quantification and characterization of bacterial outer membrane derived nano-particles with flow cytometric analysis. *Int. J. Med. Microbiol.* **304**, 1032–1037 (2014). https://doi.org/10.1016/j.ijmm.2014.07.012

169. Hoekstra, D., van der Laan, J. W., de Leij, L., & Witholt, B. Release of Outer Membrane Fragments from Normally Growing *Escherichia coli*. *Algebr. Discret. Math.* **3**, 889–899 (1976). https://doi.org/10.1016/0005-2736(76)90058-4

170. Nagakubo, T., Nomura, N. & Toyofuku, M. Cracking Open Bacterial Membrane Vesicles. *Front. Microbiol.* **10**, (2020). https://doi.org/10.3389/fmicb.2019.03026

Appendices

Supplementary Table 1. Total LC-MS/MS proteome hits for *M. album* BG8 purified OMVs

		Replicate A			Replicate B			Replicate C		
Accession	Description	PSM	peptide	score	PSM	peptide	score	PSM	peptide	score
H8GMU5	Flagellin OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1728 PE=3 SV=1	399	23	1512.12	428	23	1726.29	168	21	711.56
H8GHU9	Prepilin-type N-terminal cleavage/methylation domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1116 PE=3 SV=1	80	3	290.36	78	4	275.33	35	5	128.6
H8GNA1	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0479 PE=4 SV=1	35	17	115.46	53	27	196.88	3	3	7.69
H8GIF1	Type 1 secretion C-terminal target domain protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3821 PE=4 SV=1	30	12	89.27	19	13	54.51	#N/A	#N/A	#N/A
H8GNF7	Flagellar hook protein FlgE OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1783 PE=3 SV=1	19	8	56.21	16	9	53.75	13	9	45.08
H8GGQ6	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2279 PE=4 SV=1	14	4	45.02	26	6	87.31	8	4	25.19
H8GIA0	ATP synthase epsilon chain OS=Methylomicrobium album BG8 OX=686340 GN=atpC PE=3 SV=1	13	1	30.07	10	1	22.98	6	1	14.16
H8GJI6	LPS-assembly protein LptD OS=Methylomicrobium album BG8 OX=686340 GN=lptD PE=3 SV=1	12	1	31.37	28	6	74.49	10	1	25.47
H8GJ49	Outer membrane cobalamin receptor protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3919 PE=3 SV=1	9	7	25.16	10	7	28.65	5	5	13.78
H8GGW0	TonB-dependent siderophore receptor OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2337 PE=3 SV=1	7	7	21.15	12	10	33.06	2	2	6.32
H8GN21	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3055 PE=3 SV=1*	6	1	13.3	5	1	11.19	1	1	2.28
H8GKK7	Ca2+-binding protein, RTX toxin OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0147 PE=4 SV=1	6	6	21.79	5	5	13.87	6	6	19.79
H8GNA4	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0482 PE=4 SV=1*	6	2	15.55	4	2	9.73	4	2	10.02
H8GNM8	*Hemolysin-coregulated protein (Uncharacterized) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3106 PE=4 SV=1*	5	4	13.52	4	3	11	2	2	6.38
H8GL31	*Cell division coordinator CpoB OS=Methylomicrobium album BG8 OX=686340 GN=cpoB PE=3 SV=1*	5	5	10.9	5	5	12.56	5	5	11.49
H8GKR0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1447 PE=4 SV=1	4	1	12.03	7	2	28.1	4	3	13.69
H8GIF2	*VCBS repeat-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3822 PE=4 SV=1*	4	2	11.5	4	2	11.41	#N/A	#N/A	#N/A
H8GFV3	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0880 PE=4 SV=1	4	4	11.06	16	13	52.42	2	2	6.04
H8GI88	*Probable cytosol aminopeptidase OS=Methylomicrobium album BG8 OX=686340 GN=pepA PE=3 SV=1*	4	3	10.23	1	1	2.89	2	2	5.5
H8GL29	*Tol-Pal system protein TolB OS=Methylomicrobium album BG8 OX=686340 GN=tolB PE=3 SV=1*	4	3	11.74	5	5	17.2	5	5	13.72
H8GM80	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1666 PE=4 SV=1*	4	1	8.24	4	1	8.25	2	1	4.67
H8GFV2	*Type I secretion outer membrane protein, TolC family OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0879 PE=3 SV=1*	4	4	9.69	4	4	11.76	1	1	2.82
H8GI44	*PQQ-dependent dehydrogenase, methanol/ethanol family OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2469 PE=3 SV=1*	4	4	9.42	3	3	8.65	2	2	4.14
H8GHK8	*Gly-zipper_OmpA domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3679 PE=4 SV=1*	3	2	10.1	4	2	13.96	#N/A	#N/A	#N/A

H8GMU7	*Flagellar hook-associated protein 2 OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1730 PE=3 SV=1*	3	3	7.33	5	5	13.8	7	7	19.33
H8GNF2	Histidine kinase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1778 PE=4 SV=1	3	1	6.33	2	1	4.7	#N/A	#N/A	#N/A
H8GK80	*Outer membrane protein/peptidoglycan-associated (Lipo)protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1419 PE=4 SV=1*	3	2	6.94	4	3	12.53	2	2	5.5
H8GNG4	*Flagellar hook-associated protein 3 OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1790 PE=4 SV=1*	2	2	5.57	2	2	4.76	3	3	9.31
H8GKM9	Thiol:disulfide interchange protein DsbA OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0170 PE=3 SV=1	2	1	4.21	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GH31	ABC-type antimicrobial peptide transport system, permease component OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3659 PE=3 SV=1	2	1	4.01	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GK49	Putative secreted protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1386 PE=4 SV=1	2	1	3.95	2	1	4.2	4	1	7.83
H8GJN2	C-terminal processing peptidase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2685 PE=3 SV=1	2	2	4.51	2	2	5.22	#N/A	#N/A	#N/A
H8GPH9	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3255 PE=4 SV=1	2	2	7.69	2	2	6.74	1	1	3.74
H8GIB8	Aspartate--tRNA(Asp/Asn) ligase OS=Methylomicrobium album BG8 OX=686340 GN=aspS PE=3 SV=1	2	1	3.86	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GPX1	*Citrate lyase beta subunit OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1986 PE=4 SV=1*	2	1	4.67	5	1	11.34	#N/A	#N/A	#N/A
H8GIF8	*General secretion pathway protein D OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3828 PE=3 SV=1*	2	2	6.09	4	3	9.79	#N/A	#N/A	#N/A
H8GGQ9	*DNA-binding transcriptional regulator NtrC OS=Methylomicrobium album BG8 OX=686340 GN=ntrC PE=4 SV=1*	2	1	4.85	2	1	4.99	4	1	10.54
H8GNG3	*Flagellar hook-associated protein 1 OS=Methylomicrobium album BG8 OX=686340 GN=flgK PE=3 SV=1*	2	2	6.58	3	3	9.63	3	3	9.22
H8GNG1	Flagellar P-ring protein OS=Methylomicrobium album BG8 OX=686340 GN=flgI PE=3 SV=1	2	2	5.92	1	1	2.93	#N/A	#N/A	#N/A
H8GK31	DUF4114 domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1368 PE=4 SV=1	2	2	5.73	2	2	6.12	#N/A	#N/A	#N/A
H8GKT0	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1467 PE=3 SV=1	2	1	4.46	#N/A	#N/A	#N/A	2	1	4.4
H8GJN0	Sulfite reductase, beta subunit (Hemoprotein) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2683 PE=4 SV=1	2	1	4.05	1	1	2.03	#N/A	#N/A	#N/A
H8GPQ2	Bacterioferritin (Cytochrome b1) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0675 PE=4 SV=1	2	1	4.32	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GPP0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0659 PE=4 SV=1	2	1	5	15	1	42.99	4	1	12.02
H8GGW4	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2345 PE=4 SV=1*	2	1	5	2	1	5.68	4	1	9.66
H8GQ90	Sigma54-dependent transcription regulator containing an AAA-type ATPase domain and a DNA-binding domain OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0714 PE=4 SV=1	2	2	4.31	#N/A	#N/A	#N/A	1	1	1.91
H8GRD0	*Transaldolase OS=Methylomicrobium album BG8 OX=686340 GN=tal PE=3 SV=1*	2	2	4.63	6	5	13.77	5	5	12.22
H8GG32	*Adenosylhomocysteinase OS=Methylomicrobium album BG8 OX=686340 GN=ahcY PE=3 SV=1*	2	1	4.13	6	2	8.27	2	1	1.99
H8GQ43	Elongation factor Tu OS=Methylomicrobium album BG8 OX=686340 GN=tuf PE=3 SV=1	1	1	2.65	1	1	2.49	#N/A	#N/A	#N/A
H8GRI4	UPF0102 protein Metal_3515 OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3515 PE=3 SV=1	1	1	2	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GN32	DNA/RNA helicase, superfamily II OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3066 PE=3 SV=1	1	1	2.34	#N/A	#N/A	#N/A	1	1	2.37
H8GGQ7	Glutamine synthetase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2280 PE=3 SV=1	1	1	2.91	#N/A	#N/A	#N/A	2	2	4.95
H8GM78	Glycine dehydrogenase (decarboxylating) OS=Methylomicrobium album BG8 OX=686340 GN=gcvP PE=3 SV=1	1	1	2.29	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GHU8	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1115 PE=4 SV=1	1	1	2.42	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A

H8GQQ0	ResIII domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3375 PE=4 SV=1	1	1	1.98	#N/A	#N/A	#N/A	1	1	2.06
H8GQY4	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0813 PE=4 SV=1	1	1	2.96	#N/A	#N/A	#N/A	2	1	4.47
H8GLB5	Signal peptide peptidase SppA, 36K type OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0251 PE=3 SV=1	1	1	2.38	#N/A	#N/A	#N/A	3	1	7.1
H8GIE5	Transposase_31 domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3815 PE=4 SV=1	1	1	2.15	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GIH5	Periplasmic serine endoprotease DegP-like OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1191 PE=3 SV=1	1	1	2.26	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GRD5	3-hexulose-6-phosphate synthase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3465 PE=3 SV=1	1	1	2.4	1	1	2.18	#N/A	#N/A	#N/A
H8GGE8	EAL domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3580 PE=4 SV=1	1	1	2.03	3	1	6.15	1	1	1.92
H8GPJ5	Thiol:disulfide interchange protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0612 PE=3 SV=1	1	1	1.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GKS9	Oxoglutarate dehydrogenase (succinyl-transferring) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1466 PE=3 SV=1	1	1	1.93	1	1	2.16	2	1	4.02
H8GKK3	Outer membrane protein assembly factor BamA OS=Methylomicrobium album BG8 OX=686340 GN=bamA PE=3 SV=1	1	1	2.74	2	2	4.94	#N/A	#N/A	#N/A
H8GPQ4	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0677 PE=4 SV=1	1	1	1.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GI22	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2444 PE=4 SV=1	1	1	2.45	1	1	2.18	#N/A	#N/A	#N/A
H8GNS0	3-hexulose-6-phosphate synthase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3152 PE=3 SV=1	1	1	1.96	1	1	3.52	#N/A	#N/A	#N/A
H8GH58	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1030 PE=4 SV=1	1	1	2.12	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GQI9	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2057 PE=4 SV=1	1	1	2.09	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GN99	Large extracellular alpha-helical protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0477 PE=3 SV=1	1	1	1.9	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GJT9	Outer membrane protein/peptidoglycan-associated (Lipo)protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3992 PE=4 SV=1	1	1	2.05	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GPL2	Adenine-specific DNA methylase containing a Zn-ribbon OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0629 PE=4 SV=1	1	1	1.92	#N/A	#N/A	#N/A	1	1	1.97
H8GL99	UDP-N-acetylglucosamine 1-carboxyvinyltransferase OS=Methylomicrobium album BG8 OX=686340 GN=murA PE=3 SV=1	1	1	1.9	#N/A	#N/A	#N/A	1	1	2.58
H8GJT1	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3984 PE=4 SV=1	1	1	2.5	1	1	2.74	1	1	2.29
H8GID1	Periplasmic serine endoprotease DegP-like OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3800 PE=3 SV=1	1	1	2.94	2	2	5.25	2	2	5.11
H8GKD1	Fe2+-dicitrate sensor, membrane component OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2720 PE=4 SV=1	1	1	2.95	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GGF2	60 kDa chaperonin OS=Methylomicrobium album BG8 OX=686340 GN=groL PE=3 SV=1	1	1	3.63	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GFX0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0897 PE=4 SV=1	1	1	2.14	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GPU4	Outer membrane protein/peptidoglycan-associated (Lipo)protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1958 PE=4 SV=1	1	1	4.15	1	1	4.41	1	1	4.07
H8GQH8	Chaperone protein DnaK OS=Methylomicrobium album BG8 OX=686340 GN=dnaK PE=2 SV=1	1	1	3.27	2	2	5.25	#N/A	#N/A	#N/A
H8GH44	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3672 PE=4 SV=1	1	1	2.15	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GMZ9	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3033 PE=4 SV=1	1	1	1.96	1	1	1.91	1	1	1.99
H8GGM0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0997 PE=4 SV=1	1	1	2.55	1	1	2.01	#N/A	#N/A	#N/A
H8GQ63	Peptide chain release factor 1 OS=Methylomicrobium album BG8 OX=686340 GN=prfA PE=3 SV=1	1	1	1.93	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A

H8GRI9	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_4000 PE=4 SV=1	1	1	2.44	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GLG1	Pyridoxine 5'-phosphate synthase OS=Methylomicrobium album BG8 OX=686340 GN=pdxJ PE=3 SV=1	1	1	1.92	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GPS4	Type IV pilus biogenesis/stability protein PilW OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1937 PE=4 SV=1	1	1	2.11	2	2	4.72	#N/A	#N/A	#N/A
H8GK04	*Cation diffusion facilitator family transporter OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0094 PE=4 SV=1*	1	1	2.32	#N/A	#N/A	#N/A	4	1	10.05
H8GPY3	*Outer membrane protein/peptidoglycan-associated (Lipo)protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1998 PE=4 SV=1*	1	1	1.96	4	3	12.67	3	3	7.83
H8GN43	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3078 PE=4 SV=1	1	1	2.09	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GHZ8	Outer membrane protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1171 PE=3 SV=1	1	1	2.02	#N/A	#N/A	#N/A	1	1	2.26
H8GQU1	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3423 PE=4 SV=1	1	1	2.28	1	1	2.32	#N/A	#N/A	#N/A
H8GP62	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1882 PE=4 SV=1	1	1	2.6	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GMC0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2963 PE=4 SV=1	1	1	1.98	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GKL3	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0154 PE=4 SV=1	1	1	1.9	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GP21	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0598 PE=4 SV=1	1	1	2.45	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GIS9	Rnf electron transport complex subunit B OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2552 PE=4 SV=1	1	1	2.08	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GMG9	ABC-type transport system involved in resistance to organic solvents, ATPase component OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3013 PE=4 SV=1	1	1	1.93	1	1	1.92	#N/A	#N/A	#N/A
H8GG77	Putative stress response protein, TerZ-and CABP1 OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2261 PE=4 SV=1	1	1	1.91	2	1	4.55	#N/A	#N/A	#N/A
H8GQ61	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3338 PE=4 SV=1*	1	1	2.05	7	6	17.76	1	1	1.99
H8GFX2	Bifunctional ligase/repressor BirA OS=Methylomicrobium album BG8 OX=686340 GN=birA PE=3 SV=1	1	1	2.1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GGS6	Cation/multidrug efflux pump OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2301 PE=4 SV=1	1	1	1.93	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GFZ0	Efflux transporter, outer membrane factor lipoprotein, NodT family OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0918 PE=3 SV=1	1	1	2.17	#N/A	#N/A	#N/A	1	1	2.3
H8GNC0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0498 PE=4 SV=1	1	1	2.27	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
H8GMW7	Response regulator with CheY-like receiver, AAA-type ATPase, and DNA-binding domains OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1750 PE=4 SV=1	#N/A	#N/A	#N/A	4	1	8.36	3	1	6.63
H8GI56	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2481 PE=4 SV=1*	#N/A	#N/A	#N/A	4	4	9.84	1	1	2.73
H8GK39	Phosphomannomutase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1376 PE=3 SV=1	#N/A	#N/A	#N/A	3	2	6.35	#N/A	#N/A	#N/A
H8GNN9	Site-specific DNA-methyltransferase (adenine-specific) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3119 PE=3 SV=1	#N/A	#N/A	#N/A	3	2	6.16	1	1	2.08
H8GNH6	Sulfate adenylyltransferase subunit 2 OS=Methylomicrobium album BG8 OX=686340 GN=cysD PE=3 SV=1	#N/A	#N/A	#N/A	3	1	6.57	#N/A	#N/A	#N/A
H8GJK1	Outer membrane receptor protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2650 PE=3 SV=1	#N/A	#N/A	#N/A	3	2	9.75	#N/A	#N/A	#N/A
H8GJK6	Bifunctional NAD(P)H-hydrate repair enzyme OS=Methylomicrobium album BG8 OX=686340 GN=nnrD PE=3 SV=1	#N/A	#N/A	#N/A	3	1	8.13	#N/A	#N/A	#N/A
H8GQF7	Putative metal-dependent hydrolase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2023 PE=4 SV=1	#N/A	#N/A	#N/A	3	3	6.54	2	2	4.99
H8GJ52	Cobyric acid synthase OS=Methylomicrobium album BG8 OX=686340 GN=cobQ PE=3 SV=1	#N/A	#N/A	#N/A	3	2	6.8	#N/A	#N/A	#N/A

H8GQ31	50S ribosomal protein L14 OS=Methylomicrobium album BG8 OX=686340 GN=rplN PE=3 SV=1	#N/A	#N/A	#N/A	2	1	5.69	#N/A	#N/A	#N/A
H8GHQ5	RecBCD enzyme subunit RecD OS=Methylomicrobium album BG8 OX=686340 GN=recD PE=3 SV=1	#N/A	#N/A	#N/A	2	1	3.91	#N/A	#N/A	#N/A
H8GIS0	ATP synthase, F1 beta subunit OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2543 PE=3 SV=1	#N/A	#N/A	#N/A	2	1	5.66	#N/A	#N/A	#N/A
H8GPM4	Putative phage-type endonuclease OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0643 PE=4 SV=1	#N/A	#N/A	#N/A	2	1	4.18	#N/A	#N/A	#N/A
H8GI94	Death-on-curing family protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3762 PE=4 SV=1	#N/A	#N/A	#N/A	2	1	4.69	3	1	6.02
H8GH59	*PAS domain S-box/diguanylate cyclase (GGDEF) domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1031 PE=4 SV=1*	#N/A	#N/A	#N/A	2	1	5.01	5	1	13.31
H8GGU1	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2318 PE=4 SV=1	#N/A	#N/A	#N/A	2	2	7.39	#N/A	#N/A	#N/A
H8GL30	Peptidoglycan-associated protein OS=Methylomicrobium album BG8 OX=686340 GN=pal PE=3 SV=1	#N/A	#N/A	#N/A	2	2	4.84	#N/A	#N/A	#N/A
H8GR09	Tetratricopeptide repeat protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0839 PE=4 SV=1	#N/A	#N/A	#N/A	2	1	6.38	1	1	2.49
H8GGQ1	Uncharacterized protein involved in outer membrane biogenesis OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2274 PE=4 SV=1	#N/A	#N/A	#N/A	2	2	3.85	1	1	2.38
H8GJ24	REJ domain protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3894 PE=4 SV=1	#N/A	#N/A	#N/A	2	2	5.51	#N/A	#N/A	#N/A
H8GN53	Rhs element Vgr protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3088 PE=3 SV=1	#N/A	#N/A	#N/A	2	1	4.2	1	1	1.93
H8GNY3	Putative TIM-barrel fold metal-dependent hydrolase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0555 PE=4 SV=1	#N/A	#N/A	#N/A	2	1	4.7	1	1	2.62
H8GNN3	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3113 PE=4 SV=1	#N/A	#N/A	#N/A	2	2	8.27	#N/A	#N/A	#N/A
H8GH93	Periplasmic serine endoprotease DegP-like OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1066 PE=3 SV=1	#N/A	#N/A	#N/A	2	1	6.1	#N/A	#N/A	#N/A
H8GR73	Carbamoyl-phosphate synthase large chain OS=Methylomicrobium album BG8 OX=686340 GN=carB PE=3 SV=1	#N/A	#N/A	#N/A	2	1	3.96	#N/A	#N/A	#N/A
H8GKF8	HAMP domain-containing protein,cache domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2751 PE=4 SV=1	#N/A	#N/A	#N/A	2	1	4.81	#N/A	#N/A	#N/A
H8GID7	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3807 PE=4 SV=1	#N/A	#N/A	#N/A	2	1	4.08	1	1	2.06
H8GMR8	Probable chorismate pyruvate-lyase OS=Methylomicrobium album BG8 OX=686340 GN=ubiC PE=3 SV=1	#N/A	#N/A	#N/A	2	1	5.51	#N/A	#N/A	#N/A
H8GIA3	ATP synthase subunit alpha OS=Methylomicrobium album BG8 OX=686340 GN=atpA PE=3 SV=1	#N/A	#N/A	#N/A	2	1	6.59	1	1	2.08
H8GI24	*Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2446 PE=4 SV=1*	#N/A	#N/A	#N/A	2	1	5.39	6	1	15.75
H8GMP6	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0432 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.92	#N/A	#N/A	#N/A
H8GLR3	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2908 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.95	#N/A	#N/A	#N/A
H8GQN6	Multifunctional CCA protein OS=Methylomicrobium album BG8 OX=686340 GN=cca PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.93	#N/A	#N/A	#N/A
H8GM23	ABC transporter, substrate binding protein, PQQ-dependent alcohol dehydrogenase system OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1605 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.94	#N/A	#N/A	#N/A
H8GMH4	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3018 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.82	#N/A	#N/A	#N/A
H8GQN1	Cell division protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3353 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.94	#N/A	#N/A	#N/A
H8GIT2	EAL domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2555 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.31	#N/A	#N/A	#N/A
H8GK84	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1423 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.32	#N/A	#N/A	#N/A

H8GJJ4	Transposase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0719 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.95	#N/A	#N/A	#N/A
H8GM09	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0347 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.53	#N/A	#N/A	#N/A
H8GII0	TonB family protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1196 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.06	#N/A	#N/A	#N/A
H8GPS3	Dual-specificity RNA methyltransferase RlmN OS=Methylomicrobium album BG8 OX=686340 GN=rlmN PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.21	1	1	2.41
H8GLK6	CRISPR-associated protein Cas7/Cse4/CasC, subtype I-E OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1591 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.43	#N/A	#N/A	#N/A
H8GMH5	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3019 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.37	#N/A	#N/A	#N/A
H8GMF5	Parvulin-like peptidyl-prolyl isomerase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2999 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	4.06	#N/A	#N/A	#N/A
H8GQE7	Cytochrome c domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0773 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.33	#N/A	#N/A	#N/A
H8GNP5	Type I restriction enzyme R Protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3125 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.97	2	1	3.94
H8GGA9	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3541 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.4	#N/A	#N/A	#N/A
H8GN39	MuF_C domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3073 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.02	#N/A	#N/A	#N/A
H8GIJ6	Peptide methionine sulfoxide reductase MsrA OS=Methylomicrobium album BG8 OX=686340 GN=msrA PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.92	#N/A	#N/A	#N/A
H8GLH8	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1560 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.97	#N/A	#N/A	#N/A
H8GPP4	Stress-induced acidophilic repeat motif-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0664 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.47	1	1	2.61
H8GM37	3-oxoacyl-[acyl-carrier-protein] reductase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1619 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.07	#N/A	#N/A	#N/A
H8GIR1	GBBH-like_N domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2534 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.55	#N/A	#N/A	#N/A
H8GHC9	Translation initiation factor IF-2 OS=Methylomicrobium album BG8 OX=686340 GN=infB PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.33	#N/A	#N/A	#N/A
H8GQG9	Dihydroxy-acid dehydratase OS=Methylomicrobium album BG8 OX=686340 GN=ilvD PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.01	#N/A	#N/A	#N/A
H8GPN4	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0653 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.31	#N/A	#N/A	#N/A
H8GNL3	RHS repeat-associated core domain protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1840 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.6	#N/A	#N/A	#N/A
H8GK23	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0113 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.29	#N/A	#N/A	#N/A
H8GGF3	EAL domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3585 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.46	#N/A	#N/A	#N/A
H8GQJ9	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2068 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.67	1	1	2.69
H8GIG2	Type II secretory pathway, component PulK OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3832 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.39	#N/A	#N/A	#N/A
H8GNE5	3-isopropylmalate dehydrogenase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0524 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.41	#N/A	#N/A	#N/A
H8GI84	Valine--tRNA ligase OS=Methylomicrobium album BG8 OX=686340 GN=valS PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.98	1	1	1.96
H8GNJ7	RND family efflux transporter, MFP subunit OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1823 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.6	#N/A	#N/A	#N/A
H8GH97	Glutamyl-tRNA reductase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1070 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2	#N/A	#N/A	#N/A
H8GHI0	Elongation factor Ts OS=Methylomicrobium album BG8 OX=686340 GN=tsf PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.93	#N/A	#N/A	#N/A
H8GQT5	ABC-type nitrate/sulfonate/bicarbonate transport system, ATPase component OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3417 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.43	#N/A	#N/A	#N/A

H8GNH8	FAD-dependent dehydrogenase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1804 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	3.15	#N/A	#N/A	#N/A
H8GJ39	Acyl-CoA dehydrogenase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3909 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.09	1	1	2.59
H8GL38	Aminodeoxychorismate synthase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2828 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.95	#N/A	#N/A	#N/A
H8GNM3	TPR_REGION domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1850 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.04	#N/A	#N/A	#N/A
H8GHM2	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3693 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.73	1	1	2.4
H8GLU9	Glyceraldehyde-3-phosphate dehydrogenase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0282 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.68	#N/A	#N/A	#N/A
H8GIU9	2-isopropylmalate synthase OS=Methylomicrobium album BG8 OX=686340 GN=leuA PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.06	#N/A	#N/A	#N/A
H8GNA2	OMP_b-brl domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0480 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	3.12	#N/A	#N/A	#N/A
H8GI30	Histidine kinase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2453 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2	#N/A	#N/A	#N/A
H8GKK2	Outer membrane protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0142 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.24	#N/A	#N/A	#N/A
H8GIW8	DNA (cytosine-5-)-methyltransferase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2593 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.39	1	1	2.04
H8GN52	Type VI secretion lipoprotein, VC_A0113 family OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3087 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.81	#N/A	#N/A	#N/A
H8GM59	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1641 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.3	1	1	1.96
H8GR31	Cation/multidrug efflux pump OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2103 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.17	#N/A	#N/A	#N/A
H8GQ38	50S ribosomal protein L2 OS=Methylomicrobium album BG8 OX=686340 GN=rplB PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.85	#N/A	#N/A	#N/A
H8GLD8	LysM domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0274 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.61	#N/A	#N/A	#N/A
H8GIL3	5-bromo-4-chloroindolyl phosphate hydrolysis protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1234 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.92	1	1	2
H8GLL2	DUF2384 domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1597 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.28	1	1	2.03
H8GGZ2	Probable septum site-determining protein MinC OS=Methylomicrobium album BG8 OX=686340 GN=minC PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.91	#N/A	#N/A	#N/A
H8GQD0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0756 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.98	#N/A	#N/A	#N/A
H8GR60	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2133 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	3.01	#N/A	#N/A	#N/A
H8GLS4	Protein HflK OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2920 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	3.03	#N/A	#N/A	#N/A
H8GNE6	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0525 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.34	#N/A	#N/A	#N/A
H8GG30	Response regulator with CheY-like receiver, AAA-type ATPase, and DNA-binding domains OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2204 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.01	#N/A	#N/A	#N/A
H8GKU5	Heavy metal efflux pump, cobalt-zinc-cadmium OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1482 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.07	#N/A	#N/A	#N/A
H8GLC0	Diguanylate cyclase (GGDEF) domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0256 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.15	1	1	2.56
H8GGM3	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1000 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.95	#N/A	#N/A	#N/A
H8GKL5	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0156 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.19	#N/A	#N/A	#N/A
H8GLG0	DNA repair protein RecO OS=Methylomicrobium album BG8 OX=686340 GN=recO PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.3	#N/A	#N/A	#N/A
H8GJW4	tRNA-dihydrouridine synthase B OS=Methylomicrobium album BG8 OX=686340 GN=dusB PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.45	#N/A	#N/A	#N/A

H8GGS7	RND family efflux transporter, MFP subunit OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2302 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.97	#N/A	#N/A	#N/A
H8GMS3	Phosphoglycerate kinase OS=Methylomicrobium album BG8 OX=686340 GN=pgk PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.32	#N/A	#N/A	#N/A
H8GMX5	Methyl-accepting chemotaxis protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1758 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.15	#N/A	#N/A	#N/A
H8GPS7	Outer membrane protein assembly factor BamB OS=Methylomicrobium album BG8 OX=686340 GN=bamB PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.4	#N/A	#N/A	#N/A
H8GJ35	Sigma-54 interacting regulator OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3905 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.61	#N/A	#N/A	#N/A
H8GI74	Arginine biosynthesis bifunctional protein ArgJ OS=Methylomicrobium album BG8 OX=686340 GN=argJ PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.94	#N/A	#N/A	#N/A
H8GKT3	Methylase involved in ubiquinone/menaquinone biosynthesis OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1470 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.1	#N/A	#N/A	#N/A
H8GQH4	Acyl-CoA synthetase (NDP forming) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2042 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.81	1	1	2.47
H8GQA6	56kDa selenium binding protein (SBP56) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0731 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.41	#N/A	#N/A	#N/A
H8GMI8	DNA polymerase I OS=Methylomicrobium album BG8 OX=686340 GN=polA PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.32	2	1	4.95
H8GLR8	Major facilitator superfamily permease OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2914 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.66	#N/A	#N/A	#N/A
H8GGS9	Non-ribosomal peptide synthase/amino acid adenylation enzyme OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2304 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.08	1	1	2.22
H8GP63	Single-stranded-DNA-specific exonuclease RecJ OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1883 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2	#N/A	#N/A	#N/A
H8GNR8	Metalloendopeptidase-like membrane protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3150 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.02	#N/A	#N/A	#N/A
H8GPC6	Site-specific DNA-methyltransferase (adenine-specific) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3199 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.99	#N/A	#N/A	#N/A
H8GJ90	Amino acid adenylation enzyme/thioester reductase family protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1280 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.44	#N/A	#N/A	#N/A
H8GK75	Sulfite reductase, alpha subunit (Flavoprotein) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1414 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.39	#N/A	#N/A	#N/A
H8GPU9	UPF0753 protein Metal_1963 OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1963 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.4	#N/A	#N/A	#N/A
H8GQA8	GMP synthase [glutamine-hydrolyzing] OS=Methylomicrobium album BG8 OX=686340 GN=guaA PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.96	#N/A	#N/A	#N/A
H8GIM0	Transcriptional regulator OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1242 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.22	#N/A	#N/A	#N/A
H8GRL6	Nucleotidyltransferase/DNA polymerase involved in DNA repair OS=Methylomicrobium album BG8 OX=686340 GN=Metal_4028 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.93	#N/A	#N/A	#N/A
H8GLG6	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1548 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.73	#N/A	#N/A	#N/A
H8GN57	Aspzincin_M35 domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3092 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.93	2	2	4.31
H8GPX4	Cytochrome c peroxidase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1989 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.54	1	1	2.51
H8GPG7	P-type Ca(2+) transporter OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3243 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.19	#N/A	#N/A	#N/A
H8GPJ4	Bacterial conjugation TrbI-like protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0611 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.14	#N/A	#N/A	#N/A
H8GL42	Site-specific DNA-methyltransferase (adenine-specific) OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2832 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.44	#N/A	#N/A	#N/A
H8GRC9	Fructose-bisphosphate aldolase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3459 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	4.19	1	1	3.58
H8GIB0	Heavy metal efflux pump, cobalt-zinc-cadmium OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3778 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	1.95	#N/A	#N/A	#N/A
H8GJJ3	Adenylate kinase OS=Methylomicrobium album BG8 OX=686340 GN=adk PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.26	#N/A	#N/A	#N/A
H8GL62	Surface lipoprotein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2856 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.57	#N/A	#N/A	#N/A

H8GR02	Homoserine O-succinyltransferase OS=Methylomicrobium album BG8 OX=686340 GN=metAS PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.09	#N/A	#N/A	#N/A
H8GMH3	Putative divalent heavy-metal cations transporter OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3017 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.36	#N/A	#N/A	#N/A
H8GMV1	Regulator of stationary/sporulation gene expression OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1734 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.04	#N/A	#N/A	#N/A
H8GRB9	Response regulator with CheY-like receiver domain and winged-helix DNA-binding domain OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3448 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.45	#N/A	#N/A	#N/A
H8GQX9	Phosphate regulon transcriptional regulatory protein PhoB OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0808 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.51	#N/A	#N/A	#N/A
H8GH68	Putative ATPase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1040 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.02	#N/A	#N/A	#N/A
H8GI40	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2464 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.99	#N/A	#N/A	#N/A
H8GHU1	Putative K(+)-stimulated pyrophosphate-energized sodium pump OS=Methylomicrobium album BG8 OX=686340 GN=hppA PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.33	#N/A	#N/A	#N/A
H8GKA7	YkuD domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2692 PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.45	#N/A	#N/A	#N/A
H8GQN2	Arginine--tRNA ligase OS=Methylomicrobium album BG8 OX=686340 GN=argS PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.62	#N/A	#N/A	#N/A
H8GL01	Phosphate-starvation-inducible E OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2790 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	2.01	#N/A	#N/A	#N/A
H8GH27	Putative periplasmic or secreted lipoprotein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3655 PE=4 SV=1	#N/A	#N/A	#N/A	1	1	1.91	#N/A	#N/A	#N/A
H8GJI7	Chaperone SurA OS=Methylomicrobium album BG8 OX=686340 GN=surA PE=3 SV=1	#N/A	#N/A	#N/A	1	1	2.24	1	1	2.61
H8GMA4	CBS domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2947 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	3	1	7.4
H8GIK8	Chemotaxis signal transduction protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1229 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	2	2	5.02
H8GFU5	Isoleucine--tRNA ligase OS=Methylomicrobium album BG8 OX=686340 GN=ileS PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	2	1	4.16
H8GNN0	Type VI secretion protein, VC_A0107 family OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3108 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	2	1	4.07
H8GHV3	Amidophosphoribosyltransferase OS=Methylomicrobium album BG8 OX=686340 GN=purF PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	2	1	4.34
H8GHC3	Putative ATPase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1096 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	2	2	4.07
H8GM13	Putative transcriptional regulator OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0352 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	2	1	4.66
H8GJR6	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3966 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.57
H8GQZ7	PAS domain S-box OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0827 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2
H8GGN3	DNA repair protein radc OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1010 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	3.11
H8GKR5	NADH:ubiquinone oxidoreductase, NADH-binding (51 kD) subunit OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1452 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.49
H8GG05	RNA polymerase-associated protein RapA OS=Methylomicrobium album BG8 OX=686340 GN=rapA PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.93
H8GQ29	50S ribosomal protein L5 OS=Methylomicrobium album BG8 OX=686340 GN=rplE PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.09
H8GPI5	Outer membrane protein assembly factor BamD OS=Methylomicrobium album BG8 OX=686340 GN=bamD PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.3
H8GKF5	Anti-sigma factor antagonist OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2748 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.9
H8GJN4	Putative transcriptional regulator with HTH domain OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3927 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.05
H8GI45	Methanol oxidation system protein MoxJ OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2470 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	3.95
H8GLY3	Putative PLP-dependent enzyme possibly involved in cell wall biogenesis OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0320 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.81

H8GG42	Putative metal-binding integral membrane protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2220 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.63
H8GIL5	Fibronectin type III domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1236 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.11
H8GKU9	RNA pyrophosphohydrolase OS=Methylomicrobium album BG8 OX=686340 GN=rppH PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.3
H8GL47	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2839 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.3
H8GMC6	RNA polymerase sigma factor RpoD OS=Methylomicrobium album BG8 OX=686340 GN=rpoD PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.29
H8GKK0	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase OS=Methylomicrobium album BG8 OX=686340 GN=lpxA PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.12
H8GJB7	Lipoprotein releasing system, transmembrane protein, LolC/E family OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1309 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.45
H8GKJ9	Ribonuclease HII OS=Methylomicrobium album BG8 OX=686340 GN=rnhB PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	3.27
H8GKB0	ATPase component of various ABC-type transport systems with duplicated ATPase domain OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2695 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.91
H8GKC8	Cation/multidrug efflux pump OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2717 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.43
H8GIR3	Ubiquinone biosynthesis accessory factor UbiJ OS=Methylomicrobium album BG8 OX=686340 GN=ubiJ PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.9
H8GH32	ABC-type antimicrobial peptide transport system, permease component OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3660 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.3
H8GNX4	Urease subunit gamma OS=Methylomicrobium album BG8 OX=686340 GN=ureA PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.9
H8GJT2	Phosphate-selective porin OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3985 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.98
H8GN71	DNA gyrase subunit A OS=Methylomicrobium album BG8 OX=686340 GN=gyrA PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.37
H8GLD5	DNA topoisomerase 1 OS=Methylomicrobium album BG8 OX=686340 GN=topA PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.12
H8GMH2	GDT1 family protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3016 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.34
H8GQP1	Lon protease OS=Methylomicrobium album BG8 OX=686340 GN=lon PE=2 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.6
H8GH42	2-polyprenyl-6-methoxyphenol 4-hydroxylase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3670 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.08
H8GGL0	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0987 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.17
H8GJK4	Diguanylate cyclase (GGDEF) domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2654 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.3
H8GPL4	DNA/RNA helicase, superfamily II OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0631 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.99
H8GPZ4	Serine--tRNA ligase OS=Methylomicrobium album BG8 OX=686340 GN=serS PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.42
H8GNY7	Transcriptional regulator OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0559 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2
H8GJ71	Mutator family transposase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0017 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.61
H8GJA1	Putative addiction module killer protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1291 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.06
H8GMA8	Magnesium transport protein CorA OS=Methylomicrobium album BG8 OX=686340 GN=corA PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.54
H8GP30	Putative helicase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0607 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.95
H8GPF9	Adenine-specific DNA methylase containing a Zn-ribbon OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3235 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.3
H8GQH0	3-hydroxyacyl-CoA dehydrogenase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2038 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.66

H8GIC1	Helix-turn-helix protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3789 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.5
H8GQC6	DNA translocase FtsK OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0751 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.42
H8GG08	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_0939 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.99
H8GR39	Uncharacterized protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2111 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.93
H8GLS3	Protein HflC OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2919 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.04
H8GGF8	Methane monooxygenase/ammonia monooxygenase, subunit B OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3591 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.36
H8GPD5	6-phosphogluconate dehydrogenase, decarboxylating OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3210 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.94
H8GN04	Diguanylate cyclase (GGDEF) domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3038 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.04
H8GI72	Putative tRNA(5-methylaminomethyl-2-thiouridylate) methyltransferase with PP-loop ATPase domain OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2497 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.1
H8GR81	Aspartokinase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2155 PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.39
H8GR90	Lipid-A-disaccharide synthase OS=Methylomicrobium album BG8 OX=686340 GN=lpxB PE=3 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.45
H8GNN2	Outer membrane autotransporter barrel domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3112 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.87
H8GHD8	Putative sugar kinase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2363 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.98
H8GIT3	ATP-dependent helicase HrpA OS=Methylomicrobium album BG8 OX=686340 GN=Metal_2557 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	1.98
H8GNT1	Non-specific serine/threonine protein kinase OS=Methylomicrobium album BG8 OX=686340 GN=Metal_3163 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.51
H8GIP6	EH_Signature domain-containing protein OS=Methylomicrobium album BG8 OX=686340 GN=Metal_1273 PE=4 SV=1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	1	1	2.59

Bolded proteins are referenced in Table 2
Italicized proteins are referenced in Table 3

Supplementary Table 2. Primers used for amplification of pQSD, promotors and gene inserts.

	Forward primer (5'→3')	Reverse primer (5'→3')
pQCHsfGFP	ATGAGCAAAGGAGAAGAA	TATTGCAAGGACGCGGAAC
P_{MMO}	aggcatgttccgcgtccttgcaataCCGGCCTGTTTGAGTGCT	gttcttctcctttgctcatTTCTACCTCCTAAAAATTTAACAATCCC
P_{GAP}	aggcatgttccgcgtccttgcaataGACACCGCAAAAAACCGC	gttcttctcctttgctcatTGAGTCTCTCCAGAGTGATGAG
TolC	ATGAGGTTGAATATCGGCACTG	TCAGTTTTCCAGCAGTTTGGA
GroL	ATGGCAGCAAAAGACGTATATTTT	CTACATCATGCCGCCCATAC
OmpL	ATGAAAAAATATCAAAGCAATGATCGC	TTAGAAGCTGATCGTAAAATCCGT