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Isolation of bacterial vesicles and characterisation of their genetic cargo

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Preface

This thesis is submitted in partial fulfilment of the requirements for the degree of *Philosophiae Doctor* at the University of Oslo. The research presented here is conducted under the supervision of Professor Hanne Cecilie Winther-Larsen and researcher Anders Kristian Krabberød, with the aid of Ole Andreas Løchen Økstad.

The thesis is a collection of two papers, presented in chronological order. The common theme to them is the isolation of bacterial vesicles and their genetic transfer capabilities. The papers are preceded by an introductory chapter that relates them together and provides background information and motivation for the work.

Acknowledgements

I give my heartfelt thanks to my main supervisor Hanne Cecilie Winther-Larsen for giving me the opportunity to embark on this research project, and other supervisors and colleagues that helped me along the way; Anders Kristian Krabberød, Andreas Løchen Økstad, Antje Hofgaard, Norbert Roos, Bernd Thiede, Bente Edvardsen and Julia Tandberg. I also want to praise my colleagues at the ZEB-building (zoofysiologi, ernæringsforskning og biokjemi) for being phenomenal, and my friends from the outside world that are still awaiting my re-appearance. A special thanks to Marthe Fjelldal, Sarah Finke and Michael Larsen for taking the time to proofread the text, and to my mother and father; thanks for the continued support and guidance. Og selvfølgelig; tusen takk til min fantastiske Ida, for all kjærlighet og tålmodighet underveis.

Petter Langlete
Oslo, November 2019

List of Papers

Paper I

Petter Langlete, Anders Kristian Krabberød, Hanne Cecilie Winther-Larsen. 'Vesicles from *Vibrio cholerae* contain AT-rich DNA and shorter mRNAs that do not correlate with their protein products'. *In press, Frontiers in Microbiology. Accepted Nov. 8. 2019.*

Paper II

Petter Langlete, Hanne Cecilie Winther-Larsen. 'Assessing methods for bulk bacterial vesicle isolation'. *Submitted for publication.*

Work published during the PhD period not part of the thesis

Paper III

Julia I. Tandberg, Leidy X. Lagos, **Petter Langlete**, Eva Berger, Anne-Lise Rishovd, Norbert Roos, Deepa Varkey, Ian T. Paulsen, Hanne C. Winther-Larsen. 'Comparative Analysis of Membrane Vesicles from Three *Piscirickettsia salmonis* Isolates Reveals Differences in Vesicle Characteristics'. In: *PloS one* **11** (2016) pp. e0165099 DOI: 10.1371/0165099

Paper IV

Sravani K Ramisetty, **Petter Langlete**, Rahmi Lale, Rita S Dias. 'In vitro studies of DNA condensation by bridging protein in a crowding environment'. IN: *International journal of biological macromolecules* **103:**845-853 (2017)

Contents

List of Abbreviations

AB Antibiotic

AMR Anti-microbial Resistance

CD86 Cluster of Differentiation 86

Ch1 Chromosome 1 (*Vibrio cholerae*)

Ch2 Chromosome 2 (*Vibrio cholerae*)

CTX Cholera Toxin

DAP Diaminopilemic Acid

DNA Deoxyribonucleic Acid

EV Extracellular Vesicle

HGT Horizontal Gene Transfer

H-NS Histone-like Nucleoid-structuring Protein

IL-12 Interleukin 12

IM Inner Membrane

KGF-2 Keratinocyte Growth Factor-2

LD_{50} Median Lethal Dose

LPS Lipopolysaccharide

MDA Multiple Displacement Amplification

MHC-II Major Histocompatibility Complex class II

miRNA Micro RNA

msRNA miRNA-sized small RNA

OIMV Outer-Inner Membrane vesicle

OM Outer Membrane

OMV Outer Membrane Vesicle

PBP Penicillin-binding Protein

PQS *Pseudomonas* quinolone signal

PRR Pattern Recognition Receptor

PCR Polymerase Chain Reaction

PG Peptidoglycan

RNA Ribonucleic Acid

rRNA ribosomal RNA

SIP Salt-induced Precipitation

ser. serovar

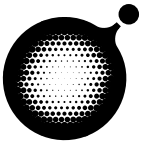
sRNA small RNA

TFF Tangential Flow Filtration

TNF-α Tumor Necrosis Factor Alpha

UC Ultracentrifugation

WT Wild Type



Chapter 1
Introduction

1.1 Bacterial Extracellular Vesicles (EVs)

All living organisms consist of cells, individually enclosed by biological membranes. Beyond providing a physiochemical barrier between the cell's interior and its environment, membranes are key players in many biological processes, such as cell–cell communication, cell-division, nutrient acquisition, metabolism, and structural support.[1] For pathogenic bacteria, the membrane is a key location for proteins involved in host–pathogen interactions and virulence.[2] While eukaryotic organisms such as fungi or humans have intracellular membrane structures (e.g. nuclei or mitochondria), the membranes of prokaryotes are –with few exceptions– limited to their enclosure.[3] The outer membranes (OM) of all microorganisms are thought to perform a process called vesiculation,[4] also referred to as "budding" or "blebbing", in which a portion of the membrane bulges out and forms a smaller, independent body (vesicle) that can be communicated to other cells of the same organism, or to other organisms altogether (Fig. 1.1).[5] The first description of this phenomenon in Gram-negative bacteria was published in 1965,[6] although interest in the field has been relatively low until recent decades.[7] As secretion of EVs has since been observed for every Gram-negative strain investigated, the ubiquity of the process has even inspired some authors to refer to vesiculation as a type zero secretion system (T0SS).[8,9]

While Gram-negative bacteria are enveloped by two membranes interspaced by a peptidoglycan (PG) layer, their vesicles were initially thought to contain only the outermost layer. This gave rise to the name *outer membrane vesicles* (OMVs),[11] despite the fact that one of the earliest publications on vesicles from *Escherichia coli* describes structures with up to three-layers.[12] Later findings have confirmed the existence of outer- and inner membrane vesicles (OIMVs),[13] indicating that the original nomenclature may be misleading. Therefore, bacterial extracellular membrane vesicles are in this thesis referred to simply as *extracellular vesicles* (EVs) generally, while OMV is used specifically for vesicles

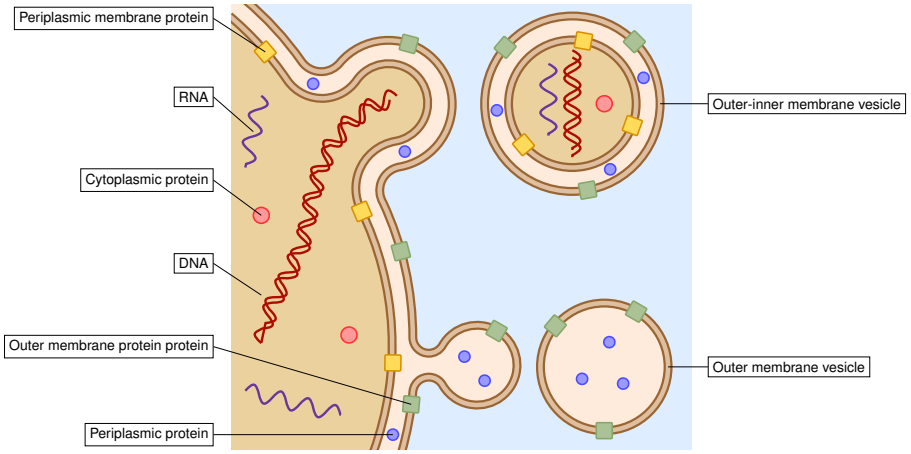


Figure 1.1: Intuitive model of vesicle budding by Gram-negative bacteria showing how the different biomolecules could be incorporated within vesicles of different membrane composition. Figure drawn in TikZ.[10]

containing *only* the outer membrane, and OIMV is used for vesicles containing *both* the outer- and inner membrane. Gram-positive bacteria have only one lipid membrane, but it is covered with a comparably thick cell wall, which initially led to doubts to whether it could secrete EVs.[4] However, later reports would confirm vesiculation for such strains as well.[14] As the original work at the core of the thesis is performed on Gram-negative bacteria, the information provided here concerns these organisms specifically, unless otherwise stated.

1.2 EV Biogenesis and Structure

EVs inherit their constituents from the mother cell, which means they are generally composed of the same material, but certain biomolecules, such as proteins and lipopolysaccharides (LPS) have been found to be enriched.[15] As mentioned, they can have one, two or even a higher number of enclosing membranes, giving rise to a series of questions regarding their biogenesis. For instance, what is the topology of a single- double- or triple membrane vesicle? The intuitive explanation for a single membrane vesicle is simply budding off of the outer membrane, but could in theory be an inner membrane (IM) vesicle lacking the OM, reminiscent of cytoplasmic membrane vesicles from Gram-positive bacteria, which can protrude out through holes in the outer cell wall.[16] A double membrane vesicle may be an OIMV, but could in theory consist of

extracellular matter engulfed by an endocytosis-like process (as observed in *Gemmata obscuriglobus*[17]), subsequently secreted from the bacteria within an outer membrane vesicle. Despite a high number of investigations on vesicles through the last decades, the specific mechanisms behind their biogenesis are still elusive, especially for OIMVs or higher-number membrane vesicles. This being noted, some progress has been made,[18] especially regarding the role of PG anchoring and stability.

1.2.1 OMV Biogenesis

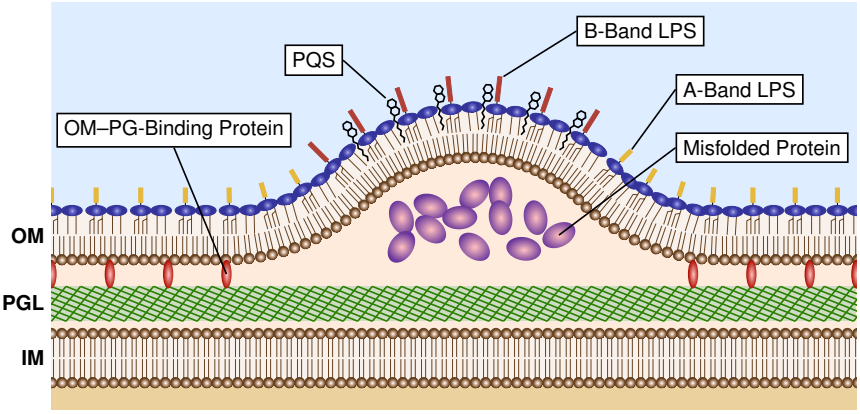


Figure 1.2: Proposed mechanisms behind OMV biogenesis. Accumulation of misfolded proteins or peptidoglycan (PG) fragments can induce turgor on the OM, and reduction of OM–PG binding allows it to bulge out from the PG. Additionally, the acquisition of *Pseudomonas* quinolone signal (PQS), B-band lipopolysaccharides (LPS) or phospholipids to the outer leaflet of the OM can induce curvature and promote vesiculation. Figure drawn in TikZ.[10]

Several mechanisms for OMV biogenesis have been proposed (Fig. 1.2), the first being based on disruption of the linkage between the OM and the PG layer. In Gram-negative bacteria, the OM is attached to a relatively rigid PG layer in the periplasmic space, keeping it in place and maintaining cell wall stability. The formation of an OMV therefore depends on the membranes' localised release from this layer, before it bulges out to detach completely. Attachment to the PG layer is facilitated by several structures, some of which are covalently bound. For instance, Braun's lipoprotein (Lpp) is one of the most abundant membrane proteins in Gram-negative bacteria,[19] and provides covalent crosslinking between the OM and diaminopilemic acid (DAP) embedded in the PG. Perhaps not surprisingly, hypervesiculation is observed in *E. coli nlpI* (*nlpI* encodes the

partly characterised OM-anchored lipoprotein nlpI) mutants, which have a lower level of Lpp–PG crosslinking.[20] Similar results are observed when knocking out the operon (*lppAB*) in *Salmonella typhimurium*.[21] The porin Outer membrane protein A (OmpA) also has periplasmic binding sites for DAP,[22] and thus helps stabilise cell wall integrity. *S. typhimurium* Δ*ompA* mutants also show increased vesiculation,[21] further supporting this hypothesis. There are some non-covalently binding agents that serve the same purpose, such as PG-associated lipoprotein (Pal), which is embedded in the OM and binds to the PG wall through the Tol multiprotein complex.[23] Knocking out either Pal, TolA or TolB all increase vesiculation in *S. typhimurium*.[21] An interesting observation in these data is that Δ*lppAB* mutants released smaller EVs than the WT, while Δ*pal*, Δ*tolA* and Δ*tolB* mutants released significantly larger EVs, indicating that their modes of OM–PG-binding are unique.

A second proposed mechanism is based on the idea that accumulation of misfolded proteins or PG fragments between the OM and PG can exert turgor pressure on the OM.[24] This pressure has been postulated to be able to create local volumes that can make OM bulge out and ultimately bud off.[25] One of the first studies that supported this hypothesis showed that a specific mimic of misfolded protein is packed into OMVs, indicating that they can be utilised by bacteria to dispose of cellular waste products.[26] The study also found that vesiculation is increased by the deletion of DegP, a periplasmic chaperone and protease that manages mis- and unfolded outer membrane proteins.[27] A later study on EVs from a hypervesiculating Δ*degP E. coli* mutant revealed accumulation of misfolded DegP substrate outer membrane porins.[28] This mechanism is somewhat related to the first, in that the accumulation of matter between the OM and PG necessarily exclude PG–OM binding bridges from a certain microdomain of the membrane as it bulges out. So the first mechanism does not necessitate chemical breakdown of PG–OM binding, but rather its dislocation.

A third mechanism is the accumulation of specific agents that induce membrane-curvature, such as B-band LPS, or *Pseudomonas* Quinolone Signal (PQS). Native vesiculation in *Pseudomonas aeruginosa* has been found to be correlated with production of B-band LPS,[29] and the resulting EVs are reported to contain this form.[30] B-band LPS is very different from A-band LPS, in that it has long O-side-chains stretching up to 40 nm from the membrane.[31] These side-chains are negatively charged,[32] and depending on the recruitment of counterions, they may repel one another to such an extent that the membrane curves. If enough B-band LPS is recruited to a localised domain of the OM, it may therefore bulge out and form a vesicle. Similarly, PQS is thought capable of

inducing curvature. EV production in *P. aeruginosa* has been found to be dependent on PQS regardless of the deletion of the PQS-receptor,[33] leading to the theory that PQS physically induces EV formation, rather than functioning as a signalling agent.[34] Additionally, PQS synthesis relies on molecular oxygen, and EV production is severely reduced under anaerobic conditions.[35] While PQS is specific for *P. aeruginosa*, three genes of the five-gene PQS synthesis operon *pqsABCDE* (*pqsA*, *pqsB* and *pqsD*) have homologues in e.g. *Vibrio cholerae*, and there may be analogous effects yet to be uncovered.

A fourth and similar proposed mechanism is also based on OM curvature, but is thought to be induced by the transport pattern of phospholipids to the outer leaflet of the OM. This hypothesis is based on the observation that deletion or repression of the VacJ/Yrb ABC transport system (retrograde transport of phospholipids from OM to IM) increased the EV production in both *V. cholerae* and *Haemophilus influenzae*, two distantly related Gram-negative bacteria.[36] The regulation of such transporters could therefore be key in order to modulate vesiculation rates in response to external factors.

The support for the proposed vesiculation mechanisms relies mostly on data from deletion mutants, while we see varying degrees of vesiculation between different WT organisms.[37] Furthermore, their vesiculation is also largely dependent on growth conditions.[38] This indicates that vesiculation is a complex process, that depends on a symphony of subcellular mechanisms and pathways, dictated by more subtle gene regulation.

1.2.2 OIMV Biogenesis

OIMVs are reminiscent of bacterial minicells,[39] albeit considerably smaller. The secretion of OIMVs is observed in an increasing number of bacterial species, such as *Shewanella vesiculosa*,[13] *Neisseria gonorrhoea*, *P. aeruginosa*, *Acinetobacter baumannii*,[40] *Ahrensia kielensis* and *Pseudoalteromonas marina*,[41] where they account for 0.23–98% of secreted EVs. The production of OIMVs is a more complex affair than what is the case with OMVs, as the inclusion of two or even three layers now have to be accounted for; OM, IM and potentially PG (Fig. 1.3). It may be that all three layers of the Gram-negative encapsulation is included in OIMVs, but based on observations in *P. aeruginosa*, some have suggested that it is rather holes in the PG layer that may allow for OIMV biogenesis. They pictured that these holes would allow the IM to protrude through the PG, into a bulge in the OM and thus construct a OIMV.[42] This theory was supported by the observation that the same EVs are enriched with PG hydrolases (autolysins),[30] but these could in theory be part of a ranged offensive mechanism, aimed at

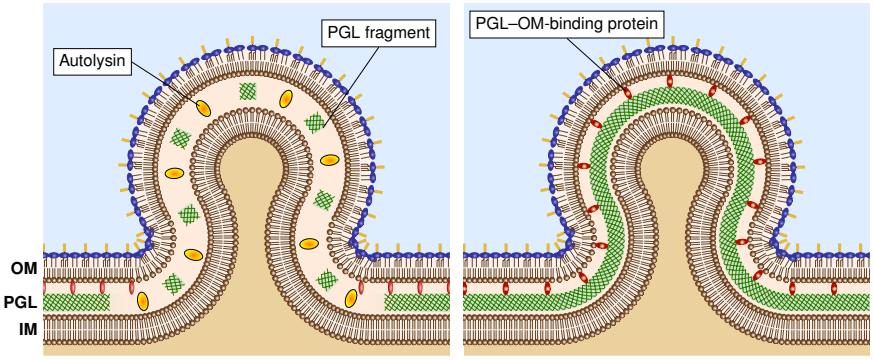


Figure 1.3: Proposed topologies of OIMV biogenesis. In addition to the induction of curvature as proposed for OMVs, some have proposed that holes in the PG layer may allow the IM to protrude out and be included in an OMV (left), while some have observed the inclusion of PG still attached to the OM (right). Figure drawn in TikZ.[10]

other strains.[43] Moreover, EVs from *Helicobacter pylori*, *P. aeruginosa* and *N. gonorrhoea* have been reported to carry and deliver PG into epithelial cells,[44] suggesting that such holes are not required for OIMV biogenesis, and that PG could rather be a common component. Indeed, some results indicate that OIMVs from *A. baumannii* can include a partial PG layer which occasionally was found detached from the IM but not from the OM, which argues against the need for holes in the PG layer to form OIMVs.[45] The same study found that the production of OIMVs appears to be distinct from OMV production, in that OMVs seem to stem from the distal ends, while OIMVs radiate from the septa of dividing bacteria. While native secretion of OIMVs has been recorded for several species, some have argued that they could rather be the result of non-native vesiculation induced by stress factors such as antimicrobial agents, or from explosive cell lysis.[46] This is based on the assumption that presence of chromosomal DNA in the vesicles should be indicative of cell death, and that antibiotics (ABs) have been shown to induce production of OIMVs in e.g. *Stenotrophomonas maltophilia*[47] and *P. aeruginosa*.[30] The presence of chromosomal DNA in EVs however, could be due to stochastically replicated DNA, or replication of mobile elements.[48] Additionally, even though OIMVs may also arise from cell lysis, results from *P. aeruginosa* indicate that both lysis-dependent and -independent mechanisms likely contribute to the OIMV population.[49]

1.2.3 Membrane Enrichment in EVs

When judging data from investigations on EVs, one particular topological concept is important to keep in mind; the inherent enrichment of membranes in smaller vesicular bodies. Although EVs contain a quantity of intravesicular cargo, such as cytoplasmic and periplasmic proteins, DNA and RNA, the increased surface-to-volume ratio compared to the progenitor bacteria implies that membrane components should be enriched in EV fractions (Fig. 1.4). The implication of this is that when bacteria secrete EVs, they are largely communicating a modified portion of their membranes to their surroundings, including any associated membrane proteins such as antigens and virulence factors. This has in fact been a frequent conclusion of investigations on pathogen-secreted EVs; that they are abundant in membrane-associated virulence factors and adhesion-related proteins.[42,50,51] While this may be true, it is a necessary consequence of membrane enrichment due to the differences in size and shape between bacteria and vesicles.

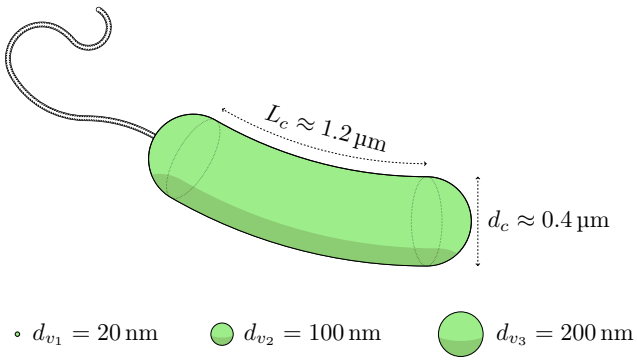


Figure 1.4: Schematic of simplified *V. cholerae* cell shape and vesicle sizes of interest. L_c is the length of the bent cylinder, while d_c is the diameter of cylinder and the the hemispherical ends. Figure drawn in TikZ.[10]

1.3 The Diversity of EV Functions

The secretion of EVs seems to be common for virtually all Gram-negative bacteria, and many have tried to elucidate what their purposes may be.[52] EV production is not free; the secretion of a vesicle can involve a significant loss of

biomass, depending on the size of the cell in question. For instance, the secretion of a single 200 nm vesicle would lead to a 2.3% loss of biomass for a *V. cholerae* bacterium,[†] while only $3 \cdot 10^{-5}$% for a human Purkinje cell.[53,54] The sacrifice of biomass required for vesicle secretion (especially for bacteria) has led to the hypothesis that EVs may provide some advantage to the organism in question, rather than being a purely stochastic process. A general, predictable function would be disposal of intracellular hydrophilic waste, but decades of research have uncovered a more complex picture, with a whole variety of highly specialised applications. As this section will describe, EVs provide "decoy" epitopes for the immune system or phages to target, can deliver toxins or cytolysins to disrupt or kill host tissues, and prime epithelia for adhesion, among other functions.

1.3.1 Host–pathogen interactions

Some of the first papers that identify EVs also note that they have the capability to modulate host immune responses, as they possess antigenic characteristics similar to those of their parent bacteria.[55,56] While the increased immune response in a host could be bad news for a pathogen, there are several ways this could be of benefit, e.g. as coughing or itching may enable the spread of infectious agents.[57,58]

Being enriched with membranes, EVs from Gram-negative bacteria are also abundant in LPS, which is a relatively toxic compound. While lower vertebrates such as frogs and fish have a comparably high tolerance for LPS, higher animals such as mammals are very susceptible to lethal shock.[59] For instance, the LD_{50} of *E. coli* LPS in mice by intraperitoneal injection is only 1.6–25.6 $\mathrm{mg\,kg^{-1}}$ body weight, depending on age.[60] Therefore, it is not surprising that it was originally coined *endotoxin*,[61] which in contrast to *exotoxin* points to its toxic activity while still being part of the bacterium. Being rich in LPS, as well as parallelly enriched virulence factors, it would be remarkable if these EVs *did not* induce potent immune responses in higher animal hosts. This renders some EV-research somewhat inconclusive; an important but often forgotten control is to investigate the effect of lysed EVs compared to intact EVs. If the same effect was observed in a host or tissue exposed to the resulting lysate, one could not conclude much about the effects of the EV superstructure, other than that it exerts the same effects as its constituents. Many experiments subject host organisms to EVs alone, or as a vaccination trial before a pathogen challenge, using buffer as a control.[62,63] These experiments have uncovered a range of

[†] Assuming a cell with length $L = 1.6\,\mu\mathrm{m}$ and width $2r = 0.4\,\mu\mathrm{m}$, shaped like a cylinder with rounded ends, with volume $V = 4/3\pi r^3 + \pi r^2(L - 2r)$

host-responses, most of which indicate the immunological awareness of a foreign pathogen. Examples are stimulation of antigen-presenting cells (APCs), increased surface expression of Major Histocompatibility Complex class II (MHC-II) and Cluster of Differentiation 86 (CD86), increased production of cytokines such as Tumor Necrosis Factor Alpha (TNF-α) and Interleukin 12 (IL-12), as well as the induction of pattern recognition receptor (PRR) responses.[62,63,64]

However, some studies have included controls for lysed EVs or LPS, and they have demonstrated that certain effects of EVs are dependent not only on their components, but also on their superstructure. For example, complete EVs from *P. aeruginosa* elicit a much greater inflammatory response than their isolated LPS,[65] indicating that the topology and combination of epitopes on the EV surfaces are decisive for their function. Similarly, EVs from *Staphylococcus aureus* induce host cell death in a dose dependent manner, but this is not observed with lysed EVs, suggesting that some of the diverse functions of vesicles are intimately associated with membrane compartmentalisation.[66]

The host modulation by EVs is not at all limited to induced expression and production of commonly recognised immune system components. For instance, it has been found that EVs from serum-resistant strains of *N. gonorrhoea* have a different protein composition than those from susceptible strains,[67] and that the former was significantly better than the latter at protecting serum-susceptible strains from serum killing when added exogenously.[68] The conclusion from this investigation was that the unique protein composition of EVs from serum-resistant strains make them recognise, bind, and remove cell-targeted bactericidal factors, and may be important for serum-resistance. It is also known that EVs from *Treponema denticola* are capable of disrupting and penetrating human epithelial monolayers, and may thus interfere with tight junctions,[69] perhaps arming the bacteria with a significant offensive mechanism towards the host. Similarly, EVs from *Porphyromonas gingivalis* cause gingipain-mediated detachment of oral epithelial cells,[70] and could in this way induce damage, immunosupression, and increase the nutrient content of its surroundings.

1.3.1.1 Infection & Biofilm

A curious application of vesicles for modulation of biofilm through the infection cycle is observed in *Xylella fastidiosa*, a xylem-colonizing plant pathogen. It was initially found that *X. fastidiosa* significantly increases its vesiculation inside its plant host, but not within an insect vector. It was concluded that the secreted EVs block potential adhesion sites in the xylem, so that bacteria do not accumulate at one place, clogging the water flow.[71] This allows the bacterial

infection to migrate further through the plant, in turn giving more potential sites for insect vectors to pick it up. When that happens, the pathogen downregulates EV production in order to adhere firmly to the insect until transferred to the next plant. It was discovered that vesiculation was reduced in tandem with the synthesis of a diffusing signal factor (DSF), and that exogenous addition of DSF would reduce vesiculation, which led to a potential solution to the problem: in *X. fastidiosa*, the gene *rpfF* is responsible for DSF synthesis, and when this gene was expressed in grape plants, the growth and mobility of the bacteria was reduced considerably during an infection. This is one of the first examples where EV-based research has provided specific solutions to a problem other than functioning as vaccines, as shall be visited further in Section 1.4.1. On the other hand, the addition of EVs has also been found to induce the formation of biofilm; when *P. aeruginosa* is subjected to mucosal fluid, it secretes EVs that are able to prime host corneal surfaces to increase bacterial adhesion by 4-fold.[72] This mechanism could thereby be highly advantageous for a pathogen seeking to establish a biofilm upon the cornea. Similarly, the exogenous addition of EVs from *Streptococcus mutans* to non-biofilm-forming mutants increases oral biofilm formation.[73]

1.3.1.2 Immune System Inhibition

In contrast to the ample results that show elevated immune responses when subjected to EVs, some studies have found that the action of specific EV components can actually lower the response elicited by bacterial antigens. For instance, it has been found that EVs from *P. aeruginosa* can deliver sRNA into human airway cells, and one such sRNA (a fragment of a methionine tRNA that is abundant in these EVs) can reduce LPS-, and EV-induced interleukin 8 (IL-8) secretion.[74] The same sRNA also attenuates EV-induced keratinocyte-derived chemokine secretion and neutrophil infiltration in mice lungs, making it an efficient weapon for the bacteria to obstruct host defences. The modulation of host immune-mechanisms by EVs is markedly variable, even between strains of the same bacterium. While EVs from *P. aeruginosa* generally induce a potent immune response, EVs from multi-drug resistant strains are actually capable of up-regulating certain anti-inflammatory cytokines.[75] The delivery of immuno-modulating sRNAs demonstrates the potential for the genetic cargo of EVs, which will be further described in Section 1.5.

1.3.1.3 Entry Into Host Cells

When investigating membrane vesicles released from macrophages infected with *Mycobacterium tuberculosis*, two very distinct populations of vesicles were found; one was carrying the host cell markers of exosomes, but the other carried *M. tuberculosis* components (lipoglycans, lipoproteins).[76] The authors proposed that *M. tuberculosis* could in fact shed EVs from within macrophages, and that they could subsequently be absorbed by surrounding cells. They concluded that EVs are the primary mechanism for *M. tuberculosis* to secrete and deliver lipoglycans and lipoproteins in order to impair macrophage functions, as well as regulate immune responses in uninfected cells.

These results demonstrate a phenomenon that has become common knowledge in EV science, namely that they have the ability to enter and exit host cells, making their range and utility even broader.[77] In fact, entry of EVs into host cells seems to be quite a ubiquitous and rapid mechanism, as it has been observed in e.g. *M. tuberculosis*,[76] *P. aeruginosa*,[78] *H. pylori*,[79] and *E. coli*,[80] the last of which was internalised in HeLa cells within 15 minutes. The mechanisms behind this effective entry are still not fully elucidated, but several have been proposed. In order to understand this process, we need to underline the important distinction between two main modes of entry. The first is membrane fusion, in which the outer membrane of the EV fuses with the plasma membrane of a host cell, and the contents of the vesicle are deposited directly into the cytoplasm.[78] The second is "intact" entry, where the complete or modified membrane structure of the EV enters the cytoplasm, either naked or within a host-membrane-bound cytoplasmic body (Fig. 1.5).[81]

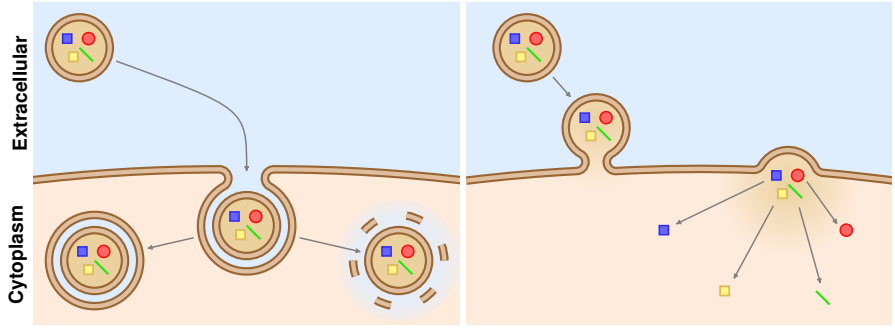


Figure 1.5: Two modes of EV entry into host cells, one being intact entry (left), the other membrane fusion (right). Intact fusion allows the co-localised transport of EV cargo, while membrane fusion results in EV cargo being deposited directly in the cytoplasm. Figure drawn in TikZ.[10]

Membrane Fusion

It is not intuitive how the membranes of bacteria and eukaryotes can fuse effectively because of their different architectures, but this has indeed been observed for EVs from *P. aeruginosa*,[78] *Aggregatibacter actinomycetemcomitans*[82] and *Legionella pneumophila*.[83] It was for the latter observed at both 4 °C and 37 °C, so the authors suggested that this may be a somewhat spontaneous process that can take place independently of energetically demanding mechanisms.[83] This being said, studies on the fusion of EVs with eukaryotic membranes are somewhat lacking, which could be due to there not being a universal mode of entry, but rather species-specific variations. Molecular simulations have indeed shown that spontaneous membrane fusion could happen in theory, but depends strictly on the thickness of the LPS layer, as longer O-antigen chains may keep the lipid bilayers too separated to fuse.[84] This means that Gram-negative bacteria with shorter antigen chains can be more probable candidates for research on this effect. One group did indeed investigate the effect of O-antigen deletion (thinner saccharide layer) on the entry potential of *E. coli* EVs, but concluded that membrane fusion was not a mode of entry in their system, and that the presence of O-antigen actually increased the rate of entry of the vesicles, by clathrin-independent endocytosis (intact entrance).[85]

Intact Entrance

Most studies on EV entry into host cells have assumed that they are engulfed and internalised in a membrane-bound compartment, which can be mediated by several mechanisms. Some have proposed that it could in part rely on macropinocytosis, i.e. actin-driven protrusions in host cells.[78] The authors behind this hypothesis found that the entry of *P. aeruginosa* EVs into airway epithelial cells is reduced by inhibition of actin polymerisation, but this may be a general inhibition of uptake, and not strictly an EV-specific result. Others have recognised that the fusion of EVs to host cells is dependent on lipid rafts,[81,86] areas of the membrane that are enriched in sphingolipids and cholesterol.[87] It is already thought that these rafts induce curvature that facilitates endocytosis and viral entry,[81] and considering the EVs' virus-like size and character, it is possible that they may enter in a similar way.[52] Entry of EVs from *H. pylori* has indeed been found to be dependent on lipid rafts, and was reduced by sequestration of cholesterol from the host membrane.[44] A similar conclusion was reached when *S. aureus* EVs were observed localised to cholesterol-rich membrane microdomains.[66] Just as membrane fusion is dependent on surface characteristics of the EVs, it was reported that the specific entry mechanism of *H. pylori*

EVs depends on their size, with smaller EVs entering by caveolin-mediated endocytosis, while larger EVs enter by macropinocytosis and endocytosis.[79]

The uptake of EVs is likely dependent on interactions between specific epitopes on the host and the EVs in question. For instance, the effective internalisation in eukaryotic cells of EVs from *E. coli*[86] and *P. aeruginosa* is not only very temperature-dependent (in contrast to membrane fusion), but the latter also depends on the bacterial secretion of aminopeptidase PaAP,[88] which associates with the EV surface. This could point to the interaction being dictated by specific, energetic processes. Interestingly, EVs from more virulent strains expressed more PaAP, while a PaAP knockout had 40% reduced association with cells, and secretion of PaAP was significantly higher for strains carrying a β-lactamase-resistant vector than others. This means that there are several distinct virulence effects that are simultaneously propagated by EVs in this infection model. Not only are the EVs internalised, but may also carry antibiotic resistance genes across host barriers, which can have implications during co-infection.[89]

After being engulfed by a eukaryotic cell, one would suspect that an EV would be degraded inside a digestive organelle such as a lysosome, stopping it in its tracks, but this is not necessarily the case. As mentioned, EVs from *M. tuberculosis* can actually be trafficked from the inside of an infected macrophage,[76] meaning they can persist and evade the interior defence mechanisms of such cells. It has also been reported that after entering host cells, EVs from *E. coli* can be internalised in non-acidified intracellular compartments and endure for several hours.[86]

1.3.2 Antimicrobial Resistance

EVs provide bacteria a double-edged weapon against antimicrobial agents; they may provide direct protection by breaking down or absorbing the dangerous substance, and they may transfer genes that could render the receiving strain resistant. For instance, EVs from colistin- and polymyxin B-resistant strains of *E. coli* provide direct protection for susceptible strains,[90] and EVs from clinically β-lactam-resistant *P. aeruginosa* may deliver β-lactamase and the virulence factor CFTR inhibitory factor (Cif) into host cells.[91,78] Furthermore, EVs from *Acinetobacter baumannii* can transfer antibiotic resistance genes to susceptible strains of *A. baylyi* and *E. coli*.[92] Addition of a small amount of EVs from polymyxin B-resistant *E. coli* gives increased protection and increased development of resistance in susceptible strains, while addition of high concentrations give immediate protection, but actually lowers the development

of resistance.[90] This has an intuitive, possible explanation; if protection against the antimicrobial is already present, there is little selective pressure towards acquiring independent resistance, although genes for this are presumably present in a significant concentration. The transfer of resistance genes by EVs has been known for quite some time, and may have significant clinical implications.[89]

1.3.3 Predation

The antimicrobial effects of cell-free filtrates of *P. aeruginosa* has been known for over 140 years,[93] but the contribution of EVs to this effect was not reported until 1996.[29] The demonstrated killing potential of certain EVs have earned them the nickname "predatory" EVs.[94] It was soon reported that EVs from 15 different strains under 8 genera of Gram-negative bacteria were capable of killing Gram-positive and -negative bacteria.[43] Ironically, while EVs from *P. aeruginosa* displayed a broad killing potential, the strain is also quite susceptible to predation by EVs from other strains. They found that although EVs kill bacteria of the same species to a low extent, higher LPS similarity does correlate with killing potential. While it was recognised that autolysins in the *P. aeruginosa* EVs were responsible for breakdown of the PG in e.g. *E. coli*, this killing is not achieved using lysed EVs, indicating that the soluble autolysins cannot exert their effect alone. This dependence on membrane encapsulation suggests that the autolysins cannot permeate the bacterial membrane, and rather depend on membrane fusion.[30]

1.3.4 Phage Defence and Susceptibility

As mentioned in Section 1.2.3, EVs are enriched with OM, the initial target of bacteriophages. Vesicles in the 50 nm size range may have a tenfold higher surface-to-volume ratio than the bacteria (Fig. 4.1), implying that if such a bacterium secreted only a tenth of its volume in vesicles, the available targets for phages in proximity would be doubled. This way, the bacterium will have made it just as likely that a phage rather attaches to –and "infects"– the vesicle instead. This mechanism has in fact been observed in *V. cholerae*[95] and *Prochlorococcus*,[96] and provides a potent defence for the bacteria in question. On the other hand, EVs have been shown to transmit susceptibility to phages in *Bacillus subtilis* and *Bacillus cereus*[97] by transferring surface proteins necessary for phage attachment, giving the bacterium and phage an alternative offensive mechanism.

1.3.5 Membrane Remodelling

In response to a new environment, bacteria change their composition, including remodelling their outer membranes.[98] For instance, *Salmonella enterica* modifies the glycoforms of its LPS in response to an acidic environment, which enables it to survive inside host lysosomes.[99] So far, there are no known methods in bacteria to chemically remove unwanted glycoforms from the OM, which –if true– implies that these would have to be removed by dilution during growth or by discarding parts of the membrane altogether. Conveniently, EVs allow for the secretion of OM enriched in unwanted components. It has indeed been reported that *S. enterica* EV production is increased during environmental changes, and that the lipid A species of the EVs tend to be the types the bacteria would want to discard in favour of new species.[100] Similar evidence is found in *V. cholerae*; production of EVs with specific membrane composition has been observed during a short time span after transition to a gastrointestinal environment,* after which the membrane protein composition of the bacteria has changed considerably. In this case, the shedding of EVs has another important function, namely providing decoy targets for the intestinal immune system, so that the bacteria themselves have an increased chance of survival and colonisation.

1.3.6 Environmental Biofilm Modulation

The role of EVs in infection-related biofilms is discussed in Section 1.3.1.1, but EVs have also been found to contribute to host-independent biofilms. For instance, it is visible in electron micrographs of *Myxococcus xanthus* biofilm that EVs occupy the gaps between bacteria, tethered to the cells and each other.[101] Furthermore, these bacteria have been found to connect with each other through longer chains of EVs, and it is hypothesised that these connections may facilitate complex signalling of proteins and other molecules.[102] EV-based communication related to biofilm is observed in *Listeria monocytogenes*, as EVs isolated under salt-stressed conditions reduce biofilm formation, and EVs isolated under energy-stressed conditions induce it.[103] The fact that EVs produced under these different conditions are distinct and promote opposite effects, implies that EVs carry complex information between the cells that can facilitate a quorum sensing-type decision-making-process about e.g. when to establish biofilms. While quorum sensing molecules such as small peptides, acyl-homoserine lactones and quinolones act as "words" in sociomicrobiology,[104] it is not unthinkable that

*Unpublished data presented by Stefan Schild, (Institute of Molecular Biosciences, Karl Franzens, Universität Graz) at the University of Oslo May 23, 2018.

EVs can analogously make up sentences, as they can contain a practically unlimited combination of proteins and signalling molecules in varying quantities. Furthermore, many of these signalling molecules can be short-lived under sub-optimal pH,[104] and vesicle encapsulation may provide additional longevity and range for these under stressing conditions. A further discussion on the role of EV-DNA and DNA in biofilm formation is featured in Section 1.5.1.

1.3.7 Selfish Vesicles

Advantageous mechanisms aside, as with any mechanism in nature, vesiculation does not necessarily yield an advantage to its facilitator. For instance, newly acquired viral infections that have just crossed the species-barrier can induce an array of detrimental effects for a host species.[105] This could also be the case with certain vesicles, as EV-carried plasmids in theory could carry genes that induce EV-production in order to facilitate their own spread, reminiscent of the viral infection cycle. Such an effect has been observed in *Halorubrum* spp, where vesiculation can be induced by a plasmid.[106] The plasmid (pR1SE) carries genes encoding proteins found in regularly shaped vesicles that also harbour the plasmid. This EV-borne plasmid is the first encountered member in a form of replicating agent at the intersection between viruses, plasmids and bacteria.

1.4 Applications for EVs

In parallel with our unravelling of the native functions of EVs, several potential applications have been identified, both in medicine and science in general.

1.4.1 EVs as Vaccines

Since EVs stem largely from the outer membranes of bacteria, they are immunological doppelgängers of their parent cells, carrying much of the same LPS and surface proteins. Naturally, the first hypothesised application for EVs was that they could be used as vaccines against their bacterial strains.[107]

1.4.1.1 Antibiotic Resistance

To fairly underline the need for efficient vaccines and the potential of EVs for this application, it is necessary to address the imminent emergence of antibiotic resistance. During his Nobel lecture in 1945, the father of antibiotics, Alexander Fleming, already predicted the rise of antimicrobial resistance (AMR).[108] While his penicillin –and the subsequently developed antibiotics– are initially effective

therapeutics against a narrow or wider range of bacteria, their effectiveness will invariably diminish over time due to bacterial evolution. Fleming himself noted that in the case of underdosing of an antibiotic (AB), all the pathogenic bacteria in a patient may not die, and rather stimulate evolution towards resistance against a higher dose. This is what we have witnessed over the last decades, as more and more of our ABs are proven ineffective against an increasing number of pathogenic bacteria.[109] This is partly because of medical mishandling; doctors may prescribe ABs when not needed, patients may take them when not needed or arrest their treatments early. Additionally, environmental exposure of ABs increase the rise of resistance, such as wastewater from AB production plants, as well as meat or fish farming.[109] To make matters worse, every bacterial species does not need to develop resistance itself, as cross-strain communication of genes is ubiquitous; as long as one bacterium has gained resistance, several other strains in proximity subjected to an AB selection pressure will swiftly acquire the genes necessary through horizontal gene transfer (HGT).[109] The prime arena for this development is the soil surrounding wastewater outlets from AB production plants, as the drug will diffuse through the dirt, effectively creating a gradient from a very low concentration of antibiotic at a distance, to a very high dose close to the plant.[110] As soil is laden with an immense variety of bacterial strains, one of them will rapidly acquire resistance as subjected to a low dose of antibiotics, and may transmit this to the others, pathogenic or not. Moreover, bacterial infections vary widely in their pathogenesis, and some respond less to antibiotics than others. For instance, some strains establish their infections within the cells of the host, like *Francisella tularensis* or *Mycobacterium avium*, which can persist in phagosomes.[111,112] There, the bioavailability of the applied antibiotic may be lower than outside the cell, depending on antibiotic types.[113,111] Effective immunity against these microbes would minimise their chances of entering the cells in the first place, reducing the use of ABs. Therefore, it is of the utmost importance to find vaccine alternatives to as many pathogenic bacteria as possible, both to decrease the use of ABs to combat the rise of resistance, and in order to have alternatives in place as the effect of ABs is dwindling.

1.4.1.2 Antigenic Properties of EVs

Over the years, EV vaccines have been proposed and developed against many pathogens, with varying degrees of success.[107] For instance, EV-based vaccines against *Neisseria meningitidis* have shown a 70–80% efficacy in trials since the 1980s onwards,[114,115] and a similar method has been used to make EV-based vaccines against *Bordetella pertussis*.[116] Previously, many candidates for EV-

vaccines were produced by detergent-based methods, but native EVs have started to gain interest in later years,[117] due to the development of hypervesiculating mutants that increase yield, controlled expression of important antigens, and attenuation of toxic components such as LPS.[118] At this point, several EV-based vaccines have shown efficacy; intranasal EV vaccines for *V. cholerae*,[119] *E. coli*[120] and *Francisella novicida*,[121] as well as EV injections for *S. typhimurium*[62] and *Brucella melitensis*[122] have yielded protective immunity in mice. Impressively, an oral vaccine consisting of a mix of EVs from *Shigellae* species *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* yields infant mice simultaneous protection against all of them.[123]

Modified EVs

The immunizing potential of EVs is not limited towards the strains from which they are secreted. For instance, EVs from modified *E. coli* expressing Omp22 from *A. baumannii*, can actually elicit protective immunity against *A. baumannii* when injected subcutaneously.[124] Similarly, EVs from *S. enterica* delivering pneumococcal proteins can elicit protection against *Streptococcus pneumoniae*.[125] This effect is not even limited to bacterial pathogens; *E. coli*-EVs presenting antigens against influenza H1N1 and MERS-CoV can provide immunity against these viruses,[126] and EVs from *Bacteroides thetaiotaomicron* carrying vaccine antigens from *S. enterica* ser. Typhimurium and IAV H5 hemagglutinin from Influenza A provide resistance against both of these pathogens in mice, and can as mentioned deliver KGF-2.[127] The latter example may foreshadow the potential of engineered EVs to perform several tasks simultaneously, for instance providing defence against a pathogen while delivering general therapeutics to a patient.

1.4.2 Therapeutics

EVs have shown promise as therapeutic agents in certain areas. As discussed in Section 1.3.3, they can exhibit bacteriolytic activity, making them a promising alternative to regular antibiotics.[30,128] One of the most striking examples is that natural and gentamycin-induced OMVs from *P. aeruginosa* can kill other strains of *P. aeruginosa* that have acquired permeability resistance against gentamycin.[30] These EVs have also shown promise against other bacteria; the skin-flora-associated bacterium *Staphylococcus epidermidis* can give severe infections in immunosuppressed patients, and EVs from *P. aeruginosa* inhibit its growth, providing an alternative topical antmicrobial treatment.[33] It has also been found that myxobacteria secrete EVs that are as effective as gentamycin in killing

E. coli. These EVs were reported to contain natural antimicrobial cargo, such as topoisomerase-inhibiting cystobactamids.[128]

In addition to exploiting the predatory nature of certain EVs, they have also been found effective in providing direct relief for some microbiome-associated illnesses, such as in the gastrointestinal system and epidermis. For instance, EVs of (the Gram-positive) lactobacillus, which is a natural symbiont in our gut flora,[129] have been found to relieve chemically induced digestive distress in mice when added exogenously.[130] The authors suggested that these EVs may communicate directly with the host to reduce oxidative stress in the gut. Moreover, EV-associated proteins from *Bifidobacterium longum* can actually reduce food allergy in mice by inducing apoptosis in mast cells without affecting T-cell responses.[131] Together, these results imply that it may be possible to develop EV-based therapeutics for people with irritable bowel syndrome, or provide digestive aid during antibiotic treatments that clear out advantageous bacteria of the gut flora. The microbiome balance is also important for our skin health, as it has been found that EVs from *S. aureus* can induce atopic dermatitis, and that EVs from *Lactobacillus plantarum* can antagonise this effect.[132]

1.4.2.1 Cancer therapy

Due to their ability to enter or fuse with target cells in their hosts, EVs have been proposed as drug-delivery agents,[133] and some have even suggested they have potential for eukaryote gene editing.[134] But one of the most impressive discoveries in research on EVs as drug-delivery tools is their functionality as anticancer drugs. The first paper to effectively utilise EVs against cancer cells bioengineered an *E. coli* strain to express an HER2-specific affibody, and subsequently loaded the isolated vesicles with siRNA that targeted kinesin spindle protein.[135] Injection of these EVs was found to significantly induce tumour growth regression. Similarly, EVs from LPS-depleted *E. coli* shows a highly significant antitumour response with few side-effects, which in turn lasted through subsequent injections of cancer cells.[136]

1.4.3 Optics and biosensing

Interestingly, the potential uses of EVs are not limited to medicine. In fact, engineered *E. coli*-EVs can work as contrast-enhancers and nano-heaters for optoacoustic imaging, i.e. detection of sound emitted by irradiated tissues.[137] This way, customised EVs can be used to target specific tissues that can be

locally heated and imaged at the same time. Similarly, EVs with an internal cargo of nanoluciferase and external antibody-binding proteins can be used to stain specific tissues, such as tumours.[138]

1.5 EV Genetics

1.5.1 DNA

The association of DNA with EVs (or membrane blebs) has been known since at least 1982.[139,140] It was observed that membrane protrusions from *H. influenzae* could absorb DNA as a part of transformation, and that these blebs could be isolated, carrying DNase-protected DNA cargo. In fact, their transformation potential actually inspired one group working on HGT in *H. influenzae* to name them *transformasomes* in their 1983 publication, and concluded that this was their function; a means for bacteria to absorb DNA into a DNase-protected state.[141] Although the conclusion in this case was that the DNA was attached to the EVs from the outside, and that these blebs were not actively secreted, the DNase-protected DNA content of EV samples from *N. meningitidis* was confirmed in 1989,[142] and this really marked the beginning of EV-DNA research. Since then, many articles have been published on the matter, most of which are featured in Table 1.1. This first group to detect DNA inside EVs also reported that the vesicles separated in two different density fractions, with specific gravities 1.12 and 1.3, and that only the heavier fraction provided DNase protection for the genetic cargo.* In parallel, they identified 17 DNA-binding proteins present in vesicles from *N. gonorrhoea*, and that the two fractions had their unique DNA-binding protein composition.[143] These investigations started painting a bigger picture of how certain EVs may be elaborated to transfer DNA, and that there could be a dedicated system for incorporation of DNA in EVs. Shortly, EV secretion and and their DNA content was confirmed for a whole range of additional Gram-negative strains: *Borrelia burgdorferi*, *Agrobacterium tumefaciens*, *B. pertussis*, *E. coli*, *Moraxella osloensis*, *P. aeruginosa*, *S. typhimurium*, *Serratia marcescens*, *S. dysenteriae*, *S. flexneri* and *Yersinia pestis*, while not detected for the Gram-positive strains *B. cereus*, *B. subtilis*, *S. aureus* and *Streptococcus sanguis*.[144,37] This led the authors to conclude that DNA secretion by EVs was universal for, but limited to, Gram-negative bacteria, although later findings have also confirmed EV-DNA from Gram-positive bacteria.[145] These studies investigated

*The sucrose gradient was centrifuged in a 34 mm tube for only 1 h at 200,000 × g, which may not be enough to completely settle smaller vesicles, and the separation between populations could be partly due to differential centrifugation (Section 1.6.4.1).

purified DNA, isolated from DNase-treated EV-samples, and did not localise DNA to the *inside* of EVs per se, as the DNA in theory could be protected in the membranes or in association with soluble proteins. The first investigation that utilised anti-DNA antibodies on electron microscopy sections in order to properly localise DNA was performed in 1995 on EVs from *P. aeruginosa*.[42] This constituted the first satisfactory proof to conclude that EVs do in fact harbour genetic material within their membrane encapsulation.

Thus, we have good reason to believe that native EVs *do* carry DNA, but what kind of DNA is present, and is it the case for both OMVs and OIMVs? Furthermore, is EV-DNA secreted by living bacteria, or are they the result of explosive cell lysis? During bacterial growth, DNA may be increasingly present as the cargo of lysis-derived vesicles, but investigations generally harvest their EVs in exponential phase, where this effect is supposedly minimal. And by doing this, chromosomal DNA has in fact been detected in both types of vesicles, secreted by living bacteria.[148,13] The level of DNA inclusion in EVs seems highly species-specific, as electron microscopy studies on EV populations from *A. kielensis* and *P. marina* found that 35–98% of the EVs carried double-stranded DNA (dsDNA).[41] At the time of the first publications on the matter, it was assumed that DNA was largely confined within the cytoplasm of the bacteria, making its inclusion in EVs a topological conundrum. The first authors to localise DNA in *P. aeruginosa*-derived EVs suggested that it could be transported over the periplasmic membranes through the systems related to transformation or conjugation.[42] Similarly, some suggested that free DNA in the medium may be internalised from the outside in a mechanism similar to transformation,[150] and later it was in fact discovered that DNA could be absorbed into the periplasm of *V. cholerae* from its surroundings, where it is bound by the competence protein ComEA.[151] This process would allow EVs from *V. cholerae* (or other bacteria with homologous functions) to acquire DNA not only from lysed cells of its own strain, but DNA freely floating in its environment. Ironically, *V. cholerae* secretes several DNases that severely lowers transformation frequencies, which would antagonise this mechanism of DNA uptake.[152] This being said, parallel findings suggested that OIMVs do indeed comprise a significant portion of EVs, and for *S. vesiculosa* $M7^T$, that they harbour the majority of the EV-DNA.[13] This did provide an explanation as to how some of the DNA could be included even without traversing membranes, but not how chromosomal DNA could be shed without resulting in the death of the bacteria. However, there are several mechanisms that could lead to certain parts of the bacterial genome being present in the cytosol in different concentrations. In bacteria, there are a whole range of

Table 1.1: Published reports on DNA detected in EVs, and proposed functions. Methods of detection are fluorometric quantification (FQ), transformation (T), electron microscopy (EM), gel electrophoresis (GE), sequencing (S) and quantitative differential (comparing genetic abundance to the parent bacteria) sequencing (QS). Types of DNA are plasmid (P), chromosomal (C), or specified genes (SG).

Strain	DNase	Method	DNA types	Function	Year^ref.
Agrobacterium tumefaciens	+	GE, EM	P	ND	1990[37]
Ahrensia kielensis	+	GE, S	C, SG	ND	2014[41]
Alteromonas	+	FQ, GE	C	ND	2017[146]
Bordetella pertussis	+	GE, EM	C	ND	1990[37]
Borrelia burgdorferi	+	GE, EM	C, P	ND	1990[37]
Escherichia coli	+	GE, EM	C	ND	1990[37]
	+	FQ	C	ND	2017[146]
	+	qPCR, GE	P 3.5–15 kbp	HGT	2019[147]
Haemophilus influenzae	+	T	P	HGT	1982[139]
	+	EM	6 kbp	ND	1982[140]
	+	GE	P	HGT	1983[141]
	+	GE, EM	P	ND	1990[37]
Haemophilus parainfluenzae	+	GE, EM	P	ND	1990[37]
Moraxella osloensis	+	GE, EM	C, P	ND	1990[37]
Neisseria gonorrhoeae	+	GE, EM	P	ND	1990[37]
Neisseria meningitidis	+	GE, EM	C, P	HGT	1989[142]
Prochlorococcus	+	S	C	ND	2014[96]
	+	FQ, GE	C	ND	2017[146]
Pseudoalteromonas marina	+	GE, S	C, SG	ND	2014[41]
Pseudomonas aeruginosa	+	GE, EM	C	ND	1990[37]
	+	FQ, EM, GE	ND	ND	1995[42]
	+	GE, QS	C, SG	ND	2017[148]
Ruminococcus spp.	+	GE	C 20–90 kbp	HGT	2005[145]
Salinicola	+	FQ, GE	C	ND	2017[146]
Salmonella typhimurium	+	GE, EM	C	ND	1990[37]
Serpulina hyodysenteriae	+	GE	C, 6.5 kbp	ND	1997[149]
Serratia marcescens	+	GE, EM	C	ND	1990[37]
Shewanella vesiculosa	+	FQ, EM	C	ND	2013[13]
Shigella dysenteriae	+	GE, EM	C, P	ND	1990[37]
Shigella flexneri	+	GE, EM	C, P	ND	1990[37]
Thalassospira	+	FQ, GE	C	ND	2017[146]
Yersinia pestis	+	GE, EM	C, P	ND	1990[37]

replicative and transposing mechanisms that grant chromosome-encoded elements such as prophages, transposons and integrons their mobility,[153] not to mention the machinery that replicates the chromosome itself. These mechanisms are error prone, and may induce stochastic partial genome replication, or arrest cell division after detection of DNA damage.[154] For instance, a parts of a superintegron

structure in *V. cholerae* has been found to be present in a different number of copies from the rest of the genome.[48] Another striking example is that the DNase/proteinase K-protected extracellular DNA from *Serpulina hyodysenteriae* consists of 6.5 kbp random fragments of chromosomal DNA, rather than a specific sequence.[149] This size specificity also brings us to an important question; what is the potential DNA capacity of a typical EV? Intuition states that there is necessarily an upper bound to the length of an included fragment, as DNA is a rather rigid polymer, with a minimum persistence length P of about 40 nm (dependent on salt concentration),[155] which is longer than smaller vesicle diameters.* The inclusion of plasmids in EVs does correlate negatively with plasmid length, with plasmids in the 3.5–7 kbp size range being included 2–3 times more in EVs than plasmids in the 10–15 kbp range,[147] although linear 20–90 kbp stretches of DNA have been found in EVs from *Ruminococcus albus*.[145] Considering that the length of DNA per base pair is around 3.34 Å,[156] these DNA strands would have linear lengths L_0 of about 6.7–30 µm. The diameter of a diffusing DNA strand of this length can be approximated via the mean square end-to-end distance $\langle R^2 \rangle$:[157]

$$\langle R^2 \rangle = 2PL_0 \left[1 - \frac{P}{L_0} \left(1 - e^{-L_0/P} \right) \right] \tag{1.1}$$

Assuming θ solvent conditions (under which the DNA polymer acts as a free chain,[158]) the hydrodynamic radius R_h can be approximated to:[157]

$$R_h = \frac{0.665}{\sqrt{6}} \langle R^2 \rangle^{\frac{1}{2}} = \frac{0.665}{\sqrt{6}} \left(2PL_0 \left[1 - \frac{P}{L_0} \left(1 - e^{-L_0/P} \right) \right] \right)^{\frac{1}{2}} \tag{1.2}$$

Assuming a persistence length of 40 nm, R_h would be about 197–421 nm for a 20–90 kbp fragment. A simple random walk simulation assuming an ideal chain gives about 35% lower numbers, with R_h being 129–273 nm (Section A.3), although this does not include self-avoidance, which would increase R_h somewhat. Uncompacted, these fragments would be larger than most vesicles, and would require some DNA-condensing machinery in order to physically fit, or would otherwise have to be packed actively into the vesicles in an energy-dependent process reminiscent of a bacteriophage.[159] Circular plasmids could be supercoiled or relaxed, and these effects may contribute to compaction.[160] All things considered, it is remarkable that linear DNA in the 10 kbp scale can effectively be secreted and DNase-protected in EVs, although this may not be the most typical DNA content. In a study on marine bacteria, it was reported that

*The persistence length is the minimum required length of a certain polymer before the directions of the ends no longer correlate.

the DNA fragment size distributions in EVs were quite unique to the four strains investigated.[146] For instance, *Prochlorococcus* has a series of discrete DNA fragment sizes between 50 bp and 10 kbp, while *Thalassospira* has a broad distribution over the same span, peaking somewhere between 100 and 500 bp. It seems that DNA is a common content of EVs, but that the specific genes and quantities vary widely between species.

It is known that EVs can harbour plasmid DNA from its parent bacteria. However, there is little knowledge as to which parts of the chromosomal DNA are included. Presently, there are only a few studies that have undertaken sequencing of EV cargo.[41,96,148] One reason for this may be because DNA can be so minute in EV samples that statistically significant data requires isolation of DNA from considerable volumes of bacterial culture medium.[146] Notably, DNA from EVs from cultured *Prochlorococcus* revealed that about 50% of the genome was accounted for, but by using multiple displacement amplification (MDA) to amplify the sequence, quantitative data on the genes were lost.[146] Another study sequenced EVs from *P. aeruginosa*,[148] and detected enrichment of chromosomal DNA encoding e.g. virulence-related proteins ExoS and its chaperone SpcS,[161] membrane nitrite reductase operon *narGHIJ/K1/K2* carrying genes relevant to type II secretion, biofilm formation and motility,[162] as well as cold response gene *capB*,[163] and antibiotic resistance gene *pcs*.[164] However, this study did not undertake any analysis of sequence-dependencies in the observed enrichment (such as motifs for DNA–protein binding), which is still to be elucidated. So far, it seems that the characteristics of DNA included in EVs vary widely between bacterial strains. One way to understand the sequence variation of the DNA cargo would be to assess the potential function.

Potential advantages of secreting DNA through EVs are multiplex. One of the first functional studies on this reported that EVs can facilitate the transfer of plasmid-borne ampicillin resistance from resistant to susceptible *E. coli*.[165] Later, EVs from *A. baumannii* were found able to transfer carbapenemase genes.[89] The transfer of plasmids by EVs has been shown to work between three donor and five recipient strains of Gram-negative bacteria, suggesting that this is a general HGT mechanism.[166] Furthermore, while plasmid packing efficiency was similar for the donors, the transmission efficiency was dependent on the recipient, but did not correlate with relatedness between recipients. This could imply that while vesiculation is universal among Gram-negative bacteria, the evolution of specific EV-related functions (including their use in HGT) is not, and rather has developed independently for each strain that may benefit from this in their specific niche. Interestingly, plasmids are not the only "infective" genetic element

that can transmit through EVs, as viral genomes can be incorporated in EVs from *Thermococcus nautilus*.[167] This implies that EVs could yield viruses with an alternative, perhaps less specific infection route.

The transfer of DNA with vesicles is not only related to the microbial community, but can also apply to transkingdom HGT. For instance, *P. aeruginosa*-derived EVs deliver DNA into human host cells, where it can be detected by PCR in the nuclear fraction.[148] Naturally, this has implications for EV-based vaccines, as the potential for genetic modification is unclear.

The role of DNA in EVs does not only relate to its capability for genetic storage, as it plays a vital role as an extracellular component in biofilms.[168] It was reported that (the Gram-positive) *S. mutans* upregulates its production of extracellular DNA during biofilm formation, and that one of its important DNA secretion pathways is the lysis-independent secretion of EVs.[169] This could point to EVs being a rather efficient method of transporting DNA out of the cell for this purpose, overcoming the hydrophobic barrier that membranes present. For Gram-negative bacteria, vesiculation would allow DNA translocation across at least the OM, and potentially the IM and the physical barrier of PG. It has been found that the DNA associated with biofilm formation is not simply random genomic DNA, but complex, short fragments,[73] which could be specifically associated with EVs.

1.5.2 RNA

The mere presence of RNA in EVs has been known for some time, starting with *N. gonorrhoea* in 1989,[142] although the nature of this RNA was not determined. Some time would pass before EV-RNA was subjected to a reasonable qualitative investigation, starting with sequencing of EV-RNA from *Prochlorococcus* MED4 in 2014.[96] This study reported that EVs contained RNA from 95% of all open reading frames in the genome covering 89% of all nucleotides. However, the quantitative values of the transcripts were not compared to the RNA composition of the parent bacteria, providing little information on enrichment of certain sequences or functional RNAs in EVs. That being said, this was the first study in a series of recent investigations aimed at identifying RNA cargo in EVs from different strains,[170] as shown in Table 1.2.

Intuitively, one could assume three things to be valid for EV-RNA; firstly, more abundant RNAs in the cell should be more abundant in EVs, secondly, RNA with rapid turnover would be depleted, and thirdly, larger RNAs would be excluded due to the size restriction of EVs. Intuition then states that stable forms of abundant RNAs would dominate the sample, such as rRNA and tRNA.[180]

Table 1.2: Published reports on RNA detected in EVs, and proposed functions. Table is modified and supplemented from published versions.[171,170]

Strain	RNase	Method	RNA species	Function	Year^ref.
Aggregatibacter actinomycetemcomitans	+	Northern blot, qPCR	msRNA	Reduces IL-5, IL-13, IL-15 secretion	2017[172]
Escherichia coli	-	Sequencing	rRNA, tRNA, sRNA	ND	2015[173]
	-	Sequencing	mRNA, tRNA, sRNA, rRNA	ND	2016[174]
	-	Quantification	ND	ND	2017[175]
Mycobacterium smegmatis	-	Quantification	ND	ND	2017[175]
Neisseria gonorrheae	+	Gel electrophoresis	ND	ND	1989[142]
Porphyromonas gingivalis	-	RT-qPCR	mRNA,16S rRNA	ND	2015[176]
	+	Northern blot, RT-qPCR	msRNA	Reduces IL-5, IL-13, IL-15 secretion	2017[172]
Prochlorococcus marinus	-	Sequencing	89% genome coverage	ND	2014[96]
Pseudomonas aeruginosa	+	Sequencing	rRNA, tRNA, sRNA, mRNA, tmRNA	Reduce IL-8 mRNA, IL-8 secretion	2016[74]
Streptococcus pyogenes	+	Sequencing	tRNA, rRNA, mRNA	ND	2016[177]
Streptococcus sanguinis	+	RT-qPCR	msRNA	ND	2016[178]
Vibrio cholerae	+	Sequencing	mRNA, sRNA, intergenic	ND	2015[179]
Treponema denticola	+	Northern blot, RT-qPCR	msRNA	Reduces IL-5, IL-13, IL-15 secretion	2017[172]

Indeed, it was found that rRNA constitutes up to 99.4% and 94% of all EV-RNA in *Prochlorococcus*[96] and *E. coli*[174] respectively, as well as unspecified majorities of EV-RNA from *P. gingivalis*[176] and *Streptococcus pyogenes*.[177] tRNA is also abundant in *E. coli*-EVs.[173] In general, prokaryotic RNA content is generally over 85% rRNA,[181] which can indicate a degree of rRNA enrichment in EVs.

As this is an expected result, it is perhaps more interesting to find specific RNAs of non r/tRNA character that are enriched in EVs. As mentioned, coding

RNA has been found in EVs from *Prochlorococcus*, and subsequent analyses have detected this form of RNA in EVs from *P. aeruginosa*[74] and *S. pyogenes*.[177] The former determined that mRNA coding for e.g. DNA-binding protein HU subunit beta *hupB* is enriched in EVs. The latter study revealed an enrichment of *adcR* mRNA, which is central to zinc availability responses.[182] The authors therefore hypothesised that this mRNA could be involved in communication among the bacteria.[177] Intergenic RNA is also present in EVs from *V. cholerae*, albeit with no hypothesised function.[179]

These findings do not necessarily inspire any coherent theory as to which mRNAs are included in EVs, and what their functions are. But some interesting effects have been uncovered recently, regarding the functional, smaller RNA species included in EVs. There are two general types of these RNAs; bacterial small RNA (sRNA) and microRNA-sized small RNAs (msRNAs).

The sRNAs are generally 50–400 nt long regulating agents in bacteria.[183] In *V. cholerae*, a number of sRNAs are known to regulate virulence and quorum sensing by e.g. affecting transcription.[184] However, the territory of sRNA is not at all limited to the immediate bacterial proximity, as they can be present in high abundance in both the urine and plasma of healthy humans.[185] This not only points to a high level of stability or these sRNAs, but also a level of mobility, which could in theory be facilitated by EV encapsulation. The modulary effects of some RNAs have been known and investigated for decades, since the discovery that feeding *Caenorhabditis elegans* with dsRNA can lead to silencing of certain genes.[186] The detection of sRNA in human body fluids does therefore raise some questions as to whether these may have any regulatory effects on our genetic expression. For instance, the known sRNA *csrC* from *E. coli* has been found to be transported into liver cells,[174] although the regulating effects were not investigated. msRNAs are similar to sRNAs and somewhat analogous to eukaryotic miRNAs,[172] in that they, when fully matured, have lengths of about 15–22 nt and can hybridise with host mRNAs and suppress translation and transcription of genes.[172] These types of RNAs have been detected in EVs from e.g. *S. sanguis*[178] as well as in *E. coli*, where many are cleavage products of tRNA, rRNA and tmRNA.[173] The 15–22 nt msRNAs found in the periodontal pathogens *A. actinomycetemcomitans*, *T. denticola* and *P. gingivalis* were capable of suppressing IL-5, IL-13, and IL-15 expression in T-cells.[172] This is reminiscent of the briefly mentioned immunomodulary effects of sRNA in *P. aeruginosa*-EVs in Section 1.3.1.

While identifying enriched RNAs in EVs seems straightforward, mapping which types of RNAs are EV-specific is not, as RNA degradation is an ongoing

process during the EV-isolation. In fact, the turnover of bacterial RNA can be very quick, especially for mRNAs, which can have half-lives in the minute range.[187] After an elaborate isolation protocol and RNase-treatment that ensures a pure product with minimal extravesicular material, the remaining isolate may be dominated by the RNAs that are most stable in themselves, and not necessarily RNAs that are specifically packaged into EVs. However, utilizing EVs for the secretion of RNAs could in theory provide protection from degradation from e.g. host- or environmental RNases.[188] EV encapsulation may therefore allow longer range trafficking of interfering- or immunomodulary RNAs to a host organism, or to other bacteria in proximity.

1.5.3 The Membrane Connection

For DNA/RNA to be enriched in EVs it is intuitive to picture some association with the inner or outer membrane. For a nucleotide strand to be enriched in EVs, its concentration must be higher than in the cytosol, which necessitates that a certain amount of force has to be exerted upon it, localizing it to the site at which the EV is forming. These proteins should be directly or indirectly associated with the membrane, as well RNA/DNA, and any sequence specific binding of such proteins could provide potential motifs that can be added to preferred genetic cargo in the case of bioengineered vesicles. There are several candidate DNA-binding proteins with transmembrane domains in bacteria. For instance, *V. cholerae* has 10 according to UniProt,[189] such as several OmpR/PhoB-type domain-containing proteins and transcriptional activators, although the best characterised one so far is ToxR, a homologue of CadC in *E. coli*.[190] Similar proteins exist that rather bind to RNA. One example is an RNA recognition motif (RRM) -containing protein predicted by PSORTb[191] to be located in the inner membrane of *V. cholerae*.[192]

1.5.4 Sequence Analysis

Several methods exist, which yield quantitative sequence-dependent data from a DNA or RNA sample. If the genome is known, primers could be constructed for qPCR-based methods, which can provide quantitative comparisons between the abundance of different sequences. This is a very sensitive approach, but the data yield is limited by the number of primer pairs constructed and capacity for qPCR reactions. What makes this protocol especially practical for EV samples that may be low in DNA or RNA is that it can be performed on EV lysates, with no need for purification, which inevitably leads to loss of material and

perhaps the introduction of biases, e.g. towards different fragment lengths or AT-content. If the DNA sample is concentrated enough to be visible on an agarose gel or bioanalyser,[193] it can be subjected to restriction enzymes to yield a fragmentation table that can reveal whether or not certain sequences or lengths are enriched in the sample.[149] However, genetic sequencing is the only method that will characterise the total DNA or RNA in a sample. There are several sequencing technologies available, the most common being Illumina,[194] Pacbio,[195] and –increasingly– Nanopore sequencing.[196] They all have their advantages, but unless *de novo* genome assembly is required, Illumina is the current standard for quantitative sequencing.[197] This method has proven to be highly replicable for differential expression analysis, which is used to assess enrichment of specific genetic sequences in a sample.[198] In short, such an analysis consists of mapping DNA or RNA sequencing reads from each sample to a reference genome (Fig. 1.6), and calculating the number of times each base pair in the reference genome is represented in the read data (read coverage for that base pair). These numbers are normalised according to the coverage across the genome by one of several algorithms, and expression numbers can be compared between different samples.

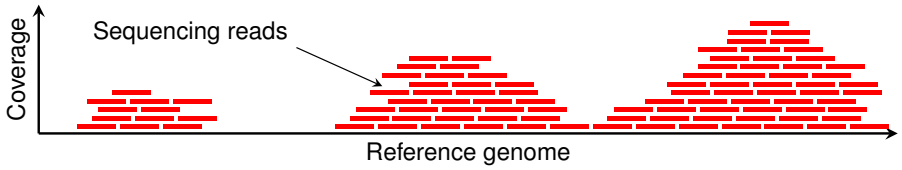


Figure 1.6: Illustration of the mapping of sequencing reads, and the resulting differences in read coverage. Figure drawn in TikZ.[10]

1.6 Methods for EV Isolation

The temporal and material requirements of isolation protocols capable of acquisition of sufficient quantities of EVs (for certain downstream applications) can be substantial. For sensitive analyses such as mass spectrometry or qPCR, simple ultracentrifugation of small volumes (5–100 mL) will often suffice,[199] depending on the vesiculation of the strain, growth conditions, and time of harvest. For quantitative whole-genome sequencing of EV-DNA on the other hand, more elaborate protocols may be necessary.[96] The reason for this is the minute quantity of DNA that is actually DNase-protected in EV-samples.[146] There are ways to amplify the total DNA in a sample, such as MDA, lowering

the necessary sample size, but this procedure introduces biases that may be systematic, and can therefore lead to replicable, but false peaks in sequencing coverage along the genome.[200,201] These systematic errors may depend on the genetic fragment composition of the sample, and thus introduce false positives in estimated enrichments or depletions between whole bacteria and EVs. Therefore, in order to yield higher quantities of DNA fit for quantitative DNA sequencing, two solutions remain; using a hypervesiculating mutant or culturing larger volumes. If the project aims to investigate the characteristics of vesicles as close to a wild-type situation as possible, hypervesiculating mutants are less appealing, and the only solution would be to increase the culture volume. This, of course, also requires an EV-isolation setup that can handle the large volumes of medium. The first couple of steps of EV isolation are the same, disregarding the subsequent concentration method (Fig. 1.7). Firstly, bacterial culture is grown to the desired density, after which the bacteria are removed from the medium by sterile filtration, usually preceded by cell pelleting by centrifugation. After this, a dilute amount of EVs, dead cell debris, and potential extracellular constructs such as flagella, pili, and endogenous bacteriophage particles remain in the filtrate. The object of a successful isolation protocol is to concentrate the EVs, change their suspension liquid, and remove as much unwanted material as possible. The most common EV-isolation protocol when working with decilitre volumes is based on pelleting by ultracentrifugation (UC). For volumes of multiple litres, only two methods are practical in a laboratory setting; tangential flow filtration (TFF) and salt-induced precipitation (SIP).

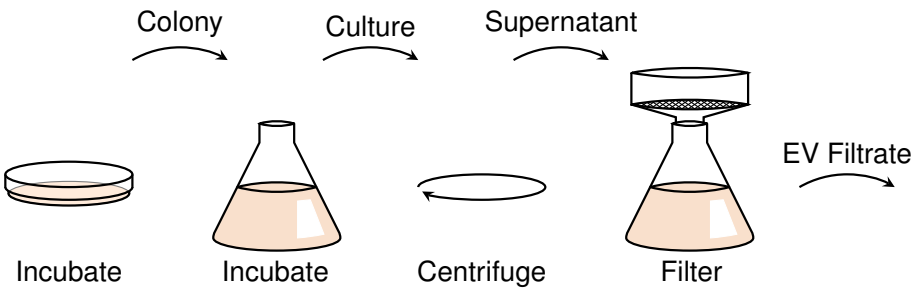


Figure 1.7: The initial steps of vesicle isolation; bacterial culture is sterile-filtrated, usually preceded by centrifugation to remove a majority of the bacteria. Figure drawn in TikZ.[10]

1.6.1 Ultracentrifugation

Being the most common method for EV isolation, UC has its advantages and drawbacks. The ultracentrifuge is a common instrument in laboratories worldwide, making the method accessible to many, and most EV-research to date has been conducted with it, giving solid grounds for comparison. Furthermore, assuming a thorough sedimentation, there is little loss of material, and the pellet can be resuspended directly in whichever buffer is preferred for subsequent experiments. The main drawback to this method is the volume bottleneck, as one typical rotor holds six tubes of less than 40 mL of liquid, and one centrifugation requires ~ 2 h to satisfactorily pellet the EVs. Taking into account ~ 20 min required for balancing the tubes and starting the centrifuge, this makes for < 96 mL of culture per hour, setting an effective upper limit at ~ 1.5 L per day per centrifuge. Another element to consider is the size bias of the method, as smaller particles sediment slower than larger particles, according to Stokes equation:[202]

$$V_t = \frac{a(\rho_p - \rho_l)D^2}{18\mu} \tag{1.3}$$

Where V_t is the sedimentation speed, a is the centrifugal acceleration, ρ_p is the density of the particle, ρ_l is the density of the liquid, D is the diameter of the particle, and μ is the viscosity of the liquid. This effect could potentially lead to a bias towards larger vesicles in the pellet, and depletion of smaller EV in the final isolate.

1.6.2 Tangential flow filtration

While regular filtration depends on a moderate pressure difference across a membrane that pushes solvent through, TFF relies on the movement of liquid tangentially upon the filter, with a very low pressure difference across the membrane itself. This reduction in pressure inhibits the push of particles into the filter, where they would quickly clog the pores and render the filter useless. With tangential flow, effluent can escape through the membrane while particles larger than the cut-off remain in the retentate, where it can continue circulation, until the particle concentration is adequate. With sufficient cleaning between runs, the method allows for the concentration of > 100 L of sterile filtrate per filter, and > 20 L in one go, depending on EV- and protein density in the starting material. The size exclusion for the technique is somewhat customisable, with filter cut-off ranging from 10–750 kDa, although even the highest cut-off is very

small compared to vesicles. For instance, a globular protein would have a mass

$$M \sim \rho \cdot \frac{4}{3}\pi r^3 \tag{1.4}$$

where M is the weight cut-off, ρ is the density of protein ($\sim 1350\,\mathrm{kg\,m^{-3}}$)[203] and r is the radius of the protein. Solving for r we get

$$r = \sqrt[3]{\frac{3M}{4\pi\rho}} = 1.43 \rightarrow 6.04\,\mathrm{nm} \qquad (10\,\mathrm{kDa} < M < 750\,\mathrm{kDa}) \tag{1.5}$$

So in contrast to UC, TFF applies a quite low cut-off, meaning soluble proteins and other debris will be co-purified along with the vesicles. Another drawback to the technique is the comparably complex instrumentation (Fig. 1.8). The setup

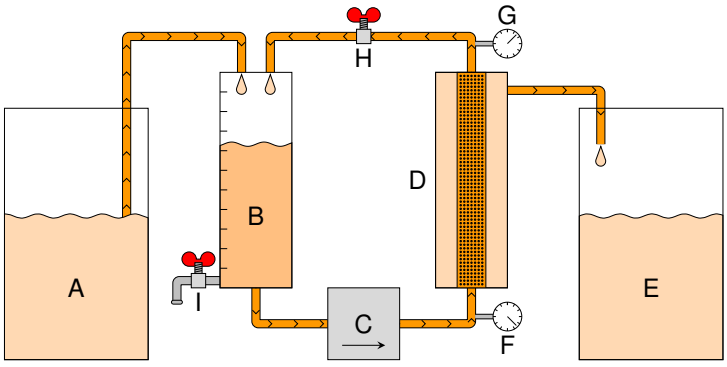


Figure 1.8: Schematic setup of tangential flow filtration concentration, drawn in TiKz.[10] **A)** Vessel with input material, i.e. bacteria-free supernatant. **B)** Reservoir, where the retentate will increase in particle concentration over time. **C)** Peristaltic pump. **D)** Hollow filter cartridge, retentate stays in the hollow center, effluent will seep to the outside. **E)** Effluent container (waste). **F)** Input pressure gauge. **G)** Output pressure gauge. **H)** Pressure valve (determines pressure over the filter membrane). **I)** Output valve, for collection of concentrate.

itself includes a rather expensive filter-holder, pressure gauges and filter unit, as well as tubing, pumps and large vessels for starting liquid and effluent. Some expertise is required to correctly adjust inlet- and outlet pressures in order to maximise efficiency and filter durability, as well as some knowledge on compatible agents for washing and sterilisation. Another setback to the technique is an inevitable loss of some starting material that is deposited in the filter. Ironically, this makes the required starting culture volumes even larger if one wants the same yield as one would have with UC.

1.6.3 Salt-induced precipitation

This technique, also called *hydrophobic interaction chromatography*, *antisolvent crystallization* or *salting-out*, relies on the addition of salts to precipitate nanoparticles out of solution.[204] This works because of the shielding effects of the salt ions upon the hydrophilic areas of proteins, which in turn increases the relative contribution of hydrophobic interactions, allowing the particles to aggregate.[205] While this is a very simple and cheap method, requiring only a stirring-plate and a sufficient amount of an adequate salt, there are some major drawbacks. Different particles precipitate at different salt-concentrations, depending on size and charge distribution,[206] but co-precipitation of some extracellular proteins and debris is inevitable.[207] This makes the raw samples unsuitable for EV-specific protein analysis, but could yield good results for the total secretome of an organism. In addition, high concentrations of salt may subject EVs to a severe amount of osmotic stress, which could be detrimental to their integrity.[208] Therefore, SIP might render the sample unfit for EV-DNA or -RNA analysis, if the DNase- and RNase-protection is impaired by membrane ruptures. As the purity of the product is initially quite low, proper resuspension of the EVs is essential after concentration, to ensure efficacy of downstream protocols. However, this may not really be possible due to irreversible protein binding.[209,210] Another issue that demands proper resuspension is that nucleotides could be protected within salt-induced aggregates, and non-EV-specific RNA/DNA may survive DNase or RNase-treatment in this state. Historically, the most widely applied salt for this protocol is ammonium sulphate, as it is low density, highly soluble, protein structure stabilizing, and comparatively inexpensive.[211]

1.6.4 Post-concentration purification

The aforementioned EV isolation protocols simply focus on increasing the concentration in a sample, but this type of crude isolate can be unsuitable for a range of applications, most notably in the field of basic science on EVs. If the aim is to analyse the DNA within vesicles, and not DNA associated with soluble proteins, phages or pili, the sample needs to be depleted of such components. On the other hand; when investigating the immunizing potential of concentrated cell-depleted bacterial culture, purification could potentially be a redundant step, as the final vaccine may need to be cheap to produce (e.g. vaccines for fish, poultry or livestock), and extra steps warrant additional cost.

1.6.4.1 Density Gradient Centrifugation

The most common purification step for EV isolation is density gradient centrifugation (DGC), which separates particles according to their specific densities. Usually, the gradient is constructed using different concentrations of either regular sucrose[142] or Optiprep,[212] which at 60% (w/v) in H_2O have densities of about $1.3\,\mathrm{g\,mL^{-1}}$.*[213] This allows for significant depletion of phages and protein complexes in the sample, although smaller proteins may remain in the gradient.[214] The main problem with this is related to sedimentation dynamics, as the higher density and viscosity further down in the centrifugation medium lowers the sedimentation speed according to Stokes' law (Eq. 1.3), prolonging the time needed for satisfactory separation. It is apparent from this equation that the time needed to sediment a certain particle is directly proportional to the viscosity, and inversely proportional to the difference in density between the particle and the medium. Thus, the element of differential centrifugation is even more problematic in DGC than UC of low-viscosity solutions such as LB broth or PBS; while water has a viscosity of about 1.5 mPa s,[215] the viscosities of 50% and 60% sucrose at 5 °C are approximately 30 and 150 mPa s,[216] which is roughly 100× higher, and would therefore require 100× the centrifugation duration to cross.† However, this is only in case a stationary end result is desired; arresting the centrifugation early will only allow the largest vesicles, flagella and phage particles to separate to their respective density gradients, while smaller particles will still be in the process of traversing the less dense phases. A common duration for relatively good separation is 16–18 h, making this one of the more time-consuming purification methods.[217,175]

1.6.4.2 Size Exclusion Chromatography

While DGC separates particles by density (and to a limited degree, size), size exclusion chromatography (SEC) is a common method for separating according to size.[175] The mothod is based on letting the EV sample flow through a porous column, where smaller particles will traverse in and out of the pores while larger particles flow past them. This means that larger particles will exit the column faster than smaller ones, in an analogous (but intuitively opposite) process to gel electrophoresis.

*OptiPrep™ cell separation media, 60% (w/v), density=1.320 g/ml. Accurate Chemical, Westbury, NY, USA.

†Viscosity data on optiprep was not found, but from personal experience, it seems rather viscous.

1.6.4.3 Dialysis

A simple and relatively fast way to deplete soluble proteins and other unwanted co-isolates (e.g. RNases, DNases and proteases) is dialysis.[218] In this method, the isolate is contained within a membrane which is inserted into a large volume of a desired buffer. The dialysis membrane has pores of a certain size (molecular weight cut-off), so that buffer and smaller solutes can diffuse freely while larger particles are contained within the membrane, until the osmotic pressure has equalised the buffer conditions on each side of the membrane. This process is usually allowed to proceed for 4–12 h,[219,199] meaning that it could be a time-consuming step in an isolation pipeline.

1.6.4.4 Time Sensitivity

The main drawback with purification protocols such as dialysis or DGC is that they are time-consuming, which has its consequences. After EV isolation, which may already take a number of hours, further steps will inevitably damage the integrity of time-sensitive biomolecules, most notably RNA. The half-life of specific mRNAs can range from less than two minutes up to hours, depending on stress factors, temperature and present RNases.[187] Even when subjected to the lowest possible thermal stress, it is impossible to perfectly preserve mRNA samples over time, even less so in the presence of the natural RNases that bacteria carry. The isolation of RNA from EVs is therefore a cumbersome endeavour, where one has to carefully weight the trade-off between purity and integrity.

1.7 Model Organism

The original aim for the project was to investigate vesicle characteristics from fish pathogens *Francisella noatunensis* spp. noatunensis and *Piscirickettsia salmonis*. This was due to previous research on their vesicles as candidates for vaccines in aquaculture. However, their slow growth and the high material yield necessary for genetic sequencing made them unsuitable, and a bacterium with shorter generation time had to be selected. The ideal strain were to be common, not too dangerous to handle in decaliter volumes, and somewhat established in the field of EVs. Of these, several candidates were available, most notably different strains of *E. coli*, *P. aeruginosa* and *V. cholerae*. *E. coli* and *P. aeruginosa* are comparably well characterised species, but were ultimately eliminated in favor of *V. cholerae*. The natural habitats of *E. coli* are largely limited to the intestine of warm-blooded animals, and since genetic transfer was of interest, a strain

with a broader niche and more potential hosts or environmental presence was of interest. *P. aeruginosa* is widespread in both animals, plants and soil,[220] but as evident in Section 1.1-1.6, its EVs are subject to broad scientific attention, and it was fair to assume that similar research was already underway. Furthermore, *P. aeruginosa* is notorious for its wide array of secretion products,[221] which could give rise to unwanted contamination of EV samples. There are notable secretion products from *V. cholerae* as well,[222] but one of the available mutants had two of the main ones deleted (details below),[223] and was therefore ultimately chosen as our model. These deletions also severely reduce the virulence of the strain, making it more suitable for larger culture volumes without introducing biological hazard.[223] While the absence of certain secretion mechanisms may yield a product of higher purity, some impurities in the crude EV filtrate provides an opportunity to assess the efficiency of different isolation protocols.

1.7.1 *Vibrio cholerae*

Certain strains of *V. cholerae* are the causative agents behind the severe diarrhoeal disease cholera, with about 2.86 million cases and 95,000 fatalities annually.[224] It is a comma-shaped, Gram-negative, facultative anaerobe bacterium, natively found in brackish and salt water, while capable of surviving in fresh water.[225] Mainly a water-borne pathogen, it transmits through contaminated drinking water or foods with moderate water contact (fish, shellfish, produce),[226] while person-to-person transmission is rarely reported.[227] *V. cholerae* colonises the intestine and secretes cholera toxin (CTX), a protein that induces immense, watery diarrhoea (coined "rice-water stool"), and patients may lose 20 mL/kg/h of water during infection (36 L/d for a 75 kg person).[228] Fluid loss is thus the main cause of death, and while the mortality for untreated patients can exceed 70%,[229] relatively simple hydration therapy may lower it to less than 0.2%.[228] CTX is encoded in a bacteriophage CTXϕ, which is usually present as a prophage in *V. cholerae*, but can be found as a virion.[230] Apart from CTX, which is a soluble enterotoxin,[231] *V. cholerae* produces three other important toxins, that have been found to contribute to pathogenesis; accessory cholerae enterotoxin (ACE), zona occludens toxin (zot) and "repeats-in-toxin" (RTX). Both ACE and zot are membrane-located toxins,[232] meaning their function is restricted to direct contact between host cells and the bacteria, unless they are mounted in the membrane of EVs, which could yield longer range function.

The colonisation of *V. cholerae* in the intestine also relies on its toxin-coregulated pilus (TCP), a thin, filamentous construct on its surface. TCP is

required to attach effectively to the intestinal lumen, and gives protection against bile components, in addition to working as the CTXϕ receptor.[233]

V. cholerae is divided into two main serogroups, O1 and O139, and the O1 group is further divided into two biotypes, classical and El Tor, both of which are divided into three serotypes; Inaba, Ogawa and Hikojima (Fig. 1.9). The strain used in the thesis work is strain O395, which is an Ogawa serotype of classical O1.

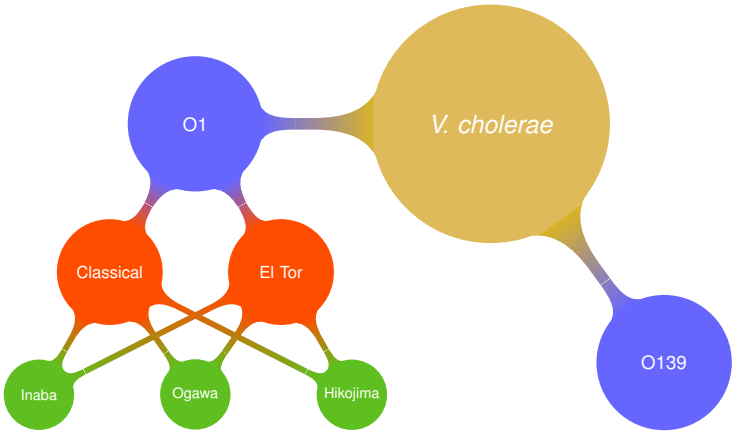
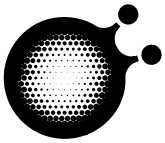


Figure 1.9: The serotype–biotype scheme of *V. cholerae* strains. Main serotypes are blue, biotypes in orange, and sub-serotypes in green. Created in TikZ.[10]

The secretion of EVs from *V. cholerae* was confirmed as early as 1967,[234] while the enterotoxicity of cell-free filtrates was known already in 1959.[235] The vesicles themselves were more thoroughly characterised by electron microscopy in 1992, and were found to react to *V. cholerae*-specific serum and to contain an electron-dense material.[236] Interestingly, *V. cholerae* has a single sheathed flagella, which for symbiotic *Vibrio fischeri* is an important mechanism to radiate EVs that induce morphogenesis of the light-emitting organ in its squid host.[237,238] There are many features that make *V. cholerae* interesting in the context of EV-based genetic transfer. Beyond the fact that its EVs have been proposed and shown preliminary success as vaccines (as discussed in Section 1.4.1), it has some very interesting genetic characteristics. Firstly, the *V. cholerae* genome is unusual in that it is divided into two chromosomes, Chromosome 1 (Ch1) and Chromosome 2 (Ch2) at approximately 3 Mbp and 1 Mbp, respectively.[239] It could therefore be of interest to investigate whether the inclusion of DNA from Ch1 and Ch2 in EVs may be different. Furthermore, it is the dynamic character of its genome that has given rise to such a span in strain pathogenicity, from

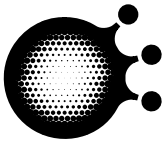
asymptomatic to devastating cholera gravis.[240,241] Indeed, every *V. cholerae* epidemic before 1992 was caused by the serotype O1, when unexpectedly, one emerged in India caused by a novel serotype, O139.[242] Although most epidemics are still caused by the O1 serotype, this event is demonstrative of the fluid nature of the *V. cholerae* genome. This dynamic is caused by a number of mechanisms, such as mobile elements, prophage-based gene storage, and frequent DNA-uptake from the environment.[243] For instance, when grown on chitin, which is an abundant material in crustacean exoskeletons in its natural habitat, *V. cholerae* is naturally competent.[244] Furthermore, CTX is located on CTXϕ, an endogenous filamentous lysogenic vibriophage encoded in copies on both Ch1 and Ch2, so this particular genetic element is especially mobile between strains, and together with its satellite phage RS1 may recombine to generate new forms of CTX.[245] There is another interesting phage structure on Ch1, namely an inverted repeat of Mu-like phage genes flanking a ∼42 kbp stretch of phage-unrelated genes. These genes include tRNAs, H-NS,[246] Cold-shock protein CspD,[247] and Potassium uptake protein TrkA,[248] and the purpose (if any) behind this specific genome architecture is unclear. It is hypothesised that Ch2 may originally have been a megaplasmid acquired by the bacteria, which subsequently inherited genes for housekeeping, metabolism, heat-shock, as well as 16S rRNA genes.[239] It also harbours two interesting mobile features in the context of vesicle-based genetic transfer, namely prophage K139[249] and a superintegron, or "integron island".[239] As inclusion in EVs is dependent on DNA fragment size,[147] the probability of inclusion of mobile fragments such as integron-carried elements or prophage is presumably higher than other regions of the genome.



Chapter 2

Aims of the Project

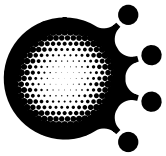
The utilization of EVs as vaccines and therapeutic agents has gained popularity, both in human medicine and veterinary sciences. Beyond their ability to transfer antibiotic resistance, the genetic character and capacity of EVs is still poorly understood, and could have implications for their use as vaccines or therapeutics. Even though sequencing has become commonplace in laboratories across the world, total quantitative DNA/RNA sequence data from EVs is still remarkably scarce, and limited to a handful of investigations that disregard sequence dependencies or the mRNA –protein correlation. This lack of data could be due to the comparatively high quantities of genetic material needed for these protocols, while EV-DNA and -RNA are scarce in bacterial cultures. The methodology behind investigations on this matter is not yet standardized, and require robust, high-throughput EV-isolation protocols. One essential investigation is therefore to quantify the yields of the available protocols directly from identical starting material. The main aims for the project were thus to investigate the nature of DNA and RNA in EVs, and assess the available isolation methods.



Chapter 3
Summary of Papers

Paper I describes an exploratory study on the DNA, RNA and protein content of vesicles from *V. cholerae*, compared quantitatively to their parent bacteria. In addition to enrichment of membrane proteins, phage proteins and phage genes, some peculiar results warrant further studies, such as the observed enrichment of certain specific motifs of RNA and DNA. No positive correlation was found between mRNA and protein quantities, providing little support for the hypothesis of post-secretion translation in EVs.

Paper II entails the assessment of two typical protocols for bulk vesicle isolation (salt-induced precipitation and tangential flow filtration), as simplifying this process would allow more thorough analyses on EVs secreted under different conditions. These methods were compared to the most common EV-isolation method (ultracentrifugation), which is not a practical procedure for large volumes. Assessment criteria were vesicle integrity according to electron microscopy, particle size according to dynamic light scattering, protein composition according to mass spectrometry, as well as DNA-, RNA-, protein- and prophage yields.



Chapter 4
Results & Discussion

EV science is a growing field with countless questions still unanswered. So far, we can safely conclude that many bacteria secrete these complex structures with an underlying purpose, be it membrane remodelling, host immune system interference, predation, biofilm modulation or phage defence. The specific roles for each bacterial strain still leave much to be investigated, and discoveries in this field will likely help us in the development of drug delivery mechanisms, therapeutics, or in our efforts to deal with the rise of antibiotic resistance. Some key questions remain, such as what the native purpose is of OIMVs in contrast to OMVs, as well as how their biogeneses differ. Furthermore, while some potential roles of the DNA and RNA inclusion in EVs have been uncovered, much of EV-borne genetic material still remains to be quantified and characterized for the majority of bacterial species.

4.1 EV Structure

In Paper I, whole-genome DNA and RNA sequencing data from EVs was compared to that of the parent bacteria, and put in context with proteomic quantities according to mass spectrometry. This is the first investigation using this methodology, resulting in several novel and statistically significant results. Several previous papers have undertaken proteomic profiling of EVs, but in our investigation, it was also quantitatively compared to the proteomic profile of the parent bacteria. This is important; as introduced in Section 1.2.3, intuition states that membranes and their associated proteins must be enriched in EVs compared to whole cells due to the higher surface-to-volume ratio. The proteins of highest enrichment (Paper I/Table S2) were generally outer membrane-located, such as Outer membrane protein K, T, U and V (OmpK, OmpT, OmpU, OmpV), outer membrane lipoprotein (YcfL) and penicillin-binding protein activator (YcfM). These specific proteins varied in enrichment in EVs from approximately 7 to infinite (only detected in EVs).

Some theoretical estimates of membrane enrichment in EVs may be useful for assessment of these numbers. Vesicles are usually spheroid and vary in size from 20 to 200 nm across,[250] while bacterial diameters can be ten times this size.[251] For instance, the shape of the *V. cholerae* bacterium is close to a bent cylinder with rounded ends, approximately 0.4 µm in diameter and 1.6 µm long[54] (Fig. 1.4). The bigger size of the bacteria in this case means that outer membrane enrichment in *V. cholerae* vesicles may be as much as >20-fold for the smallest vesicles, or about 6-fold for a typical 100 nm vesicle (Fig. 4.1). Similarly, periplasmic membranes can theoretically be enriched >4-fold (Fig. 4.1). An

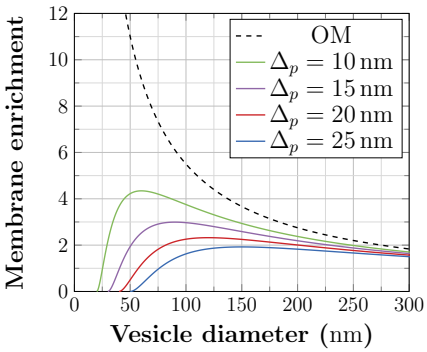


Figure 4.1: The enrichment of periplasmic membranes in *V. cholerae* vesicles compared to the progenitor cell, depending on periplasmic thickness (Δ_p) and vesicle diameter (Section A.1).

essential investigation in order to conclude whether vesicle composition is *specific* is therefore not only to compare the EV proteome to the whole cells, but to compare the balance of proteins for each subcellular location separately. As a theoretical example, an outer membrane protein may be enriched five-fold (as a fraction of total protein) in EVs compared to bacteria, but still depleted compared to the average for outer membrane proteins. The results in Paper I report that that outer membrane proteins are enriched about $2^5 (= 32)$ times compared to cytoplasmic proteins, and about $2^2 (= 4)$ times compared to total protein (Paper I/Fig. 8). This is roughly comparable to the theoretical values, at least when one considers the contribution of extracellular proteins, which may lower the number to a certain extent.

When it comes to specificity of EV proteins, the specific enrichments can mainly be asserted within each subcellular location, for instance the outer membrane or the periplasm. Table A.1 in Section A.4 lists some of the most enriched or depleted proteins of EVs according to each subcellular location

(detected by mass spectrometry as a part of the study featured in Paper I). For instance, only three outer membrane proteins are enriched more than one standard deviation in the set, being a long chain fatty acid transport protein FadL2 and two TonB-dependent proteins HutA and IrgA. Some of these proteins are visible outliers in Figure 4.2, which shows the enrichment of proteins within each location. Quite a few other outer membrane-, periplasmic- and inner membrane proteins are apparently enriched in regard to their subcellular location. For instance, a phage lambda receptor is enriched in the outer membrane, which is interesting, as EVs have been found to protect *V. cholerae* from phage infection.[95] It could be that EVs are enriched in certain phage receptors in order to increase the probability of intercepting phages that could target the bacteria. Another interesting observation is that this protein, encoded in Ch2, is genomically located directly upstream of MalE, and downstream of Lys-arg-orn-binding protein, both enriched proteins of the periplasm. As genes of related function tend to be grouped together, it is not unthinkable that some operons or genomic regions have EV-specific function and enrichment. Furthermore, the proportion $\frac{\text{proteins unique to EVs}}{\text{proteins unique to either sample}}$ is 49% for Ch1 and 71% for Ch2 ($p = 0.067$). Ch2 has been hypothesized to be associated more with virulence than Ch1,[252] and the increased presence of these proteins in vesicles may stand testament to the association of EVs with virulence.

However, this analyses is based on predicted subcellular locations, and in order to assess a high, positive enrichment of a protein in any other location than the outer membrane, one has to make sure this protein is properly localized. For instance, an outer membrane protein that is predicted to be an inner membrane protein would be more enriched in a sample than other, correctly localized inner membrane proteins. The software used for prediction in Paper I reports a precision $\left(\frac{\text{true positives}}{\text{true positives+false positives}}\right)$ of 97.3% for Gram-negative bacteria, meaning that several proteins may be wrongly localised.[191] Another thing to consider is the simplification involved in location prediction, as the real situation is not this simple. A protein may be soluble without any transmembrane domains, and still bind to- or have affinity towards the a membrane or its embedded proteins. This could render a periplasmic protein –practically speaking– an outer membrane protein, albeit connected from the inside. Such a protein would presumably be more enriched in vesicles than the average for periplasmic proteins.

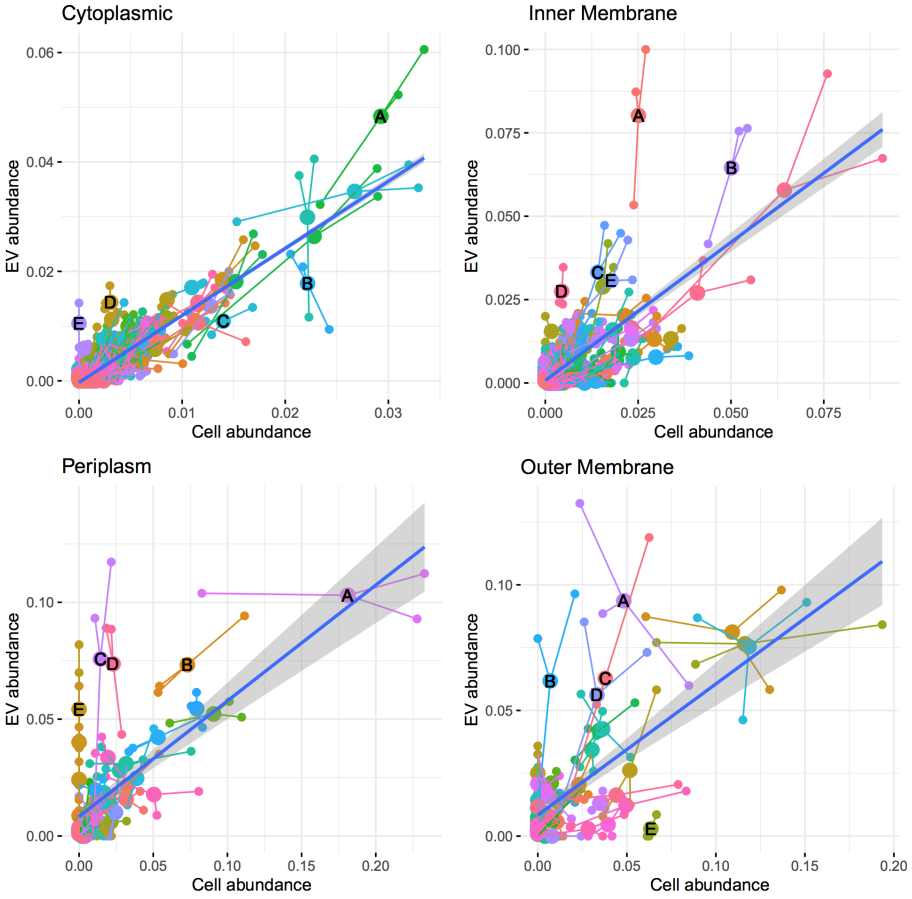


Figure 4.2: Relative abundance of detected proteins in vesicle samples plot against whole-cell samples from Paper I, according to subcellular location predicted by PSORTb.[191] Regression line with error margins are shown. Proteins of interest (A-E) for each location: **Cytoplasmic:** A: Heat shock protein 60 family chaperone GroEL (Ch1:2388533–2390167). B: Chaperone protein DnaK (Ch1:413656–415563). C: DNA-directed RNA polymerase beta subunit (Ch1:2884500–2888525). D: Na(+)-translocating NADH-quinone reductase subunit A (Ch1:2004815–2006101). E: Mu-like prophage FluMu protein gp29 (Ch1:705566–707125). **Inner Membrane:** A: LppC putative lipoprotein (Ch1:100396–102201). B: Peptidyl-prolyl cis-trans isomerase PpiD (Ch1:1622042–1623901). C: PTS system: fructose-specific IIA/IIB/IIC component (Ch1:1522544–1524445). D: Tail-specific protease precursor (Ch1:1162238–1164235). E: Cytochrome d ubiquinol oxidase subunit I (Ch1:1537994–1539580). **Periplasmic:** A: UDP-sugar hydrolase (Ch1:1873752–1875413). B: Maltose/m.-dextrin ABC transporter MalE (Ch2:327842–329023). C: Maltose operon periplasmic protein MalM (Ch2:249540–250376). D: Ch1:1532264–1533616 tolB protein precursor. E: Flagellar basal-body rod protein FlgB (Ch1:1901989–1902384). **Outer Membrane:** A: Maltoporin / phage lambda receptor protein (Ch2:248097-249335). B: Long-chain fatty acid transport protein (Ch1:604748–606034). C: OmpU (Ch1:165614–166639). D: OmpV (Ch1:962284–963057) E: N-acetylglucosamine regulated methyl-accepting chemotaxis protein (Ch1:2171660–2172916).

4.1.1 mRNA–Protein Correlation

Paper I also features a comparison between enrichment of mRNA and enrichment of their protein products in EVs. This is the first published result of its kind (Paper I/Fig. 7), and reveals that no subcellular protein location yields a positive correlation between mRNA and protein product. Curiously, there is actually a negative mRNA–protein correlation for periplasmic proteins ($p = 0.0047$). This specific analyses was undertaken in order to quantify potential intravesicular protein translation, but did not lead to evidence in support of this hypothesis. A number of other analyses were attempted to identify actual characteristics of the proteins enriched in EVs, with no positive results. For instance, no correlation was found between enrichment and protein size, or presence of signal peptides.

4.2 EV Biogenesis

The proteomic investigation in Paper I does not in itself consider the biogenesis of EVs, but the data can provide some insight regarding certain questions. A central question in the literature is OIMV biogenesis and the involvement of PG. As mentioned in Section 1.2.2, some have proposed that holes in the PG wall can allow protrusion of IM into blebs of OM, facilitating OIMV biogenesis. This would imply that peptidoglycan-associated proteins, such as penicillin-binding proteins (PBPs), could be depleted in an EV fraction compared to the whole cells.[253] However, the potential quantity of PBP binding to the fragmented PG makes this more difficult to assess. In our data (detected by mass spectrometry as a part of the study featured in Paper I), two PBPs were only detected in EVs, while two more were enriched to a statistically significant degree, the rest not detected in either fraction (Table 4.1). With no PBPs found to be depleted, our results are not supportive of the theory that holes in the PG are needed for OIMV biogenesis. However, detection of proteins by mass spectrometry is stochastic, and other proteins in whole cells may be abundant enough to render these specific periplasmic proteins undetected in the bacterial fraction. And as addressed in Section 1.2.3, periplasmic proteins are enriched in EVs by default, meaning any PBP enriched less than 4-fold are not indicative of anything more than an approximately similar proportion of PBP in the periplasmic protein fraction of EVs and bacteria.

Table 4.1: Enrichment (Enrich.) of penicillin-binding proteins (PBPs) in EVs from *Vibrio cholerae* compared to whole cells, according to liquid chromatography–mass spectrometry (MS). The genes for all these PBPs are located on chromosome 1. Three proteins were not detected (ND) in either fraction.

Start (bp)	End (bp)	p-value	Enrich. (Log_2)	Name
965698	968769	0.0001	inf	Membrane carboxypeptidase (penicillin-binding protein)
119547	121880	0.0075	inf	Multimodular transpeptidase-transglycosylase (PBP-1B/mrcB)[254]
1589229	1589993	0.0001	2.04	FIG01200265: hypothetical protein (CsiV)[255]
2361652	2364129	0.015	0.26	Multimodular transpeptidase-transglycosylase (PBP-1A/mrcA)[254]
507418	509334	ND	ND	Penicillin-binding protein 2 (PBP-2/MrdA/penA)[256]
2132789	2134531	ND	ND	Cell division protein FtsI [Peptidoglycan synthetase]
2988258	2988746	ND	ND	Rod shape-determining protein MreD

4.3 DNA Specificity of EVs

Total DNA sequencing yields large amounts of quantitative data that can be utilized in a virtually endless number of analyses. A substantial amount of work during the project has therefore been required not only to investigate the possible tools to apply, but to form scopes limited enough to fit within a publication. This being said, an initial observation in the data from Paper I is that even in the whole-cell samples, the number of copies of each portion of the genome was not the same (Paper I/Fig. S2). These anomalies were also distributed at locations remote to known replication origins. This does point to a level of stochastic replication of DNA throughout the genome, and implies that cell death is not necessary in order to incorporate chromosomal DNA into EVs, as some have suggested.[30] Furthermore, the genomic regions that where of higher abundance were different for whole cell samples and EVs. This implies that not only are parts of the genome copied or absorbed by the whole cells at a higher rate than others, but that another, identifiable set of genomic regions are preferably packaged in EVs. These regions were identified (Paper I/Fig. 2), and revealed that DNA for e.g. outer membrane proteins and DNA-binding proteins protein were enriched in EVs. Additionally, it was found that DNA with higher AT-content was generally enriched in EVs (Paper I/Fig. 3), perhaps due to the

higher AT-content of intergenic regions, which are typically the targets for DNA-binding proteins. While it is curious that DNA from many different parts of the genome (without apparent sequence similarities) were enriched to a statistically significant degree, one sequence-dependency was striking. The enrichment of DNA correlated quite well with the presence of the motif `AAAAAANATNAAA` (Figure 4.3).

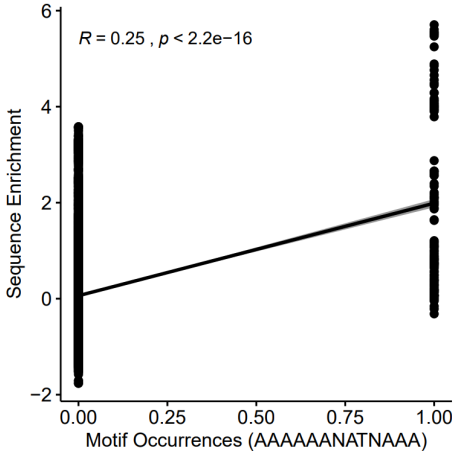


Figure 4.3: The enrichment of kbp windows of the genome, according to the number of `AAAAAANATNAAA` motifs present.

This is the binding motif of ToxR, a membrane-bound transcriptional regulator of *V. cholerae*.[257] Due to its subcellular location, this protein could provide a direct connection between DNA and the membrane, and thus EV-enrichment of sequences harbouring this motif. A follow-up pilot experiment was performed with this in mind, aiming to determine whether the presence of such a motif would increase plasmid enrichment in EVs (details on methodology in Section A.5). In short, plasmid pUC19 was modified by the addition of such a ToxR-binding motif (plasmid was named pToxR), and *V. cholerae* was transformed with either pUC19 or pToxR. The enrichment of these plasmids in EVs secreted at conditions known to induce or repress ToxR-regulated gene expression were determined by qPCR. The results of this pilot experiment are shown in Figure 4.4, and indicate that plasmid enrichment is in fact modulated by the presence of a ToxR-binding motif.

The most striking observation in the initial conditions (after overnight growth at either inducing or repressing conditions) is that the enrichment of plasmid is in general much higher in inducing compared to repressing medium, which in itself warrants further investigation. Although the initial conditions yield

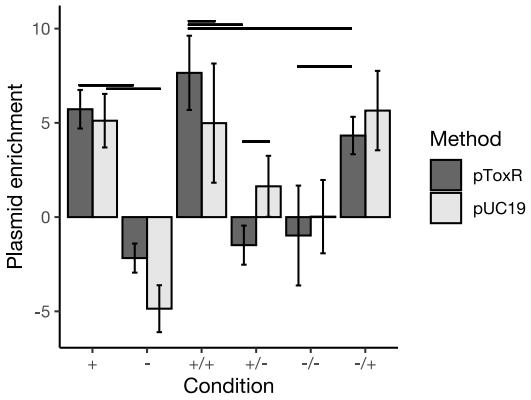


Figure 4.4: The enrichment in EVs of plasmid pUC19, and modified pUC19 with added ToxR binding motif (pToxR), at steady state ToxR-inducing and -repressing conditions (denoted + and -, respectively), and 30 min after transition between conditions (denoted with former condition and latter condition before and after slash, respectively). Two primer sets in each plasmid were pairwise compared, meaning statistical significance is poorly reflected in the standard deviations. Horizontal bars indicate a p-value less than 0.03 for a pairwise Student's t-test.

similar enrichments of both plasmids, certain transitions induce significant differences, even just the change to fresh medium of identical initial conditions. The enrichment of pToxR is actually significantly higher than pUC19 in this case. The opposite is observed in the transition from inducing- to repressing conditions, where pUC19 is actually more enriched than pToxR. Although it raises more questions than it answers, this small pilot experiment reveals significant differences between the two plasmids and encourages a more thorough investigation.

However, in this experiment, pToxR was created by deleting a 212 bp fragment and replacing it with a 33 bp fragment containing the ToxR-binding motif, making pToxR slightly shorter (9%) and less complex than pUC19. As reviewed in Section 1.5.1, the inclusion of plasmids in EVs is dependent on length,[147] making this experimental setup suboptimal. The construction of a control vector was attempted, with the exact same AT-content, length, and lack of stretches of T's or A's, but the inserts were prone to self-annealing/folding and ligation proved ineffective in closing the linearized vector. Therefore, a future control vector may need to be constructed with more lenient sequence constraints on the insert.

4.4 RNA Specificity of EVs

In Paper I, many RNAs that were highly enriched in EVs were identified. For instance the genes encoding outer membrane proteins V, K and U, as well as DNA-binding proteins such as HUα and -β (Paper I/Fig. 5). Furthermore, a significant correlation was found between purine content and mRNA enrichment (Paper I/Fig. 6A), as well as between mRNA length and enrichment (Paper I/Fig. 6B). The former has a possible explanation in that purine tracts may provide thermostability, increasing their longevity in EVs during isolation.[258] The latter has a possible intuitive explanation in the inherent size-exclusion bias of EVs. As mentioned in Section 1.5.1, plasmid inclusion in EVs is inversely proportional to size.[147] The fact that purine content may provide stability, and that purine-rich RNAs are enriched in EVs points to a relevant problem. As discussed in Section 1.5.2, the rapid turnover of RNA can lead to loss of specific RNA during the isolation process, giving rise to enrichment patterns that are not really EV-specific, per se. On the other hand, the RNA present in vesicles *in vivo* is also subjected to thermal stresses and RNases, so if any RNA cargo is of important downstream function, increased stability would be beneficial. After all, it does not make sense for a bacterium to pack a large amount of RNA in EVs if it is rapidly degraded before the EV reaches its target. The remaining RNAs in EVs may therefore be present because they are stable, and be stable because they need to be present. In fact, it is known that certain sRNAs can fold into very stable forms, and EVs may be a tool to protect these structures further from external degrading enzymes or conditions.[259] Purine-tracts are not the only sequence-dependency in mRNA degradation, as some of the RNases responsible for parts of this process are sequence-specific.[260] In order to control for intrinsic RNA turnover, it could be useful to characterize the RNA degradation pattern in the bacteria, for instance by subjecting a bacterial culture to a transcriptase inhibitor (such as rifampicin) a certain period of time before RNA isolation and sequencing.[261]

Similar to the previous findings introduced in Section 1.5.2, Paper I reports the enrichment of certain sRNAs (Paper I/Fig. S4). In addition, the RNAs of three hypothetical proteins were also enriched significantly (Paper I/Table. S1), without any protein product of said genes being detected by mass spectrometry (genes FIG01199699, FIG01200169 and FIG01200711, nucleotide sequences included in Section A.6). They can be low-abundance proteins, but the much higher RNA abundance in the extracellular fraction, as shown in Figure 4.5, makes it tempting to investigate whether they can have sRNA function. If these genes had

no predicted RNA folding or sequence similarities to human or animal genomes, potential msRNA-effects could largely be ruled out, but certain short stretches of identity were detected, as shown in Figure 4.5. This indicates that there are a number of potential RNA interference sites. For instance, FIG01199699 has a number of msRNA-sized hits in the human genome, e.g. a 16/16 nucleotide match within a gene encoding sodium/calcium exchanger 1 (NHE1), which is known to help maintain pH homeostasis.[262] FIG01200169 had a 19/19 identity with Kruppel like factor 3 (KLF3), which if impaired by RNA interference or mutation leads to the accumulation of lipids in the gut.[263] FIG01200711 has a 19/19 hit with collagen VI alpha-5 chain (COL6A5), which is regularly transcribed in the small intestine.[264] Similarly, these RNAs do have msRNA-sized sequence identities to homologous genes in mice, as well as to other genes in certain species of fish, both of which are potential hosts for the bacterium.[265,266] However, before functional studies are undertaken, these RNAs can only be assigned hypothetically function, simply inferred from bioinformatics.

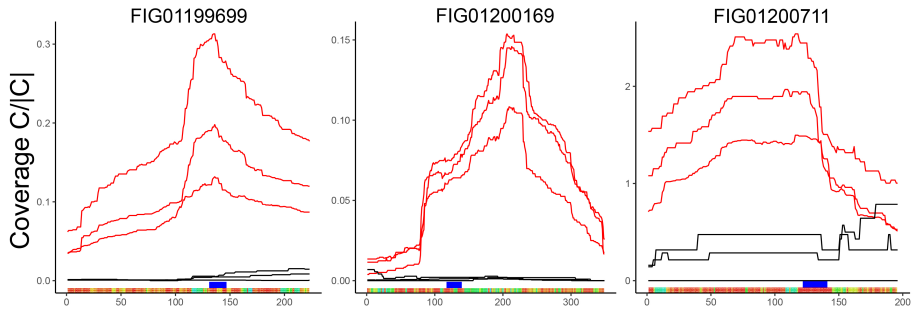


Figure 4.5: RNA sequencing coverage from EVs (red) and whole bacteria (black) over three enriched hypothetical genes (FIG01199699, FIG01200169, FIG01200711). Data from the investigation reported in Paper I. Read coverage is normalized by genome average coverage for each sample. Above the horizontal axis is base-pairing probability (red high, green low) of the RNA according to RNAfold,[267] and blue bars indicating example sequence similarity hits in the human genome according to BLAST:[268] one region in FIG01199699 maps to sodium/calcium exchanger 1 (NHE1), one in FIG01200169 to Kruppel like factor 3 (KLF3), and in FIG01200711 to collagen VI alpha-5 chain.

4.5 EV Isolation Methods

The major predicament in EV-DNA science is the DNA yield, as introduced in Section 1.6. As mentioned in Paper I, the DNA quantity obtained per liter of *V. cholerae* culture ($OD_600 \sim 1$) is only about 3 ng. However, it is possible to amplify the total DNA by multiple displacement amplification (MDA). This

would reduce the necessary amount of DNA before sequencing drastically, but the potential introduction of amplification bias had to be investigated. A pilot was therefore performed for Paper I, where five MDA replicates were produced from the original DNA samples, and sequenced in parallel with them. Sequencing on MDA-samples did in fact yield data that exhibited characteristic "bounces" in coverage, compared to high-concentration DNA-samples. (Fig. 4.6). The induction of bias in the MDA-samples led us to deem the method unfit for qunatitative sequencing, and were forced to investigate other protocols in our search for more efficient EV-DNA-isolation protocols.

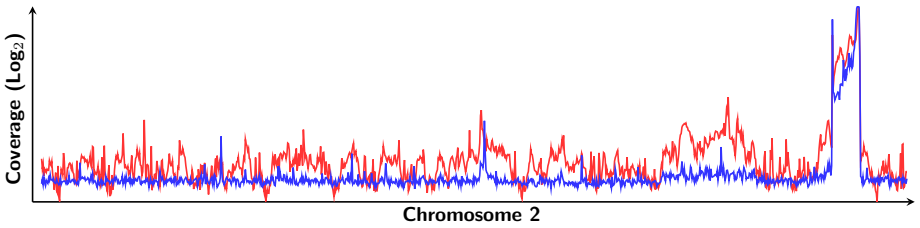


Figure 4.6: Normalized coverage (kbp average, log scale) of sequencing data from MDA-sample (red) and non-MDA, high concentration sample (blue), mapped to Chromosome 2 (∼1 Mbp) of *V. cholerae*. Long stretches of uneven amplification is visible in the MDA sample.

4.5.1 Tangential Flow Filtration and Salt-Induced Precipitation

In the search for a higher-throughput EV-DNA isolation protocol, tangential flow filtration (TFF) was utilized for larger volumes of cell-free filtrate for Paper I, but this method is a bit cumbersome and requires a more complex setup. This is why the potential of EV harvest through salt-induced precipitation (SIP) was investigated in Paper II, albeit with underwhelming results. The DNA yield with this protocol was orders of magnitude lower than with ultracentrifugation or tangential flow filtration, making the technique largely undesirable. A related comment was reported in one of the earlier papers on EVs from *H. influenzae*, in this case referred to as transformasomes. The group mentions (based on preliminary, unpublished data) that the DNA-yield from transformasomes was low after a protocol that included SIP and a DNA-isolation column, and concluded that "[...] the DNA-binding activity may have been lowered by the isolation procedures."[139] Paper II provides more definitive information on just how poorly SIP performs for the purpose of DNA isolation.

The method does however provide comparably high quantities of protein, and does deserve a proper optimization before being completely disregarded

for genetic isolation. An interesting result in Paper II is that the depletion of DNA after DNase-treatment in SIP-isolates is considerably higher than the depletion of RNA after RNase-treatment. This could suggest that a portion of the RNA in the EV-sample is protected from RNases even after the EV membrane integrity is compromised, soliciting further investigation. Ammonium sulphate was used as the precipitating agent in Paper II, but alternative materials are also being researched, such as polyethylene glycol (PEG), which could potentially provide more gentle precipitation.[269] A similar effect was observed with PEG in experiments investigating DNA condensation, where DNA in solution tended to localize to local microvolumes upon addition of higher concentrations of PEG.[270]

The technology of EV isolation for the purpose of genetic sequencing is a field in development, and the major bottleneck is the harvest of EVs from large volumes of cell-free medium (or alternatively creating a total-DNA amplification protocol that does not introduce bias). If a rapid, simple method were to be developed for this, the horizon for large-scale investigations will be extended immensely. For instance, in our case of *V. cholerae*, quantitative genetic sequencing of EVs secreted under a series of different conditions, such as ToxR-inducing conditions, or subjection to stresses such as bile salts could be assessed more thoroughly, and could reveal more about the many purposes of EV secretion.

4.5.2 Ultracentrifugation

In Paper II, TFF and SIP were compared to the current laboratory standard, ultracentrifugation (UC). But as reported in the article, several aspects of the current EV isolation standards are problematic. For instance, many determine EV size by dynamic light scattering of samples isolated by UC, which introduces size bias in two ways. One is the differential centrifugation effect, as smaller vesicles will need more time to sediment. This could tempt the operator to increase the sedimentation time or -speed, but this increases the second effect, which is the aggregation of sedimented samples. It is not clear if the aggregation involves membrane fusion between the vesicles, but the particle size in the sample is measurably higher after UC, as demonstrated in Paper II. This may not be of much importance when using EVs for e.g vaccine candidates, but does to some degree affect results in basic EV-research. The simulation data in Paper II does show that ultracentrifugation can potentially induce a bias to the average sizes of EV populations. By a similar simulation to the one presented in Paper II (details in Section A.2), we can investigate how the average diameter of UC-isolated vesicles will deviate from the real average of the sample according

to centrifugation time (Figure 4.7). We can see that the average diameter of these EVs is 10% higher after a 2 h centrifugation, with a standard deviation more than 12% lower (than the values for the original sample). Additionally, if the pellet is resuspended in a buffer and centrifuged again to wash the vesicles, this effect could be even more significant.

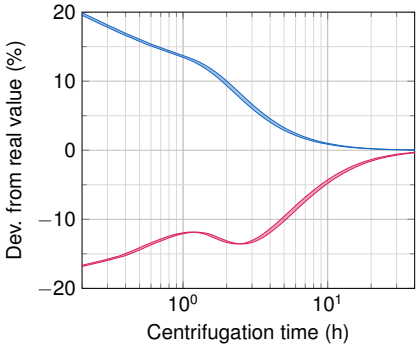


Figure 4.7: Estimated relative deviation between average diameter (blue) and standard deviation (red) of sedimented EVs and the real average diameter (43.3 nm) and standard deviation (13.4 nm) of the original sample during a 28,000 RPM centrifugation of a common 38.5 mL centrifuge tube in a SW32 Ti rotor. Vesicles were from 5–105 nm (Section A.2), with density 1107–1160 kg·m^{-3}

4.6 Future Perspectives

Some of the results from our DNA sequencing solicit further investigation. Firstly, the potential inclusion of prophage in EVs can be elucidated by subjecting EV samples to proteinase K digestion before the DNase treatment prior to DNA isolation. This could minimize the contribution of capsid-protected DNA and more appropriately determine the location of the genetic material. It could be that some level of cell lysis occurs during phage release, and with a potentially enriched prophage sequence present in the cytoplasm at this point, it may be enriched in the EVs regardless of the presence the complete phage particles. On the same note, it would be interesting to investigate whether these proteinase-treated vesicles could transfer the prophage to non-carrying bacteria, providing phage K139 with an additional transmission pathway. Clearly, this would also affect the surface proteins of the EVs, changing their native interaction patterns.

As mentioned in Section 4.4, there are some sRNA/msRNA candidates present in the EV-RNA, although a thorough investigation is warranted. This would include RNase-treating the vesicles before RNA isolation, and subjecting cell

lines of interest (for example intestinal cell lines) to specific RNA candidates and quantifying reduced levels of protein translation. Furthermore, investigating the natural turnover pattern of RNA in the bacteria may provide a basis of comparison for the EV-RNA.

The effects of membrane-located or -associated DNA- and RNA-binding proteins should also be elucidated, with investigations such as the pilot described in Section 4.3. Understanding where nucleotide-binding proteins are located, and which sequence affinities they have, could provide potent tools for customizing nucleotide enrichment in EVs. For instance, this could allow deliverance of therapeutic or interfering RNAs to eukaryotic cells or silencing RNA to infectious bacteria. Furthermore, we could enrich certain DNAs for therapeutic, vaccine, or transformation purposes.

In Paper I, *V. cholerae* was grown under common laboratory standards, such as 37 °C and typical LB broth. An interesting future project would therefore be to investigate different conditions, such as different pH, temperature and addition of low concentrations of antibiotics or bile salts that could potentially induce other patterns in EV secretion. The DNA-, RNA- and protein characteristics of EVs secreted in an intestine-like environment may be different from those secreted in an aquatic environment, and could reflect the differing requirements of the niches.

Lastly, the work in this thesis is largely undertaken on *V. cholerae*, making extrapolation of the results challenging in some ways. It would therefore be of interest to repeat the analyses of Paper I on EVs of other Gram-negative bacteria, in order to detect more general patterns.

4.7 Conclusions

Solid exploratory research is absolutely essential to gain a proper overview of complicated systems such as bacterial genetic transfer and vesiculation, and provides ideas for novel projects in the field. Paper I is the first study of its kind, in that it investigates the sequence-dependency of enriched RNAs/DNAs in EVs, and looks at the correlation between mRNA and protein enrichment. The data obtained suggest not only that EV-DNA/RNA is specific, but that bacterial chromosomal DNA is also present in cells in uneven quantities. Although many targets for further research was obtained, follow-up experiments would have to be conducted in order to confirm and quantify the role of each candidate. In an attempt to streamline sample acquisition for genetic work on vesicles, Paper II considers two large scale EV-isolation protocols, and gives reason to discourage

the use of ammonium sulphate as a precipitating agent in EV-isolation.

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Papers

Paper I

Vesicles from *Vibrio cholerae* contain AT-rich DNA and shorter mRNAs that do not correlate with their protein products

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Vesicles From *Vibrio cholerae* Contain AT-Rich DNA and Shorter mRNAs That Do Not Correlate With Their Protein Products

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Extracellular vesicles secreted by Gram-negative bacteria have proven to be important in bacterial defense, communication and host–pathogen relationships. They resemble smaller versions of the bacterial mother cell, with similar contents of proteins, LPS, DNA, and RNA. Vesicles can elicit a protective immune response in a range of hosts, and as vaccine candidates, it is of interest to properly characterize their cargo. Genetic sequencing data is already available for vesicles from several bacterial strains, but it is not yet clear how the genetic makeup of vesicles differ from that of their parent cells, and which properties may characterize enriched genetic material. The present study provides evidence for DNA inside vesicles from *Vibrio cholerae* O395, and key characteristics of their genetic and proteomic content are compared to that of whole cells. DNA analysis reveals enrichment of fragments containing ToxR binding sites, as well as a positive correlation between AT-content and enrichment. Some mRNAs were highly enriched in the vesicle fraction, such as membrane protein genes *ompV*, *ompK*, and *ompU*, DNA-binding protein genes *hupA*, *hupB*, *ihfB*, *fis*, and *ssb*, and a negative correlation was found between mRNA enrichment and transcript length, suggesting mRNA inclusion in vesicles may be a size-dependent process. Certain non-coding and functional RNAs were found to be enriched, such as VrrA, GcvB, tmRNA, RNase P, CsrB2, and CsrB3. Mass spectrometry revealed enrichment of outer membrane proteins, known virulence factors, phage components, flagella and extracellular proteins in the vesicle fraction, and a low, negative correlation was found between transcript-, and protein enrichment. This result opposes the hypothesis that a significant degree of protein translation occurs in vesicles after budding. The abundance of viral-, and flagellar proteins in the vesicle fraction underlines the importance of purification during vesicle isolation.

Keywords: *Vibrio cholerae*, extracellular vesicles, bacteriophages, ncRNA, extracellular genetics, extracellular proteomics

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1. INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound bodies regularly secreted by Gram-negative bacteria (Listgarten and Lai, 1979). At large, EVs consist of the same proteins, RNAs, DNAs, metabolites and lipopolysaccharides as their originator cell, but some reports indicate that specific proteins are enriched (Haurat et al., 2011; McMahon et al., 2012). EVs display highly diverse characteristics in shape (McCaig et al., 2013), single-, or double membrane structure (Pérez-Cruz et al., 2013), and typically vary in diameter by an order of magnitude (20–200 nm) (Chatterjee and Chaudhuri, 2011). Several bacterial mechanisms have been proven to be associated with the secretion of EVs, such as biofilm formation, nutrient acquisition and secretion of virulence determinants into host cells (Kulp and Kuehn, 2010). For example, EVs inhibit the adhesion of the pathogen *Xylella fastidiosa* inside xylem vessels, enabling wider spread throughout host plants (Ionescu et al., 2014), and they arm *Vibrio cholerae* with a defense mechanism against bacteriophages (Reyes-Robles et al., 2018). Interestingly, EVs can also transfer membrane portions that contain phage receptor proteins, and in this way propagate susceptibility to certain phages (Ofir and Sorek, 2017). This raises the question whether some phages could induce production of EVs for this very purpose. Other findings indicate that certain plasmids may induce the production of EVs, and thereby facilitate their own spread (Erdmann et al., 2017).

EVs are known to contain mRNA and non-coding RNA (ncRNA), and it has been demonstrated that they can deliver their RNA cargo into eukaryotic cells (Dauros-Singorenko et al., 2018). While some inclusion of RNA is expected when volumes of the intracellular space is incorporated during vesicle formation, the mechanisms behind DNA inclusion in EVs is still unclear. When not undergoing chromosome replication, it is generally assumed that bacteria contain a similar quantity of any part of their chromosomes. The same is not necessarily true for vesicles, which could be secreted in order to communicate specific DNA sequences into the environment. While the genetic transfer capability of certain EVs is established, it is of interest to gain an overview of which DNAs and RNAs that are specifically enriched, and understand how these might affect their environment. The genetic content of EVs from several bacteria have previously been sequenced (Biller et al., 2014; Sjöström et al., 2015; Bitto et al., 2017), revealing that specific genome regions and transcripts are significantly more abundant than others. These data, however, were not quantitatively compared to coverage discrepancies in sequence data from the parent bacteria. It is not known whether the genetic content of vesicles is actively transcribed or translated after secretion, calling for a precise proteomic profile of the vesicles in question. Being that EVs have a lower surface area to volume ratio, intuition states that vesicles should contain a higher proportion of membrane-associated proteins than their parent cell, but they may also be enriched with non-membrane proteins of vesicle-specific function. The proteomes of EVs from several bacteria have been mapped previously, revealing that they include proteins from all subcellular locations, while mainly membrane proteins, such as those related to biofilm formation, virulence and antimicrobial resistance, are enriched (Altindis et al., 2014; Lagos et al., 2017).

Due to their compositional similarity to their parent and non-replicative nature, EVs have been proposed as vaccines against many pathogens (Acevedo et al., 2014), and is being used commercially against e.g., *Neisseria meningitidis* (Holst et al., 2009). As vaccine candidates, it is important to assess the capacity of EVs for genetic transfer, since they may potentially be administered in environments that frequently contain other pathogens, such as hospitals, aquaculture facilities or livestock farms. They are known to enable cross-species transfer of virulence genes, including antibiotic resistance (Yaron et al., 2000), which may call for some restrictions when it comes to strains, antibiotic markers and growth conditions that are fit for the production of EV-based vaccines. Furthermore, the production of vesicles carrying specific DNAs or RNAs may be of interest in therapeutic or microbiological applications, underlining the importance of identifying motifs or other attributes that may increase genetic enrichment. While some basic research on vesicles requires purification steps such as density gradient centrifugation, this may not be cost-efficient for some industrial or pharmaceutical purposes, e.g., vaccines meant for farmed fish. It is therefore of interest to analyse the complete EV isolate, so that any non-EV material included in a potential vaccine is not ignored.

The main aim of this work was to investigate the potential preferential inclusion of genetic material in EVs, using *V. cholerae* O395 mutant TCP2 (Mekalanos et al., 1983) as a model organism. *V. cholerae* is naturally competent when grown on chitin, an abundant material in its natural environment (Meibom et al., 2005), indicating that it maintains a level of interspecies genetic communication. Furthermore, *V. cholerae* continues to be a detrimental pathogen, meaning that the results may carry strain-specific medical relevance. The TCP2 mutant lacks a complete CTXϕ bacteriophage, including the genes encoding both subunits of the cholera toxin that leads to the debilitating diarrhea associated with cholera. CTXϕ is a temperate phage, and its genomic DNA may at times be present in wild-type O395 strains in quantities sufficient to overshadow other DNA fragments during sequencing. In addition, the two copies of toxin co-regulated pilin precursor gene *tcpA* are deleted, which could have given rise to excessive non-vesicular extracellular matter. The TCP2 genome harbors a streptomycin resistance gene, and the use of a selective medium minimizes the risk of interference by foreign DNA from potential contamination. The genome of *V. cholerae* is divided into chromosome 1 (ChI) at approximately 3.0 Mbp, and chromosome 2 (ChII) at 1.1 Mbp, which may give further insight in specific packaging of genetic material into the EVs (Sjöström et al., 2015). In general, ChI harbors the larger portion of essential genes, while ChII encodes a larger proportion of hypothetical proteins (Cameron et al., 2008). ChII includes a ∼35 kbp K139 prophage (Reidl and Mekalanos, 1995), as well as a superintegron containing hundreds of seemingly species-specific gene cassettes (Rowe-Magnus et al., 1999), which may also be differentially included in EVs.

2. METHODS AND MATERIALS

2.1. Cultures and Media

All cultures were grown using LB medium or LB agar at 37 °C with 200 µg/mL streptomycin, and liquid cultures were grown at 100 rpm shaking. Frozen stock of *V. cholerae* was plated and grown overnight. Two 100 mL starter cultures were inoculated from plate colonies and grown overnight. Twenty-five milliliters of starter culture was added to each of four or eight 2.5 L volumes of medium, and grown overnight to late log phase, i.e., $OD_{600} \approx 1$ (**Figure S1**), when contamination from lysed cells is minimal (Sjöström et al., 2015). The process was performed three times to yield independent biological triplicates ($n = 3$).

2.2. EV Isolation

The EV isolation protocol is largely the same as described for marine samples (Biller et al., 2014). A minimum of 10 L *V. cholerae* culture was centrifuged (using Andreas Hettich Bottles: 0551, part no. 4AJ-7900519) at 4,000 × *g* for 30 min, transferred to cleaned bottles and centrifuged at 4,000 × *g* for another 30 min. The bacterial pellets were kept at −20 °C until DNA, RNA and protein isolation. The supernatant was filtered through 0.45 µm and then 0.2 µm filters (durapore cat no. HVLP14250 and qvwp14250), using a 142 mm filter holder (millipore corporation 01730) and a peristaltic pump. The volume was then concentrated to approximately 25 mL using a 100,000 NMWC hollow cartridge filter (Ge healthcare 56-4101-72) at 15 psi inlet pressure. Three hundred milliliters of PBS was added and this dilution was again concentrated to approx 150 mL. The volume was split in 10 mL aliquots and kept at −20 °C (< 7 days) to reduce RNA degradation while performing replicates. The whole volume was filtered through a 0.45 µm filter and then ultracentrifuged at 100,000 × *g* for 2 h (SW 32 Ti rotor). The supernatant was discarded and another volume of filtrate was added before another centrifugation (100,000 × *g*, 2 h). The supernatant was discarded and the tubes were filled with PBS and centrifuged again (100,000 × *g*, 2 h). Each pellet was resuspended in approximately 1.5 mL PBS and kept at −20 °C (< 7 days) to reduce RNA degradation until RNA isolation. This isolate is henceforth referred to as the extracellular vesicle fraction (EVf), and each of all three EVf replicates was used for DNA, RNA, and protein analysis, in order to minimize the effect of biological variations between the techniques. The protein content of the isolates was measured to be approximately 11.8 mg/mL. The samples were inspected by negative-stain electron microscopy to assess vesicle integrity and presence of contaminations. A ∼ 100 µl portion of the bacterial pellet was resuspended in 5 ml PBS. This isolate is henceforth referred to as the whole cell fraction (WC).

2.3. DNA and RNA Preparation

Before DNA was isolated, the vesicle isolate was split in 100 µL aliquots, 4U DNase was added to each aliquot and incubated at 37 °C for 30 min. Another 4U was added and the incubation repeated, before the DNase was inactivated at 75 °C for 15 min. RNase was not deemed fitting before RNA isolation, due to the fact that RNases are not as easily inactivated as DNases, and excessive heat or chemical treatment could reduce the integrity of the vesicles or their RNA cargo before isolation. Furthermore, some secondary structures may provide certain RNAs with protection from RNAses, making the definite localization of these difficult (Blenkiron et al., 2016).

2.3.1. DNA Isolation

All DNA quantification was performed using Qubit™ dsDNA HS assay kit. For EVf-DNA isolation, 200 µL aliquots were treated with the Qiagen EZNA mini kit type 1, according to manufacturer's protocol, with the exception of adding four volumes of supernatant lysate to the same HiBind® DNA Mini Column, and eluted in 30 µL kit-provided nuclease-free water. This kit preferentially isolates fragments under 45–50 kbp in length, with an effective cutoff at 150 kbp (Qiagen, 2012). Therefore, this kit was also used to isolate DNA from WC, to ensure similar conditions for both samples, and to avoid coverage lost to genomic DNA (gDNA). Similarly, 200 µL aliquots of WC were used for DNA isolation, as described for EVf-DNA above. The DNA isolation yielded 60–70 ng/µL for WC-DNA samples, and approximately 0.4–0.6 ng/µL for the EVf-DNA samples. This corresponds to approximately 1.75 ng DNA isolated per mg of protein in the EVf, and 3 ng EVf-DNA per liter bacterial culture. The samples were cleaned with Agencourt® AMPure® XP according to manufacturer's protocol. gDNA for genome assembly was isolated from the bacterial pellet with QIAGEN Genomic-tip 100/G according to manufacturers protocol.

2.3.2. RNA Isolation

All RNA quantification was performed using Qubit™ RNA HS assay kit. RNA was isolated from EVf with Allprep® DNA/RNA Mini Kit (Qiagen cat. 80204) treating 200 µL aliquots as starting material, and adding 500 µL RLT buffer. Four to six of these volumes were passed through a single DNA column, and the flowthrough from 2 to 3 of these columns were treated according to protocol, and passed through a single RNA column. RNA was eluted in 30 µL RNase-free water, resulting in approximately 7 ng/µL. This corresponds to approximately 23 ng RNA per mg of protein in the EVf, or 40 ng EVf-RNA per liter bacterial culture. RNA from WC was isolated in the same manner, and yielded approximately 70 ng/µL. The EVf-RNA samples and 1 µg of the each of the WC-RNA samples were prepared with Truseq mRNA stranded kit (Illiumina), quality controlled with NGS kit on Fragment Analyser (ATII), and amplified with KAPA Library Quantification Kit (Roche), all according to manufactures protocol.

2.4. Sequencing and Analysis

DNA and RNA samples were sent to Norwegian Sequencing Centre to be sequenced on Illumina HiSeq 4000, 2x150 paired-end run with 350 bp insert size.

2.4.1. Reference Genome Assembly

The 5.1 M gDNA reads were assembled *de novo* using SPAdes (v3.10.1). They were also aligned to an O395 reference genome (GenBank: GCA_000016245.1) with the burrows-wheeler aligner (BWA, v0.7.8)(Li and Durbin, 2010), resulting in 99.68% of the

reads being successfully mapped. The alignment was inspected in Geneious (v10.1.3)[1] to identify regions with no coverage. Four regions of zero coverage were identified, and replaced with the corresponding sequence data from the SPAdes assembly. After correction, the number of aligned reads increased with 2.6 k reads to 99.73%, with no gaps in coverage. The modified genome was annotated using RAST (Job# 492506) (Aziz et al., 2008). Putative phage genes were predicted using PHAST (Zhou et al., 2011).

2.4.2. Coverage Analysis

The raw EVf-DNA and WC-DNA reads were trimmed using trim-galore (v0.3.3), aligned to the O395 TCP2 genome using BWA, and replicates removed using picard-tools (v2.10.4)[2]. Cuffdiff (through cufflinks v2.2.1) was used for enrichment analysis for DNA and RNA, analogously to an RNA differential expression analysis. Cuffdiff uses a genome annotation file for expression analysis, and since intergenic regions could be of interest, placeholder annotation files were created, assigning an identifier to every kbp of the genome. Ten such files were created with 100 bp offsets, with starting bp = 0, 100, 200..., in order to construct a sliding window enrichment table after enrichment analysis. The GC-content of each sliding window was calculated using bedtools (v2.17.0). The process was identical for RNA data, with the exception that duplicate reads were not removed, and the RAST-annotated genome was used for expression analysis. The enrichment of very abundant ncRNAs was also calculated using per-base coverage provided by bamtools (v2.4.0) (Barnett et al., 2011), normalizing ncRNA coverage by the average RNA read coverage over the full genome for each sample.

2.5. Protein Analysis

Protein was quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific™ 23225) according to manufacturer's protocol. In-solution digestion and liquid chromatography mass spectrometry (MS) was performed on EV and WC samples as previously described (Aqrawi et al., 2017), with the exception that database searches were performed on a protein database with 3920 entries constructed from the RAST-annotated *V. cholerae* TCP2 genome using transeq through EMBOSS (Rice et al., 2000) (v6.5.7). Data were analyzed using Scaffold (v4.8.4, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm, while protein identifications were accepted if they could be established at greater than 99% probability and contained at least 2 identified peptides. Using Scaffold, a *T*-test and multiple test correction with Benjamini–Hochberg was done using WC as fold change reference category. The subcellular location of each gene was found using PSORTb version 3.0.2 (Yu et al., 2010).

2.6. Electron Microscopy

Freshly formvar-coated 200 mesh grids were used for both negative stain- and immunogold electron microscopy, and all micrographs were captured with a JEOL 1400 plus microscope at 100 keV. For negative stain images, grids were placed on droplets of EVf for 1 min, washed 3 times on PBS for 1 min, and fixed on 4% paraformaldehyde (PFA) in PBS for 2 min. Finally, the grids were washed 10 times on H_2O for 1 min, and stained on 4% uranyl acetate (UA) for 2 min. Excess UA was dried off using a filter paper, and the grids were left to dry for 10 min before imaging.

2.6.1. Immuno-Gold Labeling

10 µL of EVf-sample was diluted in 12% gelatine at 37 °C to a total volume of 200 µL. Thirty microliters droplets of the solution were placed on parafilm in room temperature to solidify for 10 min. The solid droplets were fixed in 4% PFA/PBS at 4 °C overnight, then submerged in sucrose and cut into ∼0.5 mm cubes, which were placed on silver rods and flash-freezed in liquid nitrogen. The samples were sectioned in a cryomicrotome at −120 °C to a thickness of approximately 45 nm, and picked up using a loop dipped in 2% methyl cellulose (MC) at 4 °C and transferred onto the grids. The grids were suspended on a droplet of PBS on ice for 3 min, and placed on a droplet of 1:1 Antibody (MAB030 anti-dsDNA clone BV16-13 from Sigma-Aldrich)/1% fish skin gelatine (FSG) for 50 min. The grids were then washed 5 times on PBS for 3 min, before being transferred to a droplet of 1%FSG/proteinA-gold for 20 min. The grids were washed 3 times on PBS for 3 min, followed by 10 times on H_2O for 1 min. Finally, the grids were stained on 4% UA for 2 min, after which the grids were picked up with a loop, excess liquid removed and left to dry for 10 min before imaging.

3. RESULTS AND DISCUSSION

3.1. Electron Micrographs Reveal DNA Associated With EVs From *V. cholerae*

Micrographs reveal that the extracellular fraction of *V. cholerae* contains both double- and single membrane vesicle structures (**Figure 1A**). Some of the visible filamentous structures are likely viral-, flagellar-, or pilin constructs, similar to previous observations (Kondo et al., 1993), but certain endogenous phages of *Vibrio* species can be difficult to distinguish from vesicles (Lorenz et al., 2016), making precise EM characterization of EV samples challenging. No complete tailed phage structures were visually confirmed in our samples. The ultrathin-section micrographs labeled with dsDNA antibodies reveal the presence of DNA within the outline of membrane vesicles (**Figures 1B1–B3**). The background labeling is insignificant, confirming that DNA in the EVf is largely confined within vesicular bodies or embedded in their membranes, although some DNA may still reside within phage particles too small to effectively access during thin-sectioning. This observation is similar to previous localization of DNA in vesicles from *Streptococcus mutans* (Zheng et al., 2009) and *Pseudomonas aeruginosa* (Bitto et al., 2017). The nature of double-membrane vesicle budding allows transport of cytoplasmic matter, including chromosomal DNA. However, regarding the secretion of single-membrane vesicles, intuition states that their interior should originate from the periplasmic space, and therefore not

[1] *Geneious: Suite of Molecular Biology and NGS Analysis Tools.* Available online at: https://www.geneious.com/

[2] *Picard Tools: Command Line Tools for Manipulating High-Throughput Sequencing (HTS) Data.* Available online at: http://broadinstitute.github.io/picard

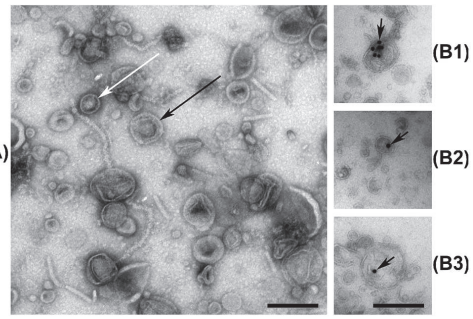

FIGURE 1 | (A) Negative stain electron micrograph of the vesicular fraction of *V. cholerae*, with white and black arrows indicating examples of single-, and double membrane vesicles, respectively. Some filamentous structures are also visible. **(B1–B3)** Ultrathin-section electron micrographs of the vesicular fraction of *V. cholerae* labeled with anti-dsDNA antibodies and gold nanoparticles, indicated by black arrows. Black bars are 200 nm long.

contain considerable quantities of DNA of chromosomal origin. Some unknown mechanism(s) may therefore transport DNA-fragments out into the periplasmic space before budding, or freely diffusing DNA may be absorbed into the periplasm from the extracellular space, before or after budding (Renelli et al., 2004; Mashburn-Warren et al., 2008; Seitz et al., 2014). A so far unreported explanation could be that the outer membrane may be shed during or after budding, due to perforations in the outer membrane, post-budding stress, or some other uncharacterized mechanism. A process reminiscent of this has been reported in the Gram-positive *Bacillus subtilis*, where vesicles form in prophage-induced holes in the peptidoglycan wall (Toyofuku et al., 2017).

3.2. EV-Associated DNA Is Characteristically Different From Whole Cell DNA

Sequencing of WC-DNA yielded 3.7–19.6M reads per sample, of which 97.0–99.3% were successfully mapped to the genome. Sequencing of EVf-DNA yielded 3.7–34.6M reads per sample, of which 97.2–98.6% were successfully mapped to the genome. The mapped read coverage reveals certain regions of the genome that are more abundant across all replicates, some characteristic to either EVf- or WC-samples (**Figures S2A,B**).

3.2.1. Phage DNA

The highest peak in coverage in EVf-DNA spans the K139 prophage, and in WC-DNA spans the superintegron (**Figure S2B**). The ratio between sequencing coverage of ChII and ChI in WC did not differ significantly when including or excluding the K139 prophage (**Figure S2C**), while the difference was considerable for EVf (**Figure S2D**). This stands testament to the abundance of the K139 prophage DNA in EVf, and suggests that the prophage DNA escapes the bacteria into extracellular DNase-protected states shortly after synthesis. Cuffdiff determined several significantly enriched kbp partitions of the genome (**Figure 2**). The K139 prophage was the most enriched DNA in EVf (**Figure 2L**), (some kbp partitions up to 2,300×, and on average 246×), and insert size estimation in Geneious revealed a high number of reads mapped approximately -34.5 kbp apart in ChII, suggesting that the phage is undergoing a significant amount of rolling-circle amplification. Within the ∼700–800 kbp region of ChI, PHAST detected two inverted copies of a Mu-like prophage. Between these prophages, a ∼43,200 bp region encodes a series of genes associated with metabolism and regulation, such as *hns* (Ramisetty et al., 2017), *hisD* (Chiariotti et al., 1986), *cspD* (Yamanaka et al., 2001), and *atoS* (Theodorou et al., 2012). Mu is a dsDNA bacteriophage (Bukhari and Ambrosio, 1978), but its lack of DNA enrichment suggests that it is not a replicating phage under the present growth conditions. Some other partial prophages were detected by PHAST; a triple repeat of phage replication protein Cri (**Figures 2C,E**), as well as remnants of the partially deleted CTXϕ phage (**Figures 2D,I**) were highly enriched in EVf, while not nearly as dramatic as the K139 phage genome. Although K139 DNA is enriched in EVf, the phage structures may not be abundant in electron micrographs, as the DNA density of the capsid is ∼425 Mbp/μm^3 (assuming a roughly spherical capsid 54 nm across and 35 kbp genome Reidl and Mekalanos, 1995), while the average DNA density the of *V. cholerae* interior is ∼22 Mbp/μm^3 (assuming a cylindrical cell shape with hemispherical ends, 1.6 μm long and 0.4 μm in diameter, harboring a 4.1 Mbp genome Baker et al., 1983). Additionally, since many vesicles may not contain DNA, only a few complete capsids could potentially amount to the same DNA quantity as hundreds of vesicles.

3.2.2. ToxR Binding Motifs

There are a number of non-prophage regions that are highly enriched in EVf. The most enriched of these sequences harbor known binding sites for ToxR (**Figures 2A–D,F,G,I**), a transcriptional regulator located in the cytoplasmic membrane. Interestingly, an association between EVs and proteins regulated by ToxR has been reported previously (Altindis et al., 2014). ToxR is known to regulate e.g., transcription of *ompU*, *ompT*, and *acfA* by binding to an upstream binding motif (AAAAAANATNAAA) (Kazi et al., 2016), and the upstream regions of these three genes were all highly enriched, (**Figure 2A,B,F**). Additionally, the most enriched DNA region of ChI (**Figure 2G**) contains this motif, upstream of a putative membrane protein. This protein, a homolog of lysoplasmalogenase YhhN (Jurkowitz et al., 2015), could potentially also be under the regulation of ToxR. All the sites in the genome matching this motif were identified (**Figure S3A**), and we observe the consensus motif for the most enriched (>4×) regions, AAAAAAMATMAAA (M signifying an amine; A or C) (Cornish-Bowden, 1985). DNA mapped to this refined binding motif was significantly enriched (∼5.6×) in EVf (**Figure S3B**), while the most enriched (∼10.6×) unambiguous motif was AAAAAAAATAAAA. ToxR also regulates *ctxAB* by binding to a "toxbox" region (TTTTGAT

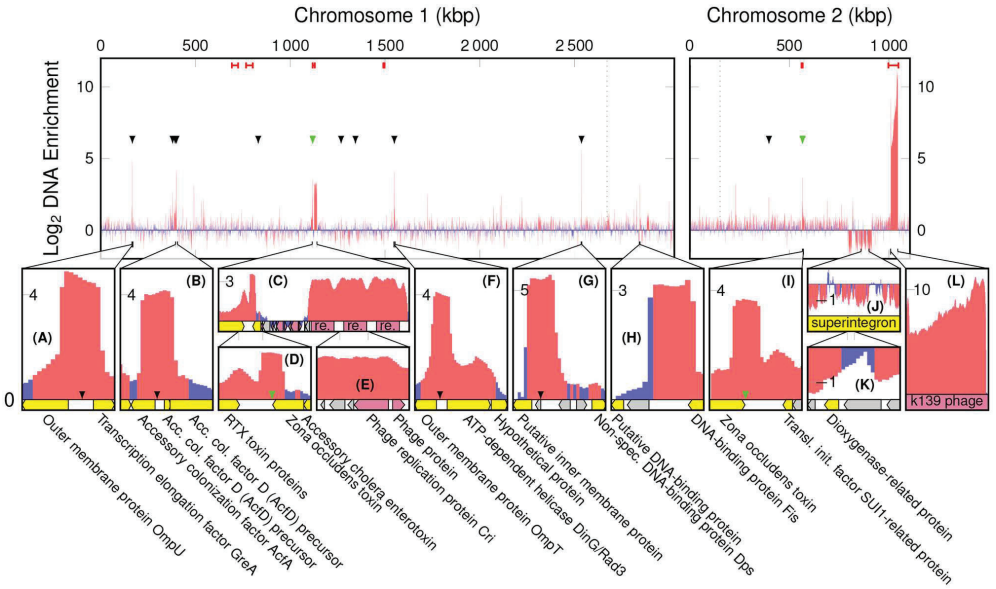

FIGURE 2 | Log_2 enrichment of kbp partitions of the *V. cholerae* genome according to cuffdiff, red denoting statistically significant enrichment. Red bars at the top are regions in which PHAST recognized phage genes, black arrowheads indicate a ToxR binding motif of the form AAAAAAMATMAAA, and green arrowheads indicate a toxbox region (TTTTGAT heptad repeat). Dotted lines are replication origo of the chromosomes. The bottom panels **(A–L)** display regions of interest, in which yellow tracks denote characterized genes, gray hypothetical, and red phage components.

tandemly repeated 3–8 times) (Miller et al., 1987), and while both copies of *ctxAB* is deleted from the TCP2 genome, their toxbox regions are intact and significantly enriched, directly downstream of the gene encoding Zona occludens toxin (*zot*) (**Figures 2D,I**). Aside from *zot*, RTX cytotoxin proteins were enriched (**Figure 2D**), which is similar to a previous report demonstrating that DNA encoding cytotoxin ExoS was abundant in EVs from *P. aeruginosa* (Bitto et al., 2017). The enrichment in EVf of sequences containing ToxR binding motifs may have its explanation in that ToxR is membrane-associated, and its binding can thus provide a direct connection to the cytoplasmic membrane, which in turn may be enriched in the vesicles. Additionally, ToxR binding could protect the motif itself from DNases, which may also contribute to enrichment (Goss et al., 2013).

3.2.3. Restriction Sites

One of the few highly enriched regions that do not contain the ToxR binding motif, harbors a 12× repeat of the motif 5′-TCTAGAATCC-3′ (**Figure 2H**). This sequence provides a number of restriction sites for restriction enzymes XbaI and TfiI, between a putative DNA-binding protein and DNA-binding protein Fis. Restriction sites could potentially increase the probability of DNA inclusion in vesicles, as loose ends and smaller DNA fragments may be more likely to enter protrusions in the bacterial membrane during vesiculation.

3.2.4. Superintegron

Although a less prominent phenomenon, some regions of DNA were significantly depleted in EVf. Most notably, relatively lengthy parts of the superintegron structure in ChII (**Figures 2J,K**). This region is high in coverage in WC-samples, which stands in contrast to previous *V. cholerae* sequence data from fecal samples (Sealfon et al., 2012). This could be explained by the fact that in the present study a different DNA isolation kit was used, tailored for shorter fragments. The discrepancy may be due to the mobile nature of transposons (Marin and Vicente, 2013), as excised fragments may be enriched in the WC sample with our kit.

3.2.5. GC-Content

The enrichment of kbp genome partitions and their GC-content was found to correlate negatively, both for the K139 phage DNA ($p = 1.5 \cdot 10^{-99}$) and the rest of the genome ($p = 2.1 \cdot 10^{-14}$) (**Figure 3**). There are several factors that may be contributing to this phenomenon; firstly, AT-rich regions of the genome are more accessible (Gomes and Wang, 2016), and a higher density of protein binding to these regions could increase their association with the bacterial membranes, or provide protection against

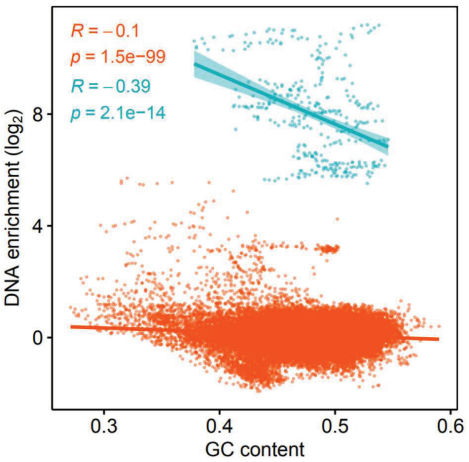

FIGURE 3 | Log_2 DNA enrichment of kbp partitions of the *V. cholerae* genome and GC-content. Blue color denotes windows within the K139 prophage, while orange denotes windows of the rest of the genome, respectively.

DNases. Protein binding sites are preferentially located between protein coding regions (Ishihama, 2010), and the intergenic GC content of the TCP2 genome is only 41.4% while it is 48.5% for protein-coding regions, according to the RAST-annotation.

3.3. EV-Associated RNA Is Characteristically Different From the Whole Cell Transcriptome

The EVf-RNA sequencing resulted in 23.6M–78.2M reads per sample, whereof 99.5–99.9% were mapped to the genome. WC-RNA sequencing resulted in 23.7–96.3M reads per sample, of which 96.8–99.6% were mapped to the genome. The mapped read coverage reveals that similar to the DNA data, certain regions display higher coverage across all replicates, some being characteristic to either EVf- of WC-samples (**Figures 4A,B**). According to cuffdiff, a total of 214 annotated transcripts were significantly enriched in the EVf, distributed irregularly across both chromosomes (**Figure 5**).

3.3.1. Ribosomal RNA

A large portion of sequencing reads stem from ribosomal RNA, and since they are all encoded in ChI, transcriptional products from ChII are very scarce in comparison. Counting only read coverage mapped to protein coding sequences (CDS), the difference is not that pronounced, but EVf maintains a significantly lower coverage in ChII than in ChI (**Figures 4C,D**). Results from previous RNA sequencing of *V. cholerae* reveal that genes on ChI are more frequently transcribed than those on ChII during growth in rich media, although the difference is less pronounced when grown in rabbit intestine (Xu et al., 2003). This upregulation of certain genes on ChII in intestine led to the suggestion that ChII-genes may be more important during infection, e.g., in response to nutritional stresses. The difference in ChII/ChI coverage ratio when counting total RNA or only CDS is smallest in EVf, implying that rRNA is depleted in the extracellular fraction.

3.3.2. Functional-, and Non-coding RNA

A series of conserved functional and non-coding RNAs are visible as peaks in mapped read coverage, most notably RNase P type A, tmRNA and CsrB1 in ChI, and CsrB3 in ChII (**Figures 4A,B**). CsrB3 is the most abundant form of RNA from in ChII, and in this study amounts to ∼70% of RNA mapped to this chromosome for both EVf and WC. The most consistently enriched of these are tmRNA and RNase P (**Figure S4**). The protein component of RNAse P has been found to be anchored to the inner membrane in *Escherichia coli*, which could partly explain its abundance in EVf (Miczak et al., 1991). The abundance of tmRNA has been observed in the extracellular milieu of *E. coli* previously (Ghosal et al., 2015), and this enrichment could partly be due to its binding to elongation factor Tu, commonly found in vesicle preparations (Blenkiron et al., 2016). sRNAs CsrB1, CsrB2, CsrB3 (Nguyen et al., 2018) and GcvB are also significantly enriched (**Figure S4**). CsrB sRNAs have been found to take part in virulence and biofilm formation, and to be regulated by quorum sensing (Lenz et al., 2005). In *E. coli* CsrB RNAs is known to antagonistically regulate the action of carbon storage regulator CsrA (Liu et al., 1997). CsrA binds to the 5' untranslated regions (UTRs) of mRNA, recognizing GGA motifs in apical loops of RNA secondary structures. These motifs are also present on CsrB sRNAs, effectively sequestering the CsrA protein, allowing translation of the formerly inhibited mRNAs (Dubey et al., 2005; Duss et al., 2014). Interestingly, CsrA was only detected in WC, suggesting that other factors may be responsible for the enrichment of CsrB sRNAs in EVf. GcvB is known to regulate several genes in *E. coli*, e.g., RNA polymerase sigma S (RpoS), which enables the bacteria to survive under lower pH (Jin et al., 2009). This sRNA is dependent on binding to Hfq for its regulatory effect (Pulvermacher et al., 2009), and this may contribute to its enrichment, as Hfq preferentially associates with the bacterial membrane (Diestra et al., 2009).

3.3.3. Phage mRNAs

While enrichment in EVf-DNA was characterized by enrichment of CTXϕ and K139 phage genes (**Figure 2C,L**), the inverted Mu-like prophages in ChI are more pronounced in RNA enrichment (**Figure 5D**). Mu-like phage DNA was not particularly enriched, but several of its transcripts are, including tail sheath and capsid genes. Similarly, mRNA for many structural proteins of phage K139 significantly (**Figures 5L,M**). Enrichment of phage RNA associated with host-relationship modulation has previously been observed in EVs from in EVs from *Salmonella enterica* (Malabirade et al., 2018).

3.3.4. Bacterial mRNAs

Cuffdiff found that a probable iron binding protein mRNA and *ompU* were enriched (**Figure 5A**), the latter of which was also

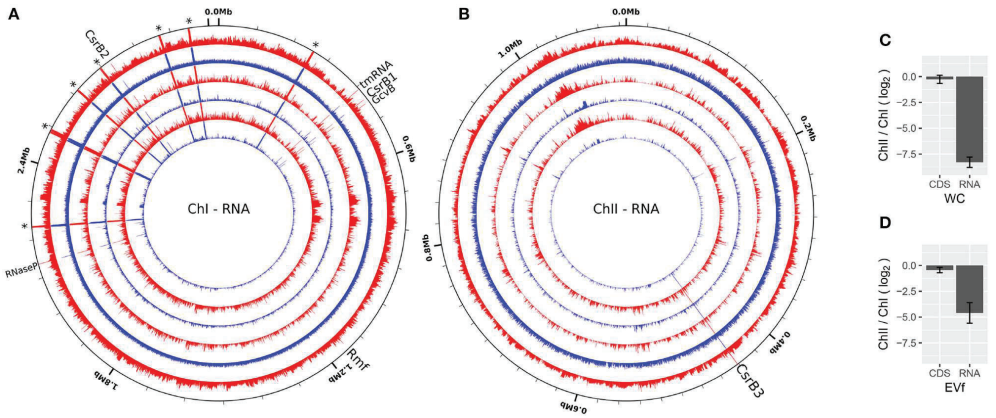

FIGURE 4 | RNA sequencing coverage over ChI **(A)** and ChII **(B)** of *V. cholerae* from EVf (red) and WC (blue) from three biological replicates (Log scale). A selection of ncRNAs are named, while asterisks denote ribosomal RNA. Made using circleator (v1.0.0) (Crabtree et al., 2014). **(C,D)** Average RNA coverage ratio ChII/ChI in WC and EVf, for total RNA, or protein coding sequences (CDS) only.

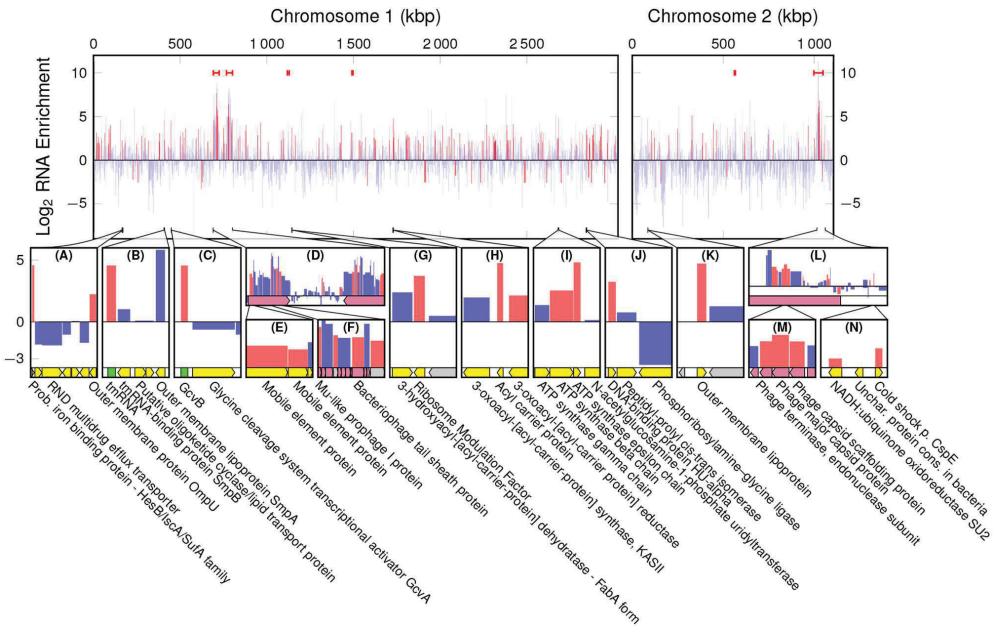

FIGURE 5 | Log_2 Enrichment of annotated RNAs in EVf, according to cuffdiff, red denoting statistically significant enrichment. Red bars are the regions in which PHAST recognized phage genes. The bottom row **(A–N)** displays regions of interest, in which yellow tracks signify characterized genes, gray hypothetical, red phage components, and green ncRNA.

associated with a coverage peak in DNA. Other highly enriched genes from ChI were *rmf* (Terui et al., 2010), *acpP* (Kutchma et al., 1999), *atpD*, *atpC* (Dunn et al., 2000), and *hupA* (Martínez et al., 2015) (**Figures 5G–K**) Interestingly, the other subunit of DNA-binding protein HU, *hupB* (Martínez et al., 2015) was enriched as well (**Table S1**). The HU dimer is in *E. coli* known to bind with high affinity to the mRNA encoding RpoS, and the ncRNA DsrA (Balandina et al., 2001), which in turn regulates transcription by overcoming the silencing effect of DNA-binding protein H-NS on the expression of RpoS. HU has also been found to regulate virulence of *Vibrio parahaemolyticus* (Phan et al., 2015). ChII harbors a number of enriched genes, e.g., an Outer membrane protein mRNA (**Figure 5K**), and *cspE* (**Figure 5N**). Cold-shock protein E (CspE) is regularly expressed at 37 °C, and was originally identified as a multicopy suppressor of a temperature-sensitive chromosome partition mutant (Yamanaka et al., 1994).

The etiology of mRNA enrichment in vesicles is not yet fully understood, but there are presumably three important mechanisms that are most important for this phenomenon; half-life, location, and size. Firstly, mRNA synthesis is likely low in EVs compared to living bacteria, meaning that mRNA with longer half-lives will be enriched in vesicles over time. It has been reported that mRNAs in thermophile prokaryotes are biased toward purine tracts, indicating that it provides thermostability (Paz et al., 2004), and in this study, a positive correlation was indeed found between purine content and enrichment of mRNAs in EVf (**Figure 6A**). Although the samples have been subjected to the lowest possible thermal stress during isolation, it could still be that some level of purine-dependent degradation has occurred in the duration of filtration and isolation. It has been observed that the uracil content of membrane mRNAs is higher in prokaryotes than in eukaryotes (Prilusky and Bibi, 2009), a pattern that coincides with the differing mRNA turnover requirements of the two branches of life. To check whether the purine–enrichment correlation in our cultures could be caused by more complex underlying sequence dependencies, the pearson correlation coefficient was calculated between occurrences of all possible RNA nonuplets and mRNA enrichment. Some of these yielded very low p-values (**Figure S5**), especially for specific purine tracts, such as NNNAAGNNN, NNNNGAAGA, and NNAAGAAGA. This indicates some level of sequence dependency of the mRNA enrichment, which in turn could be due to increased stability, or perhaps affinity to localizing-, or protecting, biomolecules. Curiously, the sextuplet that correlates most significantly with mRNA enrichment is AGAAGA, the *in-vivo* binding motif for the human mRNA splicing protein TRA2B (Änkö and Neugebauer, 2012), which shares significant RNA-binding domain similarity with an uncharacterized RNA-binding protein in *V. cholerae* (rna, 2018). In addition, this protein is likely a cytoplasmic membrane protein, according to PSORTb.

The second mechanism that could give rise to mRNA enrichment in EVf is indeed the location, affected by potential affinity to membranes or membrane proteins, such as the signal recognition particle (SRP), which is associated with the membrane through the SRP receptor (Akopian et al., 2013). Through SRP, mRNAs could be co-localized with their protein products during translation, which in turn could place them close to the cytoplasmic membrane. While this may be true for certain mRNAs, no significant enrichment was found for genes destined for any location in this study (**Figure S6**). However, mRNAs have been found to localize to the membrane in *E. coli* independent of translation (Nevo-Dinur et al., 2011), meaning some motifs or secondary structures may increase an mRNAs affinity toward the membrane regardless of their protein product. Even though a number of RNA motifs correlate positively with enrichment in this study (**Figure S5**), the folding nature of RNA makes their protein interactions more complex than what may be deduced from the primary structure alone.

A third mechanism likely to affect enrichment is size, as a portion of the vesicles are smaller than many mRNA sequences. While vesicles can be as small as 20 nm, typical RNAs can vary in size from ∼7 to 33 nm for 0.7–8.9kbp sequences, respectively (Borodavka et al., 2016). In accordance with this restriction, the correlation between mRNA length and enrichment in EVf was negative and statistically significant (**Figure 6B**). This result is similar to findings on the size-dependent inclusion of plasmids in EVs (Tran and Boedicker, 2019), and suggests that vesicles

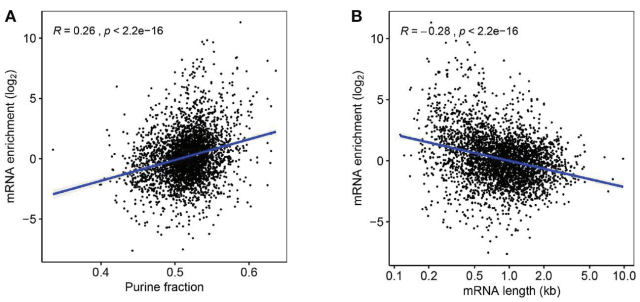

FIGURE 6 | (A) Fold-change enrichment of mRNAs in EVf, plotted against purine content. **(B)** mRNA enrichment according to transcript length.

maintain a significant size exclusion bias for genetic cargo. This correlation holds for annotated gene length, and is independent of the lengths of the actual isolated RNA fragments, information that is lost in the sequencing protocol.

3.4. The EV Proteome Is Enriched in Periplasmic-, Membrane-, and Extracellular Proteins

The results from genetic sequencing prompted us to map the proteomic profile of EVs in order to asses a possible correlation between enrichment of genes and protein products. By LC-MS, a total of 1312 proteins were detected, 670 of which in common while 222 and 420 were exclusively detected in the EVf or WC, respectively.

3.4.1. Subcellular Location

Proteins associated with the extracellular milieu, outer membrane and periplasm are significantly enriched in EVf compared to cytoplasmic proteins (**Figure 8**). The variation within each category is considerable, which indicates that the composition of proteins in each category is different from WC to EVf. For instance, one could expect all the outer membrane proteins to be enriched along with the membrane itself, but their variation from a high degree of enrichment to a high degree of depletion suggests that the mechanisms of protein enrichment are more complex than random budding of the membranes. The specific enrichment or depletion of certain membrane proteins has been observed before (Lee et al., 2008; Aguilera et al., 2014), supporting the hypothesis that vesicles are not simply random membrane blebs, but at least somewhat specifically constructed.

3.4.2. mRNA–Protein Correlation

580 genes were detected both as mRNA and their protein products in both WC and EVf. Only genes destined for the periplasm demonstrated a statistically significant mRNA–protein enrichment correlation, that being negative (**Figure 7**). These results suggest that post-budding translation of vesicle-associated mRNA is low. This does not, however, refute the possibility that mRNA cargo could be translated in a potential target cell for the EVs. Worth to mention in this context is that no genes were found as both protein and mRNA in one fraction uniquely, with the sole exception of Soluble cytochrome b562, a protein that was found in only one of three EVf replicates.

3.4.3. RNA-Binding Protein

An interesting EVf-enriched protein in the context of RNA enrichment is the methionine ABC transporter substrate-binding protein (**Table S2B**). This membrane protein, also called ProQ,

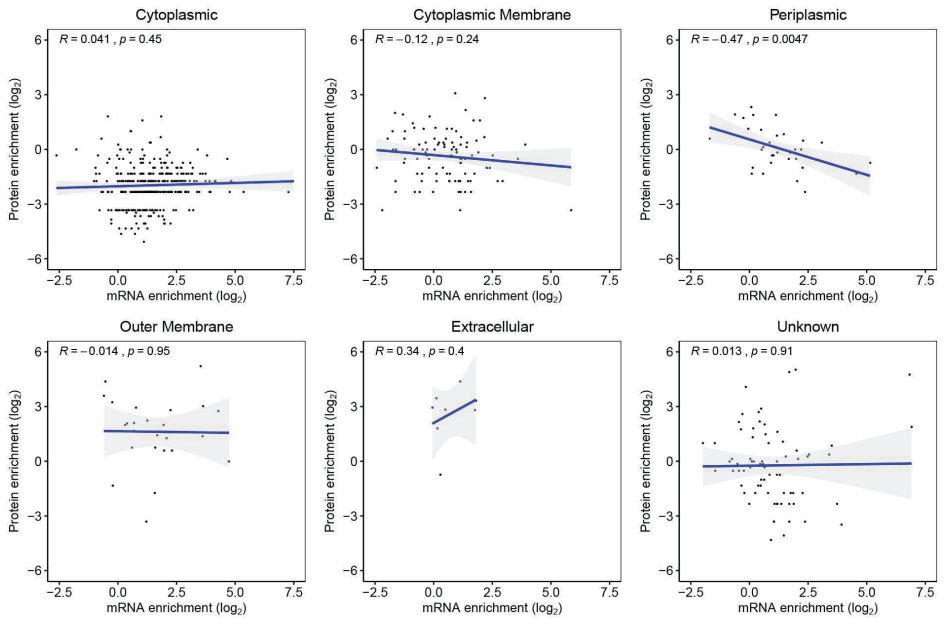

FIGURE 7 | Genes detected as both mRNA and their protein products in both EVf and WC, sorted by their predicted subcellular location. Periplasmic proteins yielded the only statistically significant regression analysis, showing a negative correlation between mRNA and protein enrichment.

has been shown in *S. enterica* and *E. coli* to bind to sRNA and mRNA, increasing their stability (Smirnov et al., 2016). This protein is enriched >8× in EVf, which is well-beyond the standard deviation of the general enrichment of cytoplasmic membrane proteins (**Figure 8**). The presence of this protein could in part responsible for the enrichment of certain sRNAs or mRNAs; for instance, it is known to bind to and stabilize *cspE* mRNA (Holmqvist et al., 2018), which was enriched >16× in EVf. This protein could act as a link between certain RNAs and the membrane, and depending on its RNA-binding affinities, be decisive for the composition of the RNA cargo of vesicles. Several CsrB sRNAs are abundant in EVf, and while these are known to bind to CsrA protein, no CsrA protein was found in the EVf. This is surprising, as one could expect that biomolecules with demonstrated affinity to each other would be co-localized. A possible explanation could be that these RNAs also bind to ProQ (Holmqvist et al., 2018), contributing to their enrichment.

3.4.4. Flagella and Phage Proteins

A number of flagellar and extracellular proteins were the most abundant in the extracellular fraction compared to whole cells. Of the 48 known flagella-associated proteins in the O395 proteome, a total of 39 were detected in the samples. 9 were found only in the bacterial fraction, including regulators such as FleN and FleQ, motor switch proteins such as FliG and FliM, and synthesis proteins such as FliS and FlgN. 17 flagellar proteins were uniquely detected in the EVf, mostly structural components such as FliD, FlaG, FlaF, FlgL, FlgK, FlgG, FlgC, FlgB, FlgF, but also the biosynthesis protein FlhF, L- and M-ring proteins FlgH and FliF, and the hook-length control protein FliK (Kim and McCarter, 2000). 13 proteins encoded by the K139 prophage were detected by LC-MS; 10 unique to EVf, 1 unique to WC, and 2 detected in both samples; tail fiber and major capsid protein, enriched 5.7× and 27×, respectively. This not nearly as dramatic as the enrichment of the prophage DNA, which according to cuffdiff was 249× on average. This indicates enrichment of phage DNA in other DNase-protected states than within phage particles, such inside- or embedded in the membrane of EVs, or possibly in other phage capsid structures. Structural components of both the Mu-like phage in ChI and K139 in ChII were enriched in the EVf, while no complete phage structures were visible in the micrographs, as has been observed before (Reidl and Mekalanos, 1995). This could imply that the K139 tail structures may be utilized by *V. cholerae* as a tailocin (Ghequire and De Mot, 2015), but this hypothesis needs testing. The high presence of flagellar and phage components in EVf sheds light on the importance of purification when working with EVs. This may be especially important in their application of vaccine candidates, when certain epitopes are of interest, and protein quantification may be used as a dose measurement. Since phages are co-isolated due to their similar sizes, they can pose a significant problem when working on vesicles specifically. One solution to this is density gradient centrifugation, but as this process may take 16 h (Olaya-Abril et al., 2014), mRNA stability is a concern.

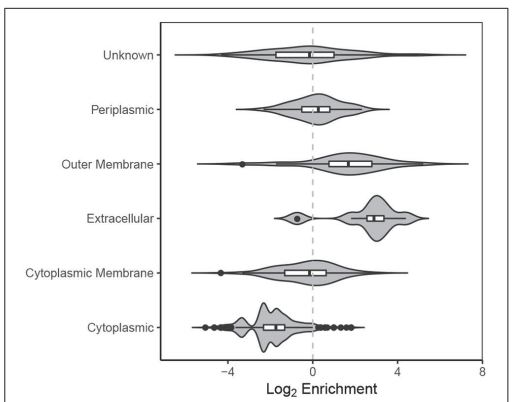

FIGURE 8 | Violin plot of $\log_2$ enrichment of proteins in EVf relative to WC and their cellular location.

3.4.5. Virulence Factors

Beyond flagellar-, and phage constituents, there were many proteins highly enriched and depleted in EVf (**Table S2**). Expectedly, among the most enriched were proteins associated with membranes or the periplasmic space. Furthermore, many of the abundant proteins uniquely detected in EVf (**Table S2A**) are associated with virulence, e.g., OmpK (Ningqiu et al., 2008), TraF-related protein (also called type IX secretion protein SprF/PorP) (Laanto et al., 2014), colonization factor AcfA (Hughes et al., 1995), and OmpT (Provenzano and Klose, 2000). Similarly, among the most enriched proteins detected in both samples are virulence factors such as TonB (Abdollahi et al., 2018), OmpU (Provenzano et al., 2001), OmpV (Liu et al., 2017), haemagglutinin biogenesis protein MshL (Hsiao et al., 2006), hemolysin Vcp, and outer membrane protein LapE of the TolC family (Smith et al., 2018). A surprising observation is the enrichments of OmpK, OmpU and OmpV, which mirror the high enrichment of their mRNAs, and may point to a degree of co-translational localization. Many of these virulence factors (e.g., AcfA, OmpU, MshL, TolC) have been observed in the proteome of EVs from *V. cholerae* previously (Altindis et al., 2014) and stands testament to the potential of EVs to modulate host–pathogen relationships. For instance, vesicles with hemolytic proteins could be used as a remote agent to damage host cells, increasing the nutritional value of the bacterial environment.

3.4.6. Iron Transport Proteins

There are several proteins associated with iron uptake that are enriched significantly in EVf. For instance, a ferrichrome-iron receptor was only found in EVf, while heme-, and sidophore receptors HutA (Henderson and Payne, 1994) and IrgA (Wyckoff et al., 2015) were highly enriched (**Table S2**). These proteins mediate iron uptake, which has some metabolic implications. If

vesicles could contribute to iron depletion of the surroundings, this would supposedly affect the bacteria negatively unless they have mechanisms for vesicle re-absorption.

3.4.7. Antimicrobial Resistance

While the bacteria need to effectively absorb iron compounds in low-iron conditions, antimicrobials are preferably effluxed out. Several relevant acriflavin-resistance proteins are highly enriched in EVf (**Tables S2A,B**). For instance, VexH, VexB, and VexD were enriched while VexK was only detected in EVf. These are members of the AcrB/AcrD/AcrF protein family, known multidrug efflux transporters (Buckley et al., 2006). The AcrB protein is located in the inner membrane, where it interacts with the outer membrane protein TolC through periplasmic protein AcrA. Deletion of *acrB* or *tolC* is associated with hypersensitivity to a range of antibiotics in *S. enterica* (Buckley et al., 2006). Another enriched protein related to resistance is YcfM, also known as LpoB, which has been identified as an activator of penicillin-binding-protein PbpG (Jean et al., 2015). A second protein from the *ycf* operon, YcfL, was among the most abundant only detected in EVf. Its location on the same operon could imply an association with drug resistance, but little is known about this lipoprotein (Alam et al., 2013). The presence of efflux pumps on vesicles could mean that the bacteria gain some benefit from them being largely void of antimocrobials, which does not contradict the hypothesis that they can be re-absorbed.

3.4.8. Adhesion and Biofilm Formation

Two other proteins previously detected in EVs from *V. cholerae* are RmbA and RmbC (Altindis et al., 2014). These were both enriched in EVf (**Tables S2A,B**) and have been found to be important for biofilm formation. RbmA is a extracellular protein that forms tandem fibronectin type III (FnIII) folds (Giglio et al., 2013), and is required for rugose colony formation and biofilm structure integrity (Fong et al., 2006). RbmC has been recognized as a hemolysin and a central agent in biofilm and pellicle formation, perhaps binding to carbohydrates in the extracellular matrix (Fong and Yildiz, 2007). It is secreted and localized on the cell surface (Teschler et al., 2015). The association of EVs with biofilm formation has been established for some time (Kulp and Kuehn, 2010), and have been found to be a major structural part of biofilm in *Staphylococcus aureus* (He et al., 2019).

3.4.9. Protein Depletion in EVf

A thorough analysis of proteins depleted in EVf is beyond the main scope of this study, but some interesting observations deserve comment. As expected, cytoplasmic proteins were significantly depleted in EVf (**Figure 8**), but many DNA-binding proteins stemming from genes that were enriched in EVf (**Table S1**) were depleted considerably (**Tables S2B,C**). For instance, HU-α and HU-β are depleted by 70 and 80%, while *hupA* and *hupB* mRNA were some of the most enriched mRNAs in EVf. Similarly, H-NS protein is depleted by 80% in EVf, while *hns* mRNA was enriched >7× on average. Ssb is depleted by 80% while *ssb* mRNA was enriched >11×, and transcription termination factor Rho is only detected in WC while *rho* was enriched >3× in EVf. While the mechanisms behind this is unknown, it could be that these mRNAs are more stable than the average, or that they somehow localize to the membrane, maybe by protein binding. DNA-binding protein HU has been found to bind to both tmRNA and RNase P RNA in *E. coli* (Macvanin et al., 2012), which were both enriched in EVf.

4. CONCLUSION

The present study is the first exploratory work that directly compares the DNA, RNA and protein content of *V. cholerae*, to that of its extracellular vesicles. Many interesting observations shed light on the general composition of the vesicle fraction, including their cargo of DNA, functional RNA, mRNA, RNA-binding proteins and virulence factors. While the observed nucleic acid enrichment patterns seem to have possible explanations in membrane protein binding, specific investigations are required to confirm the specific mechanisms involved. For instance, a future research objective is to investigate the effect of ToxR and its binding sites on the enrichment of DNA in vesicles. An alternative study could be on the effect of ProQ or other RNA-binding proteins on RNA enrichment. A series of known virulence proteins were enriched in EVf, which is to be expected, as the host-pathogen interaction is modulated in part by the outer membrane proteins of *V. cholerae*. Tail tube and tail fiber proteins of several prophages were abundant in EVf, while no complete phage structures were visible in electron microscopy. This could imply function as tailocins, but further research is required to assess this hypothesis. A discrepancy between transcript enrichment and the enrichment of their protein products in EVf argues against significant translation occurring after vesicle isolation, but the effects of mRNA turnover in this context is unclear. A future prospect is therefore to investigate the effects of translation-, transcription-, and RNase inhibitors on the bacterial culture before vesicle isolation. An interesting observation in the EVf proteome is that enrichment of genes for several DNA-binding proteins contrasted the depletion of their protein product, while for a number of outer membrane proteins, mRNA enrichment mirrored the proteomic profile. This could suggest either a certain level of co-translational mRNA localization, or perhaps differing turnover requirements for the transcripts of DNA-binding- and membrane proteins. A research prospect is to locate these mRNAs within the living bacteria, and identify the affinities of RNA-binding membrane proteins. If the mechanisms behind the observed enrichment in DNA, RNA and protein are well-characterized, this would be a big step on our way to produce customized vesicles. In turn, this could greatly expand our possibilities when it comes to the utilization of vesicles as vaccines or vessels for gene-, or drug delivery.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI, accession numbers: PRJNA586749 and PRJNA587223.

AUTHOR CONTRIBUTIONS

PL has planned and performed most of the experiments and contributed in writing of the manuscript. AK has planned and performed some of the experiments and contributed in writing of the manuscript. HW-L had contributed in planning of the experiments and in writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02708/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY FIGURES

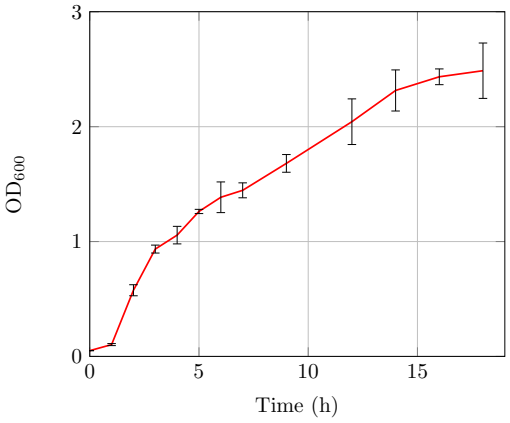


Figure S1. Typical growth curve of *V. cholerae* TCP2 in 200 mL LB volume at 37 °C. EVs were harvested at $OD_{600} \sim 1$.

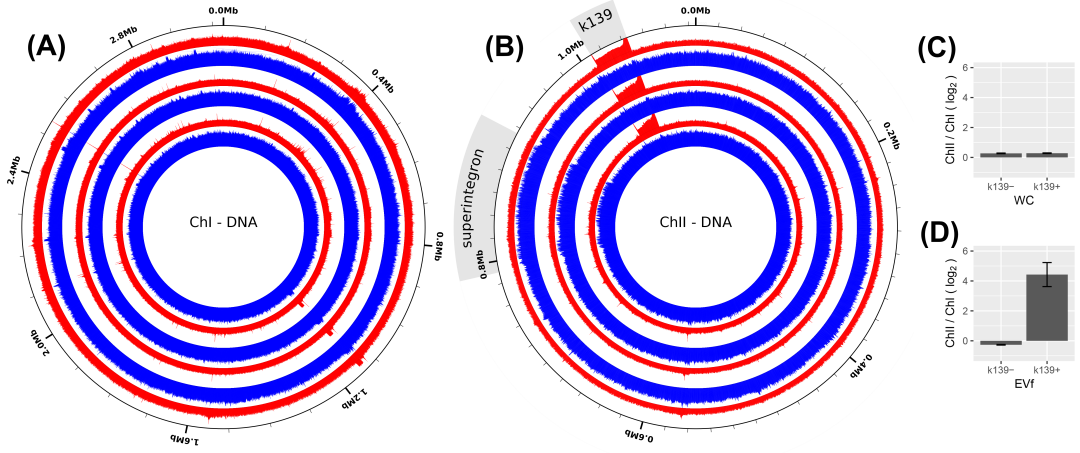
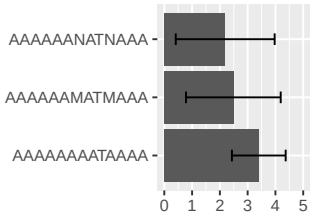


Figure S2. Normalized DNA sequencing coverage over ChI **(A)** and ChII **(B)** of *V. cholerae* from EVf (red) and WC (blue) from three biological replicates (Log scale). The K139 prophage and superintegron region are emphasized in ChII. Made using circleator (v1.0.0).[113] **(C)** and **(D):** average coverage ratio ChII/ChI in WC and EVf, respectively, K139+ and K139- denotes the inclusion and exclusion of the K139 prophage from quantification, respectively.

Ch	Position	Log2-enrichment	Significant	Sequence
1	2536386	5.51	yes	AAAAAAAATCAAA
1	166948	4.65	yes	AAAAAAAATAAAA
1	1549066	4.07	yes	AAAAAACATAAAA
1	399488	4.01	yes	AAAAAAAATAAAA
1	379445	2.64	yes	AAAAAAAATAAAA
2	397564	2.29	yes	AAAAAAAATAAAA
1	392965	2.02	yes	AAAAAACATAAAA
1	366166	1.09	yes	AAAAAACATTAAA
1	1268254	0.88	yes	AAAAAACATCAAA
1	1343873	0.69	no	AAAAAACATAAAA
1	831062	0.38	no	AAAAAAAATCAAA
1	1833342	0.19	no	AAAAAACATGAAA
1	1759734	0.06	no	AAAAAATATTAAA
consensus		>2		AAAAAAMATMAAA

(A)



(B)

Figure S3. **(A)** Positive motif hits for ToxR binding site AAAAAANATNAAA and enrichment (Log_2) in EVf of kbp genome partitions centered upon them. Log_2 enrichments greater than 2 are highlighted in gray. **(B)** Average enrichment for kbp partitions centered on AAAAAANATNAAA motifs and selected derivatives.

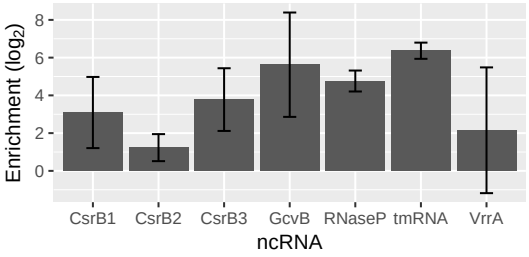


Figure S4. Average enrichment of ncRNAs in EVf (normalized by total mapped RNA reads), according to per-base coverage data.

	Motif	Estimate	p-value	mRNAs
3n	NNNAAGNNN	0.295	3.82e-71	3860
4n	GAAGNNNNN	0.295	7.37e-71	3545
5n	NNNNGAAGA	0.237	8.29e-46	2637
6n	NNNAGAAGA	0.186	1.15e-28	1220
7n	NNAAGAAGA	0.137	3.97e-16	623
8n	NAAGCGCCA	0.148	1.57e-18	133
9n	CUAUCAGCU	0.124	1.98e-13	16
A^-	NNGUUCNNN	0.162	7.51e-22	3128
G^-	ANNNCNAAN	0.188	2.93e-29	3566
R^-	CNCCCNUUC	0.080	2.44e-06	113

Figure S5. Pearson correlation estimates between mRNAs enrichment and occurrences of selected nonuplets per base of transcript length. 3n–9n denotes a minimum unambiguous length of 3 to 9 bases, respectively. A^-, G^- and R^- denotes adenine, guanine, and purine-free nonuplets, respectively. The last column is the number of mRNAs that harbors at least one copy of the nonuplet.

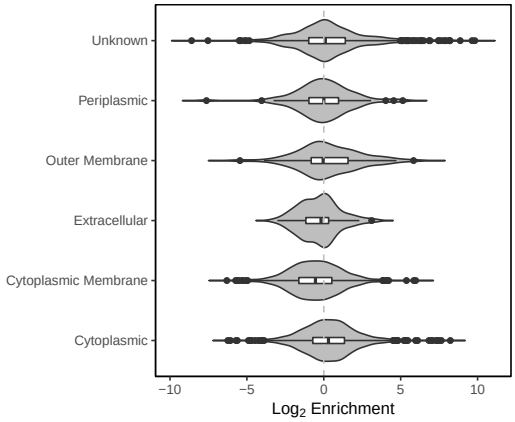


Figure S6. Violin plot of the enrichment of mRNAs in EVf compared to WC, and the subcellular location of their protein products.

Table S1. Selected non-phage transcripts of high enrichment in *V. cholerae* or its extracellular milieu, sorted from highest to lowest enrichment. **(A)** Transcripts only found in EVf. **(B)** Transcripts found in both EVf and WC, highest enriched in EVf on the top, and highest enriched in WC on the bottom. **(C)** Transcripts only detected in WC, sorted from lowest to highest abundance.

	Chr.	C_{WC}	C_{EVf}	Enrich. (log_2)	Name
(A)	ChI	0	10.8	inf	FIG01200900: hypothetical protein
	ChII	0	7.7	inf	FIG01200711: hypothetical protein
	ChI	0	7.1	inf	FIG01199699: hypothetical protein
	ChI	0	4.6	inf	Glutaredoxin-related protein
	ChI	0	4.3	inf	DNA-binding protein inhibitor Id-2-related protein
	⋮				
(B)	ChII	0.48	16.7	5.14	Periplasmic maltose/maltodextrin ABC transporter MalE
	ChI	1.40	39.5	4.82	ATP synthase epsilon chain
	ChI	2.80	76.5	4.77	Acyl carrier protein
	ChII	6.06	161.1	4.73	Outer membrane lipoprotein
	ChI	0.77	18.6	4.59	Prob. Fe binding protein - HesB/IscA/SufA family
	ChI	0.66	12.8	4.28	Outer membrane protein OmpV
	ChI	2.38	42.0	4.14	Aspartate ammonia-lyase
	ChI	0.89	15.0	4.08	Fumarate reductase subunit D
	ChII	2.04	33.4	4.03	Cold shock protein CspE
	ChI	0.98	15.9	4.02	FIG01200169: hypothetical protein
	ChII	1.28	19.1	3.90	Acetate kinase
	ChI	0.44	6.4	3.85	Outer membrane protein OmpK
	ChI	1.88	22.8	3.60	Integration host factor beta subunit
	ChI	0.92	10.4	3.50	DNA-binding protein Fis
	ChI	1.34	14.8	3.46	Single-stranded DNA-binding protein
	ChI	4.54	43.17	3.25	DNA-binding protein HU-alpha
	ChI	2.40	13.09	2.45	DNA-binding protein HU-beta
	ChI	10.87	51.5	2.24	Outer membrane protein OmpU
	⋮				
	ChI	59.2k	10.1k	-2.56	Large Subunit Ribosomal RNA
	ChI	59.1k	10.0k	-2.56	Large Subunit Ribosomal RNA
	ChI	59.1k	10.0k	-2.56	Large Subunit Ribosomal RNA
	ChI	59.4k	10.0k	-2.57	Large Subunit Ribosomal RNA
	ChI	59.2k	10.0k	-2.57	Large Subunit Ribosomal RNA
	ChI	59.4k	10.0k	-2.57	Large Subunit Ribosomal RNA
	ChI	59.3k	10.0k	-2.57	Large Subunit Ribosomal RNA
	ChI	58.0k	9.52k	-2.61	Large Subunit Ribosomal RNA
	⋮				
(C)	ChI	378.2	0	-inf	tRNA-Lys-TTT
	ChI	440.9	0	-inf	tRNA-Ile-GAT
	ChI	443.3	0	-inf	tRNA-Leu-TAG
	ChI	449.4	0	-inf	tRNA-Met-CAT
	ChI	455.4	0	-inf	tRNA-Asn-GTT
	ChI	513.8	0	-inf	tRNA-Ile-GAT
	ChI	513.9	0	-inf	tRNA-Asn-GTT
	ChII	516.8	0	-inf	tRNA-Gly-TCC
	ChI	519.1	0	-inf	tRNA-Ile-GAT
	ChI	714.2	0	-inf	tRNA-Met-CAT
	ChI	1128.7	0	-inf	tRNA-His-GTG
	ChI	1362.1	0	-inf	tRNA-His-GTG

Table S2. Proteins of high abundance in *V. cholerae* or its extracellular milieu. **(A)** Proteins only found in EVf, sorted from highest to lowest abundance. **(B)** Proteins found in both EVf and WC, highest enriched in EVf on the top, and highest enriched in WC on the bottom. **(C)** Proteins only detected in WC, sorted from lowest to highest abundance.

	Chr.	C_{WC}	C_{EVf}	Enrich. ($\log_2$)	Name
(A)	ChI	0	28.7	inf	FIG01200881: hypothetical protein
	ChI	0	19.7	inf	Outer membrane protein OmpK
	ChI	0	19.3	inf	YcfL protein: an outer membrane lipoprotein
	ChII	0	15.3	inf	TraF-related protein
	ChII	0	14.3	inf	FIG01200406: hypothetical protein
	ChI	0	12.3	inf	Hemolysin-related protein RbmC
	ChII	0	11.3	inf	FIG01199739: hypothetical protein
	ChI	0	9.7	inf	Accessory colonization factor AcfA
	ChII	0	8.7	inf	FIG01199666: hypothetical protein
	ChI	0	8.7	inf	Outer membrane protein OmpT
	ChI	0	8.3	inf	Ferrichrome-iron receptor
	ChI	0	8.0	inf	LPS-assembly lipoprotein RlpB precursor (Rare lipoprotein B)
	ChI	0	1	inf	Acriflavin resistance protein (VexK)
	⋮				
(B)	ChI	1.33	49.7	5.21	Long-chain fatty acid transport protein
	ChII	0.67	14.3	4.39	TonB-dependent heme and hemoglobin receptor HutA
	ChI	1.00	17.3	4.09	Lipoprotein YcfM - part of a salvage pathway of unknown substrate
	ChI	1.3	16.0	3.58	TonB-dependent receptor / Enterobactin receptor IrgA
	ChI	3.0	30.0	3.32	Hemolysin-related protein Vcp
	ChI	0.7	6.3	3.25	MSHA biogenesis protein MshL
	ChI	1.3	11.3	3.09	Methionine ABC transporter substrate-binding protein
	ChI	1.3	10.3	2.94	Type I secretion system - outer membrane component LapE
	ChI	0.7	4.7	2.81	Lipoprotein nlpI precursor
	ChI	7.3	51.3	2.81	Outer membrane protein OmpU
	ChI	6.7	45.0	2.77	Outer membrane protein OmpV
	ChI	1.3	8.7	2.70	RbmA protein
	ChI	1	4.7	2.23	RND multidrug efflux transporter - Acriflavin resistance protein (VexH)
	Ch2	0.7	2.7	2.00	Acriflavin resistance protein
	ChI	2.7	5.3	1.00	RND multidrug efflux transporter - Acriflavin resistance protein (VexB)
	Ch1	8	12	0.58	Acriflavin resistance protein (VexD)
	⋮				
	ChI	52.7	16.7	-1.74	DNA-binding protein HU-alpha
	ChI	8.0	1.67	-2.32	Single-stranded DNA-binding protein
	ChI	14.7	3.3	-2.32	DNA-binding protein HU-beta
	ChII	18.7	1.0	-4.32	Phosphomannomutase
	ChII	12.7	0.7	-4.32	4-alpha-glucanotransferase (amylomaltase)
	ChI	14.7	0.7	-4.32	Predicted dye-decolorizing peroxidase (DyP) - YfeX-like subgroup
	ChI	13.7	0.7	-4.32	Tyrosyl-tRNA synthetase
	ChI	13.3	0.7	-4.32	Cysteinyl-tRNA synthetase
	ChI	14.3	0.7	-4.32	Membrane alanine aminopeptidase N
	ChI	16.3	0.7	-4.64	Phosphopentomutase
	ChI	17.3	0.7	-4.64	Catalase
	ChI	20.3	0.7	-5.06	3,4-dihydroxy-2-butanone 4-phosphate synthase
	⋮				
(C)	Ch1	8.7	0	-inf	Carbon storage regulator CsrA
	Ch2	10.7	0	-inf	N-acetylglucosamine regulated methyl-accepting chemotaxis protein
	Ch2	11.0	0	-inf	Methyl-accepting chemotaxis protein
	Ch1	11.3	0	-inf	1-deoxy-D-xylulose 5-phosphate synthase
	Ch1	12.0	0	-inf	Malonyl CoA-acyl carrier protein transacylase
	Ch1	12.7	0	-inf	Glycogen synthase ADP-glucose transglucosylase
	Ch1	13.0	0	-inf	Phosphoglucosamine mutase
	Ch1	13.3	0	-inf	Transcription termination factor Rho
	Ch1	14.7	0	-inf	Methionine aminopeptidase
	Ch1	16.3	0	-inf	Uroporphyrinogen III decarboxylase
	Ch2	16.3	0	-inf	Oxygen-insensitive NAD(P)H nitroreductase
	Ch1	19.0	0	-inf	Alanine dehydrogenase

Appendices

Appendix A

Appendix

A.1 Membrane enrichment in EVs

The shape of the *V. cholerae* bacterium is close to a bent cylinder with rounded ends, approximately 0.4 µm in diameter and 1.6 µm long[1] (Fig. 1.4), with an approximate surface-to-volume ratio of

$$\begin{aligned}\frac{S_b}{V_b} = \frac{S_{sph} + S_{cyl}}{V_{sph} + V_{cyl}} = \frac{4\pi r_c^2 + 2\pi r_c L_c}{\frac{4}{3}\pi r_c^3 + \pi r_c^2 L_c} = \frac{2r_c + L_c}{\frac{2}{3}r_c^2 + \frac{1}{2}r_c L_c} \\ \approx 12.6\,\mu\text{m}^{-1} \quad (r = 0.2\,\mu\text{m}, L = 1.2\,\mu\text{m})\end{aligned} \tag{A.1}$$

where S_b and V_b is the surface and volume of the bacterium, r_c, S_{sph} and V_{sph} is the radius, surface and volume of the hemispherical ends, and L_c, S_{cyl} and V_{cyl} is the length, surface and volume of the cylindrical part of the bacterium. This number on its own does not tell us much without finding the same value for vesicles within the typical size range, which for a spherical vesicle simplifies to

$$\begin{aligned}\frac{S_v}{V_v} = \frac{4\pi r_v^2}{\frac{4}{3}\pi r_v^3} = \frac{3}{r_v} \\ \Rightarrow 30\,\mu\text{m}^{-1} < \frac{S_v}{V_v} < 300\,\mu\text{m}^{-1} \quad (10\,\text{nm} < r_v < 100\,\text{nm})\end{aligned} \tag{A.2}$$

where r_v, S_v and V_v is the radius, surface and volume of the vesicle, respectively. The ratio between the surface-to-volume ratios for vesicles and bacteria is therefore up to $300\,\mu\text{m}^{-1}/12.6\,\mu\text{m}^{-1} = 23.8$ meaning that outer membranes will be enriched up to about twenty-fold in vesicles by default.

Assessing the enrichment of the periplasmic membrane is more complex, as the periplasmic space varies in thickness from 10–25 nm, and if this thickness is inherited by double membrane vesicles, smaller vesicles will contain considerably less inner membrane than outer membrane. Modifying and combining Eq. A.1 and Eq. A.2 to solve for the periplasmic surface to total-body-volume, we end up with the ratio

$$\frac{S_{v_p}/V_v}{S_{b_p}/V_b} = \frac{(r_v - \Delta_p)^2}{r_v^3} \cdot \frac{2r_c^3 + \frac{3}{2}r_c^2 L_c}{2(r_c - \Delta_p)^2 + (r_c - \Delta_p)L_c} \tag{A.3}$$

where S_{v_b} and S_{b_p} denote the periplasmic surface areas of the vesicle and bacteria, respectively, and Δ_p is the periplasmic thickness. Eq. A.3 for various Δ_p is plotted in Fig. 4.1.

A.2 Vesicle Sedimentation Rates

The velocity of sedimenting particles in solution is given by Stokes' law:

$$V_t = \frac{a(\rho_p - \rho_l)D^2}{18\mu} \tag{A.4}$$

Where V_t is the sedimentation speed, a is the centrifugal acceleration, ρ_p is the density of the particle, ρ_l is the density of the liquid, D is the diameter of the particle, and μ is the viscosity of the liquid. The centrifugal force varies considerably with the position in the centrifuge tube:

$$a = \omega^2 r \tag{A.5}$$

Where ω is the angular velocity. The speed of the particle at a given centrifuge radius is therefore

$$V(r) = \frac{dr}{dt}(r) = \frac{\omega^2 r(\rho_p - \rho_l)D^2}{18\mu} \tag{A.6}$$

giving the integral

$$\int_r^{r_{max}} \frac{dr}{r} = \int_0^{\Delta t} \frac{\omega^2(\rho_p - \rho_l)D^2}{18\mu} dt \tag{A.7}$$

which gives the specific solution

$$[\ln(r) + C]_r^{r_{max}} = \left[\frac{\omega^2 t(\rho_p - \rho_l)D^2}{18\mu}\right]_0^{\Delta t} \tag{A.8}$$

$$\ln\left(\frac{r_{max}}{r}\right) = \frac{\omega^2 \Delta t(\rho_p - \rho_l)D^2}{18\mu} \tag{A.9}$$

Which finally gives the sedimentation time of a particle of diameter D and centrifuge radius r:

$$\Delta t = \ln\left(\frac{r_{max}}{r}\right) \cdot \frac{18\mu}{\omega^2(\rho_p - \rho_l)D^2} \tag{A.10}$$

Inserting the required variables, we can use this equation to calculate an approximate rate of sedimentation in the tube. Firstly, From gradient centrifugation, we have observed that the majority of vesicles are immobilized at the density interface $1107\,\mathrm{kg\,m^{-3}} < \rho < 1160\,\mathrm{kg\,m^{-3}}$ during optiprep, indicating that their density (including hydration layer) is within this range. Secondly, the vesicle size distribution is different from strain to strain, but lets assume it follows a Kumaraswamy distribution, as it has upper and lower bounds (We could assume that there are no vesicles with diameters lower than the thickness of two lipid bilayers, and larger diameter than the bacteria). Such a distribution could be plotted in R (shown in Figure A.1):

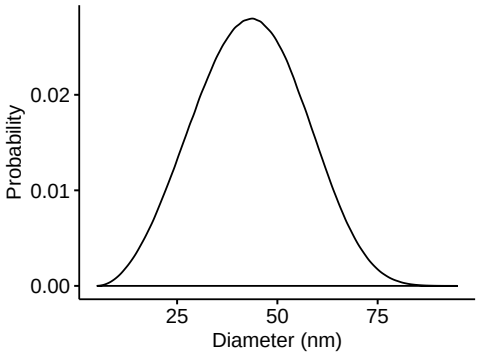


Figure A.1: A theoretical size distribution of vesicles in a sample.

```
library("extraDistr")
pdf("Rplots.pdf",width=4, height=3)
a<-rkumar(10000000,a=3,b=12)*100+5
ggdensity(a,xlab="Diameter (nm)",ylab="Probability")
dev.off()
```

This is of course not experimental data, but for demonstrative purposes. Now if we assume that that the angular velocity is $\omega = 28,000\ \mathrm{RPM} \times 2\pi/60\mathrm{s}\ \mathrm{min}^{-1}$, that the density of centrifugation liquids used in the thesis research (LB and PBS) is approximately that of water, and a solution of dynamic viscosity of $\mu = 0.001\,519\,2\,\mathrm{kg\,m^{-1}\,s^{-1}}$ for water,[2] we can numerically estimate the sedimentation times for a random population of vesicles in R with:

```
#Defining variables
#Max centrifugal radius (SW 32 Ti rotor)
rmax<-0.1525
#Min centrifugal radius (SW 32 Ti rotor)
rmin<-0.0668
#Radius of tube round bottom (SW 32 Ti rotor, 38.5 mL tube)
rtube<-0.0125
#Vesicle average size and standard deviation
v_avg<-55
v_stdev<-30
#RPM of centrifugation yields angular speed squared
rpm<-28000
omegasq<-(rpm*2*3.1415/60)^2
#Density difference between particle and medium
#This value is estimated to be between 1.1160-1 and 1.1067-1
#so we should calculate for both:
d_rho<-(1.1067-1)*1000
d_rho2<-(1.1160-1)*1000
```

```
#Nanometers per meter
nano<-1000000000
#Viscosity of medium
mu<-0.0015192
factors<-(omegasq*d_rho*(1/nano)^2/(18*mu))*3600
factors2<-(omegasq*d_rho2*(1/nano)^2/(18*mu))*3600
#Constructing a million vesicles and finding sedimentation time:
i<-1
while (i<1000000){
    #Vesicle radius:
    vesd<-rkumar(1,a=3,b=12)*100+5
    #Centrifugal radius (position in tube):
        #r<-rtruncnorm(n=1, a=5, b=300, mean=55, sd=30)
    r<-runif(1,rmin,rmax)
    #Vesicle volume
    vol<-4*pi/3*(vesd/2)^3
    #Testing if vesicle is "outside" the round bottom
    #by checking if a random number between 0 and 1 is
    #higher than the fraction between the area of a cross-
    #section at that r and the largest cross-section (pi*rtube^2).
    #Set vesicle diameter to 1 nm if so, to be discarded in next test
    if(r>(rmax-rtube)){
        if(runif(1,0,1)>(1-((r-rmax+rtube)^2)/rtube^2)){vesd<-1}
    }
    #Vesicles less than 5 nm in diameter are discarded
    if(vesd>5){
            tid1<-log(rmax/r)/(factors*vesd^2)
                tid2<-log(rmax/r)/(factors2*vesd^2)
            i<-i+1
            write(c(tid1,tid2,vol),file="sediment_times_rkumar.dat",append=TRUE)
    }
}
```

A one-liner in Bash[3] gives the deviation between the diameter of the sedimented EVs and the real average EV-diameter in the original sample (in percent).

```
cat sediment_times_rkumar.dat | sort -g | awk 'BEGIN{limit=0.1} {sum+=$3;num++}
     ($1>limit){print $1,$2,(((sum/num)*3/(4*3.14159265))^(1/3)*2/47.0985-1)
    *100;limit+=0.1+2*($1>10)}' > sediment_times_rkumar_condensed.dat
```

The data from this calculation is shown in Figure 4.7.

A.3 DNA Size Simulation

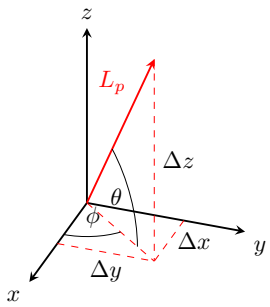


Figure A.2: Illustration of a step in a random walk, where two randomly selected angles (θ,ϕ) determine the subsequent position on the polymer. Drawn in TikZ.

A crude way to approximate the size of free DNA is the random walk, where the DNA strand is assumed to be a series of freely hinged segments of persistence length L_p. For DNA, L_p is close to 40 nm, depending on solvent conditions.[4] Each additional segment points in a random direction (a step in the random walk), and the final end-to-end distance is calculated, and the average square of this distance is calculated for a large number of segments. Using the geometry displayed in Figure A.2, this can be done for a million random DNA fragments using R:

```
#define average hydrodynamic radius with irrelevant value (averageRh),
#DNA length in bp (lengthbp) and DNA persistence length in nm (Lp)
averageRh<-0
lengthbp<-90000
Lp<-40
#Calculate DNA length in nm (lengthnm),
#and number of segments (Nseg)
lengthnm<-lengthbp*0.334
Nseg<-lengthnm/Lp
#Create 1M random polymers and calculate average hydrodynamic radius
for(i in 1:100000){
#create random walking angle in x/y plane
a<-runif(Nseg,0,2*pi)
#create random angle from x/y-plane (z-direction)
b<-runif(Nseg,-pi/2,pi/2)
#x-, y-, and z-walk distance is Lp*sin(a)cos(b), Lp*cos(a)cos(b) and Lp*sin(b)
#Calculate mean square end-to-end distance:
e2eMeanSquare<-sum(Lp*sin(a)*cos(b))^2+sum(Lp*cos(a)*cos(b))^2+sum(Lp*sin(b))^2
#Calculate hydrodynamic radius (Rh) assuming theta solvent
Rh<-0.665/sqrt(6)*sqrt(e2eMeanSquare)
```

```
#Calculate average Rh
averageRh<-(averageRh*(i-1)+Rh)/i
}
print(c("Length (bp):",lengthbp,"R_h:",averageRh))
```

Which for 90 and 20 kbp gives:

```
"Length (bp):"  "90000" "R_h:"  "272.69134249194"
"Length (bp):"  "20000" "R_h:"  "128.518193821593"
```

A.4 Intralocational enrichment of protein

Table A.1: Enrichment or depletion (in standard deviations) of selected proteins in *V. cholerae* EVs according to their subcellular location predicted by PSORTb.[5]

Chr	Start (bp)	End (bp)	Name	Deviation (sd)
Outer Membrane				
1	604748	606034	Long-chain fatty acid transport protein	+1.90
2	574154	576235	TonB-dependent heme and hemoglobin receptor HutA	+1.47
1	22921	24879	TonB-dependent receptor/Enterobactin receptor IrgA	+1.04
1	2171660	2172916	N-acetylglucosamine regulated methyl-accepting chemotaxis protein	-2.62
2	982518	984164	Methyl-accepting chemotaxis protein/hemolysin secretion protein HylB	-1.78
2	658613	660376	Methyl-accepting chemotaxis protein	-1.56
Periplasmic				
2	249540	250376	Maltose operon periplasmic protein MalM	+1.99
1	2583744	2584745	Cytochrome c551 peroxidase	+1.64
1	2638123	2638725	Periplasmic thiol disulfide interchange protein DsbA	+1.61
1	1532264	1533616	tolB protein precursor	+1.61
2	234775	235611	Lys-arg-orn-binding periplasmic protein precursor	+1.46
1	1758323	1758907	Superoxide dismutase [Fe]	-2.15
1	376496	376990	Thiol peroxidase - Tpx-type	-1.62
1	1484137	1485102	TRAP-type transport system	-1.25
1	1960971	1961480	Outer membrane protein H precursor	-1.25
1	2144138	2146042	5'-nucleotidase / 3'-cyclic-nucleotide 2'-phosphodiesterase	-1.25

Chr	Start (bp)	End (bp)	Name	Deviation (sd)
Inner Membrane				
1	458427	459236	Methionine ABC transporter substrate-binding protein	+2.61
1	183286	184197	Lipoprotein nlpI precursor	+2.40
1	1162238	1164235	Tail-specific protease precursor	+1.92
2	632746	635904	Acriflavin resistance protein	+1.79
2	691696	693534	Protein-export membrane protein SecD	+1.73
1	1534719	1535159	Tol biopolymer transport system TolR protein	+1.64
2	107415	108632	Sodium/dicarboxylate symporter	+1.47
1	1669338	1670213	FIG01200138 hypothetical protein	+1.47
2	273527	275386	ABC transporter ATP-binding protein	+1.36
1	2748829	2749776	Lipid A biosynthesis lauroyl acyltransferase	+1.18
1	2199428	2200324	Signal peptidase I	+1.18
1	1942140	1943399	FIG004599 Hypothetical protein	+1.14
1	1761404	1762618	Cytochrome c heme lyase subunit CcmH	+1.14
1	100396	102201	LppC putative lipoprotein	+1.14
2	441983	443296	Putative hemolysin	+1.03
1	2702131	2705241	RND multidrug efflux transporter / Acriflavin resistance protein	+1.03
1	1535162	1535845	MotA/TolQ/ExbB proton channel family protein	+1.03

Chr	Start (bp)	End (bp)	Name	Deviation (sd)
Inner Membrane cont.				
1	1366288	1366956	Phage shock protein A	-2.23
1	1948882	1949220	Nitrogen regulatory protein P-II	-2.23
1	2472674	2474518	GTP-binding protein TypA/BipA	-2.23
1	1152224	1153234	Dihydroorotate dehydrogenase	-1.47
1	1863873	1865744	Methyl-accepting chemotaxis protein	-1.47
1	2558614	2559762	Uncharacterized protein EC-HemY clustering with hemC/hemD	-1.47
1	516815	517690	Magnesium and cobalt efflux protein CorC	-1.47
1	85450	86835	Signal recognition particle subunit Ffh SRP54	-1.47
1	880208	881818	Methyl-accepting chemotaxis protein	-1.47
1	938486	940117	Methyl-accepting chemotaxis protein	-1.47
1	956006	957637	Methyl-accepting chemotaxis protein	-1.47
2	1075118	1077115	GGDEF family protein	-1.03
1	107711	108628	ABC-type multidrug transport system/ATPase component	-1.03
1	1335448	1337193	Response regulator VieA	-1.03
1	168900	170846	Cell division protein FtsH	-1.03
1	1804682	1805392	Succinate dehydrogenase iron-sulfur protein	-1.03
1	2200416	2202209	Translation elongation factor LepA	-1.03
1	2751913	2753592	Methyl-accepting chemotaxis protein	-1.03
1	394726	396606	Accessory colonization factor AcfB	-1.03
1	882049	882561	Glycine cleavage system regulatory protein	-1.03
1	927563	929452	Methyl-accepting chemotaxis protein	-1.03
2	204538	206427	Methyl-accepting chemotaxis protein	-1.03
2	348707	350581	Methyl-accepting chemotaxis protein	-1.03
2	934290	935426	D-alanyl-D-alanine carboxypeptidase	-1.03

Chr	Start (bp)	End (bp)	Name	Deviation (sd)
Cytoplasmic				
2	657274	657747	FKBP peptidyl-prolyl cis-trans isomerase/Macrophage infectivity potentiator	+3.44
1	761253	761990	FIG008443 hypothetical protein	+3.44
2	322932	323606	Transcriptional regulator VpsT	+3.24
1	2722657	2724828	ATP-dependent DNA helicase UvrD/PcrA	+2.71
1	196365	197636	ATP-dependent RNA helicase SrmB	+2.71
1	2783197	2784612	Long-chain-fatty-acid–CoA ligase	+2.41
1	537534	538448	Cell division inhibitor Slr1223 YfcH in EC	+2.33
1	2860465	2861781	ATP-dependent RNA helicase RhlB	+2.14
1	2004815	2006101	Na(+)-translocating NADH-quinone reductase subunit A	+2.14
1	1999156	2000160	Thiamin biosynthesis lipoprotein ApbE	+2.03
2	250529	251686	FOG TPR repeat protein/SEL1 subfamily	+1.80
1	446174	447625	tRNA S(4)U 4-thiouridine synthase	+1.80
1	2255749	2256216	Aspartate carbamoyltransferase regulatory chain PyrI	
	+1.80			
1	2134858	2135808	rRNA small subunit methyltransferase H	+1.80
1	2000306	2001532	Na(+)-translocating NADH-quinone reductase subunit F	+1.80
1	1666933	1667745	Septum site-determining protein MinD	+1.80
1	1414674	1415684	Purine nucleotide synthesis repressor	+1.80
1	1092624	1093376	Fumarate and nitrate reduction regulatory protein	+1.80
1	2917097	2918077	HflC protein	+1.80
1	2771656	2773428	Polysaccharide deacetylase	+1.66

Chr	Start (bp)	End (bp)	Name	Deviation (sd)
Cytoplasmic cont				
1	1978897	1980006	3-4-dihydroxy-2-butanone 4-phosphate synthase	-2.82
1	1226780	1228954	Catalase / Peroxidase	-2.44
1	2059672	2060892	Phosphopentomutase	-2.44
1	1158608	1161214	Membrane alanine aminopeptidase N	-2.14
1	1542419	1543798	Cysteinyl-tRNA synthetase	-2.14
1	162816	164003	Tyrosyl-tRNA synthetase	-2.14
1	1855476	1856384	Predicted dye-decolorizing peroxidase DyP	-2.14
2	135729	137909	4-alpha-glucanotransferase (amylomaltase)	-2.14

A.5 ToxR-motif investigation

An short insert sequence containing a ToxR-binding motif was constructed from two synthesized primers, with overhangs corresponding to the restriction patterns of EcoRI and NdeI, with phosphorylated 5'-ends:

```
5' AATTGTTGTATTTTTTATTTTTTTTTTATCGAG      3'
3'     CAACATAAAAAATAAAAAAAAAAATAGCTCAT   5'
```

An analogous construct of equal length and AT-content, without longer stretches of either A's or T's was also produced to construct a control vector:

```
5' AATTAATTGTTAACAATTGTTAACAATTGTTAA      3'
3'     TTAACAATTGTTAACAATTGTTAACAATTAT    5'
```

Plasmid pUC19 was digested with EcoRI and NdeI. The backbone (largest fragment) was purified from a gel and ligated with the synthesized ToxR-binding-insert, and the resulting plasmid was assigned the name pToxR (Figure A.3).

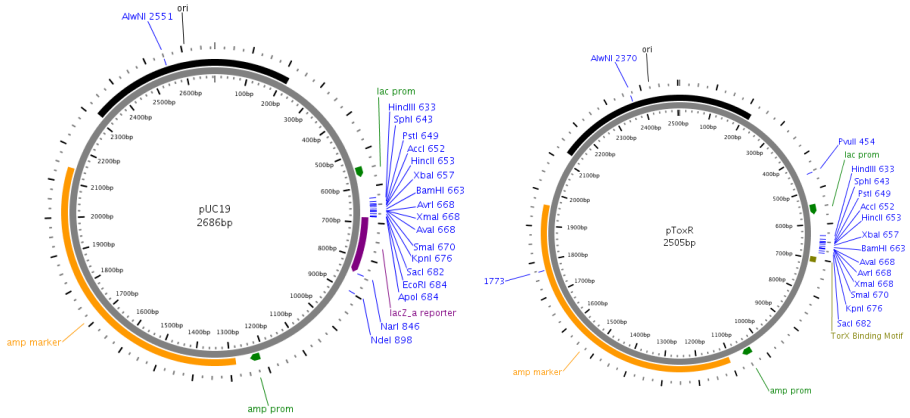


Figure A.3: Maps of pUC19 plasmid and modified plasmid pToxR with a ToxR binding motif inserted between restriction sites for EcoRI and NdeI. Restriction sites for EcoRI and NdeI are lost during ligation due to loss of palindromic sequence.

Analogously, a ligation of the backbone with the control insert was attempted, but with no success. This could be because of the self-complementary patterns in the primers used to construct the insert.

V. cholerae TCP2 (as used in Paper I) was successfully electroporated with pUC19 or pToxR. The conditions that are known to induce ToxR-regulated

Table A.2: Primers used for qPCR for relative quantification of pToxR plasmid and chromosomes of *V. cholerae*.

Contig	Position	Direction	Sequence	Abbreviation
Ch1	767k	F	TCGACGATCGGTTTGGTGTT	ch1_767k_F
Ch1	767k	R	GCGATCACGTGCAACAGATC	ch1_767k_R
Ch1	60k	F	TGTGTTTGCTGAGCATTGGC	ch1_60k_F
Ch1	60k	T	GCCTTGCAGAATAGAACGCG	ch1_60k_R
Ch2	6k	F	CCGCCCGGAATGACTTGATA	ch2_6k_F
Ch2	6k	R	TTCTGGGTAGCGTCAATGCA	ch2_6k_R
Ch2	1M	F	GCTCCCAATCGAAATCTCTGG	ch2_1M_F
Ch2	1M	R	CCGCAAGATCGAGACCGTAT	ch2_1M_R
pToxR	650	F	GTCGACTCTAGAGGATCCC	pUC19ToxR_650_F
pToxR	750	R	GGCATCAGAGCAGATTGTACT	pUC19ToxR_750_R
pToxR	2k	F	AGATGGTAAGCCCTCCCGTA	pUC19ToxR_2k_F
pToxR	2k	R	CAGTGAGGCACCTATCTCAGC	pUC19ToxR_2k_R
pUC19	1856	F	AGCCCTCCCGTATCGTAGTT	p1856_F
pUC19	1955	R	TGCTTAATCAGTGAGGCACCT	p1955_R
pUC19	2482	F	GAGCGAACGACCTACACCGA	p2482_F
pUC19	77	R	CGCTTACCGGATACCTGTCC	p77_R

gene expression are 30 °C with a starting pH of 6.5 and 85.5 mM NaCl, while repressing conditions are 37 °C with a starting pH of 8.5 and 171 mM NaCl.[6] The enrichment of pToxR and pUC19 relative to chromosomal DNA at steady states of these conditions, as well as shortly after transitions between these conditions, was quantified using qPCR as follows: 5 mL volumes of LB medium modified to either ToxR-inducing, or -repressing conditions was inoculated with 100 µL of overnight culture (regular LB inoculated with single colony, grown at 37 °C) and grown for approximately 8 h at the corresponding temperature (30/37 °C), until an OD∼1. A 500 µL portion of the culture was filtered (0.45 µm) and reserved for qPCR. The remaining culture was centrifuged at 4,000 × g at corresponding temperatures (30/37 °C) for 10 minutes, the supernatant discarded, and fresh medium of the same or another condition was added. These cultures were grown for 30 min before 500 µL of the culture was filtered (0.45 µm) and reserved for qPCR. Before qPCR, 50 µL volumes of the filtrates were subjected to 2U DNase, incubated for 30 min at 37 °C, subjected to another 2U DNase, incubated for 30 min at 37 °C, before the DNase was inactivated at 75 °C for 15 min. Each sample was lysed by addition of 5 µL 1% triton and heated to 99 °C for 10 min. 2 µL of this lysate was used as a DNA template for 20 µL qPCR reactions with LightCycler® 480 SYBR Green I Master mix (cat.# 04707516001), according to manufacturers protocol. The primers used are listed in Table A.2.

A.6 Enriched genes encoding hypothetical proteins

A few hypothetical genes were detected only in EV samples (and not in whole bacteria) as reported Paper I. These could in theory be sRNA/msRNA that is communicated through EVs. FIG01200169: hypothetical protein (Ch1:879765:880115)

```
ATGAGCAAATTAACGGCAGATATCGAAGCGAATCTAGCGCTGTTTATTCAGGAAACCCAAGAAA
GCCAACTTGTTTGGGGTTTACGCAATGAAGAAGGTTGGCTCTCTTGTGAGTCGACTGAATTTGA
AGAAAGTGAAGTGATGCCTTTCTGGTCATCCAAAAAAGATGCGCAAATTCATAACGTTGAAGAG
TGGGCTGACTTTGAAGTGGTAGAAATCCCGCTGGATGTTTTTGTAGAAGATTGGTTAATCACTC
TTGATGAAGATGGTGTTCTGGTCGGAACGAACTGGAATGCCAACCTCGAAGGTAAAGAATTAGA
GCCATCACAACTGGCCAAACTCTACCTATAA
```

FIG01200711: hypothetical protein (Ch2:148369:148566)

```
ATGTCCATTAATTCTATTGACCATGATGACATGACGAACATTGCCAACAAATGGGATTCAATTG
AAGAAATTGAATCCCAGCGACCAACAAAGAACTTGAAGTCAGCGGAAGCTCGACGCCGTATTGA
AACGCTACGTGAGATACGCGAAAGCGGCCTGACTATCGAGGAAGCTAAAGAGCTGGGTTTGTTG
CACTAG
```

FIG01199699: hypothetical protein (Ch1:1019319:1019543)

```
ATGTTTTGGGACACACTCGAACGTGTTAATCGTCTGCGTCAGCAAGCGATGAATAACCCTGAAT
TTCTTCAATCTGCCAAACAGCATGAGGAAACTTTGCAGCACGTTGAACAATACTTTGAACCGAA
GAAATATCGCAAAAGTAACCAAAAACGCCAAAAAACCTTGGCCGATATCTATGATCAAGCCGAT
TTTGGTGAACGTACTGATGATGTCACTCATTAG
```

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