

The Genomic Content of Outer Membrane Vesicles (OMVs) in Freshwater Cyanobacteria

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Claudia Maria Taubenheim, geb. Walda
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Erstgutachter: Prof. Dr. rer. nat. Tal Dagan

Zweitgutachter: Prof. Dr. rer. nat. Ruth Schmitz-Streit

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Claudia Taubenheim

Im Laufe dieser Arbeit wurden mit Zustimmung des Betreuers folgende Beiträge veröffentlicht:

Claudia Taubenheim, Jan Taubenheim, Nils Hülter, Tal Dagan

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1 Abstract

Outer membrane vesicles (OMVs) are secreted by many bacterial species, where they are involved in diverse extracellular functions as well as intercellular communication. They contain DNA which has been reported to be a source for lateral gene transfer at a similar efficiency as transduction. However, it is only poorly understood what the natural genetic content of OMVs comprise and how it changes with different environmental conditions. Here, I searched for the existence of OMVs in several cyanobacteria and studied their genomic content. Additionally, I assessed OMVs abundance and genomic content in the unicellular *Synechocystis* sp. PCC 6803 under various environmental conditions.

I isolated vesicles from stationary growth cultures. Transmission electron microscopy showed OMVs in *Synechocystis* sp. PCC 6803, as well as in the filamentous *Anabaena* sp. PCC 7120 and *Chlorogloeopsis fritschii* PCC 6912 as discrete, spherical blebs having a bilayer membrane with a diameter ranging between 20 and 150 nm. Some of the isolated OMVs contained DNA and sequencing revealed the presence of most of the genome including the chromosome and plasmids (if existing) in all three cyanobacteria. Additionally, a temperate phage was identified in *Chlorogloeopsis fritschii* PCC 6912 via TEM and sequencing.

For *Synechocystis* sp. PCC 6803, I found that the OMV abundance is significantly higher in bacteria cultured under heat- or high salt- stress in comparison to the control conditions. Sequencing revealed that two small cryptic plasmids – pCA2.4 and pCB2.4 – were highly overrepresented in the vesicle DNA independent of the growth conditions compared to a genomic DNA sample. I additionally identified overrepresented genes dependent on the growth condition in vesicles.

I hypothesize that the enrichment for small plasmids in the vesicles is a stochastic process and a result of their high abundance in the cell, their packed topology, high diffusibility, and location. Further, I speculate that various mechanisms involved in cell cycle, transcription, or gene amplification might increase the likelihood of specific sequences to be packed in vesicles. My observations implicate that OMVs are a transfer mechanism of small plasmids by providing a nuclease protected DNA space and may serve as a mechanism to maintain plasmids and/or important genes for the population.

2 Zusammenfassung

Viele Bakterien produzieren sogenannte "Outer membrane Vesicles (OMVs)"- äußere Membranvesikel. Diese sind an diversen extrazellulären Funktionen und der interzellulären Kommunikation beteiligt. Sie können DNA enthalten und frühere Studien zeigten, dass sie lateralen Gentransfer mit einer Effizienz vergleichbar zur Transduktion ermöglichen können. Es ist allerdings nur wenig bekannt, was der natürliche genomische Inhalt der Vesikel ist und wie sich dieser unter verschiedenen Bedingungen ändert. In dieser Arbeit untersuchte ich die Existenz der OMVs und deren DNA Gehalt in verschiedenen Cyanobakterienspezies. Zusätzlich quantifizierte ich OMVs und analysierte Vesikel-DNA in verschiedenen Wachstumsbedingungen in dem unizellulären *Synechocystis* sp. PCC 6803.

Die Vesikel isolierte ich aus Kulturen in der stationären Wachstumsphase. Transmissionselektronenmikroskopie zeigte die Existenz von OMVs sowohl in *Synechocystis* sp. PCC 6803 als auch in den filamentösen Spezies *Anabaena* sp. PCC 7120 und *Chlorogloeopsis fritschii* PCC 6912. Die OMVs zeigten sich als diskrete, runde Entitäten mit einer doppelschichtigen Membran und einem Durchmesser zwischen 20 und 150 nm. Die Sequenzierung des vesikulären Inhalts offenbarte die Existenz von DNA innerhalb der Vesikel, wobei fast das gesamte Chromosom und die Plasmide (sofern in der Spezies vorhanden) in allen drei Cyanobakterien identifiziert werden konnte. Außerdem identifizierte ich einen temperenten Phagen in *Chlorogloeopsis fritschii* PCC 6912 mittels TEM und Sequenzierung.

In *Synechocystis* sp. PCC 6803 stellte ich im Vergleich zu Kontrollbedingungen, signifikant höhere OMV Mengen in mit Salz- bzw. Hitzestress behandelten Bakterienkulturen fest. Die Sequenzierung der Proben zeigte eine starke Überrepräsentation der zwei kleinen Plasmide pCA2.4 und pCB2.4 im Vergleich zur genomischen DNA und unabhängig von der jeweiligen Wachstumsbedingung. Zusätzlich identifizierte ich überrepräsentierte Gene, abhängig von den jeweiligen Wachstumsbedingungen.

Ich stelle die Hypothese auf, dass die Anreicherung der kleinen Plasmide in den Vesikeln ein stochastischer Prozess ist, der durch ihre hohe Anzahl in der Zelle, ihrer komprimierten Topologie, ihrem hohen Diffusionsvermögen und ihrer zellulären Lokalisation bedingt wird. Weiterhin spekuliere ich, dass verschiedene Mechanismen des Zellzyklusses, der Transkription oder Genamplifikation die Wahrscheinlichkeit erhöhen könnten, dass spezifische DNA Sequenzen in OMVs akkumulieren. Meine Beobachtungen implizieren, dass OMVs einen Transfermechanismus für kleine Plasmide und DNA Fragmente darstellen könnten, indem sie einen Nuklease-geschützten Raum schaffen, der für die Erhaltung der Plasmide und wichtiger Gene in der Population dient.

3 Introduction

3.1 Cyanobacteria in a nutshell

Cyanobacteria are photoautotrophic, gram-negative bacteria and the only microorganisms capable of oxygenic photosynthesis (Stanier *et al.* 1971; Hamilton *et al.* 2015). They are thought to reach back 3.5 billion years ago and might have been the main contributors of molecular oxygen to the great oxygenation event (GOE) where the atmosphere of the earth was enriched with O₂ (reviewed in (Bekker 2015)). However, the actual appearance of oxygenic photosynthesis is recently under intensive debate as some studies postdate the emergence of cyanobacteria after the GOE (Shih *et al.* 2017; Betts *et al.* 2018).

Although monophyletic, cyanobacteria are phenotypically and physiologically very diverse. Depending on their morphology, their ability to fix nitrogen, form specialized cells, and their way to perform cell division, they are traditionally classified into five sections although this is not necessarily supported by phylogenetic date. (Rippka *et al.* 1979) (see Figure 1).

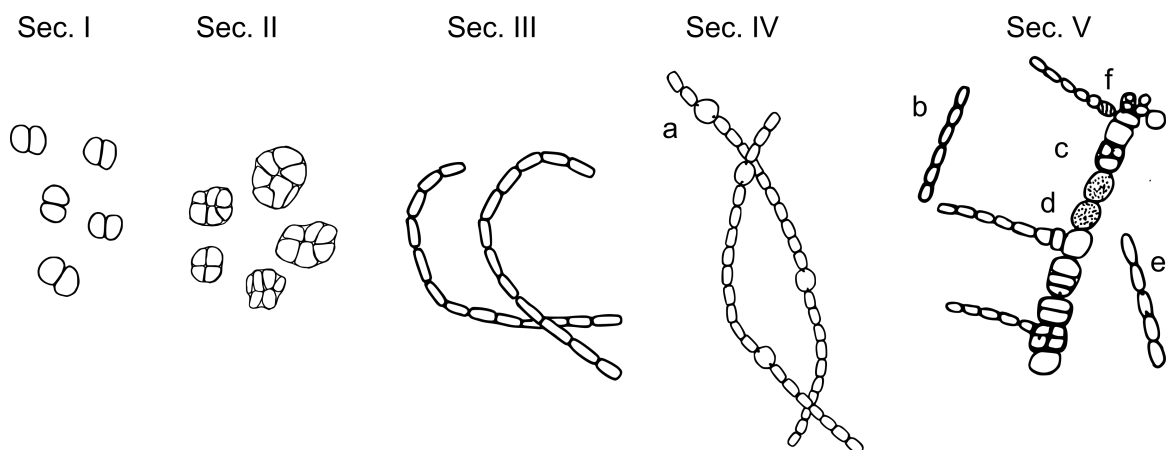


Figure 1 Schematic drawing of cyanobacteria from Section (Sec.) I – IV with generic morphologies. a & d: heterocyst, b: hormogonium, c: akinets, e: trichome, f: necridia. Sec. V inspired by <https://www.spektrum.de/lexikon/biologie/fischerella/24614>.

Section I (*Chroococcales*) are unicellular, round, cylindrical or elliptical bacteria that mainly divide by binary division or budding. They form aggregates that cohere due to sheath or mucous polysaccharides. Examples are *Synechococcus* sp. or *Synechocystis* sp. Section II (*Pleurocapsales*) such as *Pleurocapsa* or *Dermocapsa* are likewise unicellular, but divide by multiple fission of a vegetative cell without following growth, leading to small reproductive cells, so-called baeocytes that are eventually released by the rupture of the parental cell wall (Rippka *et al.* 1979). Cyanobacteria from section III (*Oscillatoriales*), e.g. *Spirulina*, *Oscillatoria* and *Pseudoanabaena* grow filamentous in uniseriate chains (trichome, Figure 1e- found in bacteria from Sec. III - V)) and reproduce by trichome-breaking, where cell division occur in one plane. They do not show specialized cell types (Rippka *et al.* 1979). Section IV and V cyanobacteria (IV: *Anabaena*, *Scytonema*, *Calothrix*; V: *Fischerella*, *Chlorogloeopsis*) are likewise

multicellular, but while section IV bacteria divide in only one plane, section V bacteria exhibit true branching by division into several planes. Section IV (*Nostocales*) and V (*Stigonematales*) are able to produce special cell types such as heterocysts (Figure 1a & d) that have a thick cell wall, thus providing a specific compartment for the oxygen sensitive nitrogen fixation. Other cell types may be akinetes, thick-walled resting cells (Figure 1c), hormogonia (Figure 1b) – short, initially uniseriate, unbranched, non-differentiated, and mobile cell chains that are important for bacterial cell growth, and necridia, cells deriving from programmed cell death and enabling the formation of hormogonia (Rippka et al. 1979; Herrero et al. 2016).

Cyanobacteria are globally relevant primary producers due to their ability of using sunlight and carbon dioxide as energy sources (Scanlan 2014). They can be found in a diverse range of environments reaching from soil in deserts to rocks in the Antarctica, basically wherever light is accessible (Garcia-Pichel et al. 2001; Pandey et al. 2004).

Additionally, they can be found in all aquatic habitats from hot springs to hypersaline ponds, in freshwater as well as in the marine environment where cyanobacteria are responsible for a large fraction of the photosynthetic output in the open ocean (Rippka et al. 1979; Field et al. 1998; Dadheech et al. 2013). They can grow endolithic a few millimeter beneath the rock surface at one of the driest deserts in the world while other species are colonizing hot springs with pH 4 and 70°C as described for *Synechococcus lividus* (Wierzchos et al. 2006; Whitton and Potts 2007).

Moreover, cyanobacteria can be free-living or being in close symbiosis for example with plants where they provide organic components or fixed nitrogen in *Cycadaceae*, *Gunnera*, and the aquatic *Azolla* (Adams et al. 2006). A well-known symbiosis occurs in lichens that comprise a fungi and algae or cyanobacteria of mainly *Nostoc* species providing energy via photosynthesis and fixed nitrogen (Rai et al. 2007). Interestingly, also animals such as *Ascidiae* and *Porifera* live in close symbiosis with cyanobacteria (López-Legentil et al. 2011; Webster and Taylor 2012).

It is acknowledged for a long time that chloroplasts are the product of an endosymbiosis of a cyanobacterial ancestor with an eukaryotic ancestor cell (Martin and Kowallik 1999). Phylogenetic studies showed that a filamentous, nitrogen-fixing species of section IV and V is most likely the ancestor of plastids (Deusch et al. 2008; Dagan et al. 2012).

In the following paragraphs, I give a more detailed introduction to the organisms, I mainly worked with during this study.

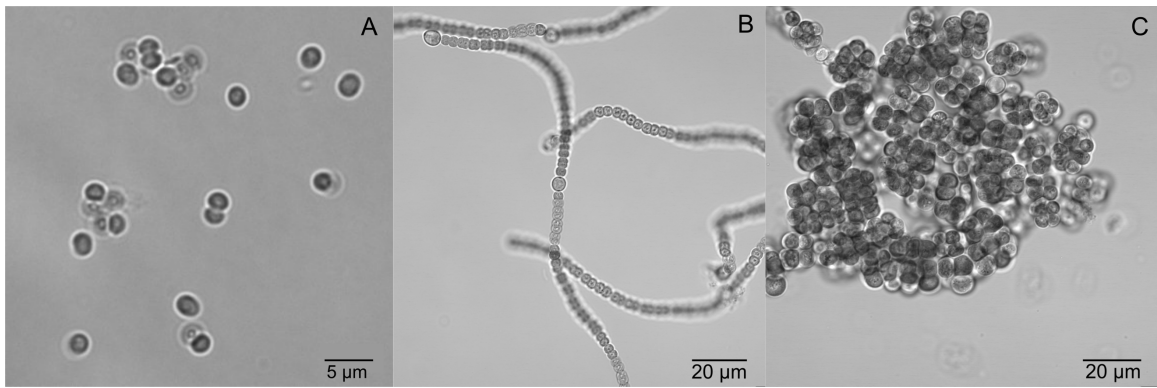


Figure 2 Light microscopic images of cyanobacteria used in this study. (A) *Synechocystis* sp. PCC 6803, (B) *Anabaena* sp. 7120, (C) *Chlorogloeopsis fritschii* 6912. Image credits B & C: Dr. Karina Stucken.

3.1.1 *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 is a unicellular, coccoid or spherical and non-nitrogen fixing cyanobacterium (see Figure 2A). The strain, I used in my thesis is non-motile and glucose-tolerant. *Synechocystis* sp. PCC 6803 is well studied, both in fundamental as well as in biotechnology studies and many molecular tools are available. This bacterium was first isolated in 1968 from a freshwater lake in California, USA (Stanier *et al.* 1971) and it is naturally competent, where DNA integration occurs via homologous recombination (Grigorieva and Shestakov 1982).

In 1996, the whole genome was sequenced as the fourth organism ever (Kaneko *et al.* 1996). It has a 3,57 megabases chromosome, 4 large plasmids (pSYSA (103 kb), pSYSG (44 kb), pSYSM (120 kb), and pSYSX (106 kb)) and 3 small plasmids (pCA2.4: 2.4 kb; pCB2.4: 2.35 kb; pCC5.2: 5.2 kb (Kaneko *et al.* 1996; Kaneko and Tabata 1997; Kaneko *et al.* 2003). On the large plasmids, 397 genes were predicted with 77% of unknown function, although some gene clusters could be found that are likely to be involved in biosynthesis and transport of yet unknown polysaccharides. Among the genes with known functions are parA and parB which are important for distributing of plasmids during cell division (Motallebi-Veshareh *et al.* 1990; Kaneko *et al.* 2003). Other genes important are a toxin-antitoxin system genes and a CRISPR-Cas system on pSYSA (Kopfmann and Hess 2013; Scholz *et al.* 2013). The three small plasmids possess no known phenotypically relevant functions for the bacterium and are therefore referred to as “cryptic” plasmids. Only pCA2.4 consists of one gene with a confirmed function: *slr9101* (*repA*), which leads to the assumption of a rolling circle replication mechanism of this plasmid (Yang and McFadden 1993) (see Box 1 for a short definition). Additionally, single stranded plasmid DNA was detected in *Synechocystis* sp. PCC 6803 (Yang and McFadden 1993; Xu and McFadden 1997) as well as in other cyanobacteria (Kurokawa *et al.* 1994), supporting the assumption that plasmid replication in cyanobacteria is by rolling circle replication. The second ORF on pCA2.4 as well as the three ORFs on pCB2.4 and six ORFs on pCC5.2 are hypothetical proteins with no similarities to any known proteins so far

(Yang and McFadden 1993; Yang and McFadden 1994; Xu and McFadden 1997). The copy numbers for the three small plasmids have been found to range from 0.4 to 7.4 per chromosome under different growth conditions ((Berla and Pakrasi 2012; Armshaw et al. 2015)).

Box 1

Rolling circle mechanism

In many bacteria, small plasmids sized roughly from 1.3 kb to 10 kb, replicate by the rolling circle mechanism. The replication starts with a nick at the double stranded strain origin (DSO) initiated by replication proteins (rep). The 3' end elongation is catalyzed by polymerase III using the unnicked strand as template without the need of a RNA primer. The single stranded, nicked DNA is displaced and ligated to a circular ssDNA where Okazaki fragments are used for complementary strand synthesis. Several ssDNA copies of the plasmids can be produced continuously by this mechanism (reviewed in (Ruiz-Masó et al. 2015)).

3.1.2 *Anabaena* sp. PCC 7120

Anabaena sp. PCC 7120 is a freshwater cyanobacterium of the order *Nostocales* (see Figure 2B). The exact origin of this strain is unclear, but it has been reported initially in 1971 at the Iowa State University as *Nostoc muscorum* (Adolph and Haselkorn 1971). However, later it was included to the Pasteur collection in Paris (PCC) and reassigned to the genus *Anabaena* (Rippka et al. 1979). It is a model organism for the investigation of its filamentous growth as well as for the mechanisms underlying the development of heterocysts in nitrogen deprived environments where *Anabaena* sp. PCC 7120 separates the process of O₂ producing photosynthesis from the O₂ sensitive nitrogenase. Up to every tenth cell can be a heterocyst that provides fixed nitrogen to the neighboring cell in exchange for photosynthetic products (Kumar et al. 2010; Herrero et al. 2016). The genome consists of a 6.4 Mbp large chromosome and 6 plasmids ranging from 5,584 to 408,101 bp. Overall, *Anabaena*'s genome contains roughly 6100 protein coding genes and was fully sequenced in 2001 (Kaneko et al. 2001).

3.1.3 *Chlorogloeopsis fritschii* PCC 6912

Chlorogloeopsis fritschii PCC 6912 is a multicellular, heterocyst forming and akinetes producing strain of section V, initially found in soil of rice fields in India and later conveyed to the Pasteur collection (PCC) (Mitra 1950; Rippka et al. 1979) (see Figure 2C). It grows in filaments without producing lateral branches leading to a multiseriate phenotype (Dagan et al. 2012). The strain harbors a 7.8 Mb chromosome with around 7,400 CDS. Additionally, an intact prophage of approximately 90 kb was identified (Dagan et al. 2012).

3.2 Membrane vesicles are produced by all life forms

3.2.1 Membrane vesicles in Eukaryotes are important for communication and stress responses

All forms of life produce membrane vesicles. These vesicles are referred to as extracellular vesicles (EVs) in eukaryotes, membrane vesicles (MV) in Gram (+) bacteria and Archaea and outer membrane vesicles (OMVs) in Gram (-) bacteria. In eukaryotes, the term “extracellular vesicles” include multiple types. For example, exosomes that derive from multivesicular bodies in the cell with a size of 40-100 nm and appear normally homogeneously formed (*Mathivanan et al. 2010*). In a yet not fully understood mechanism, eukaryotic parasites such as *Leishmania donovani* shed so called microvesicles directly from the cell membrane leading to a heterogeneous population of 100-1000 nm sized so called shedding membrane vesicles (*Silverman et al. 2008*). A third example are apoptotic blebs, produced by cells during programmed cell death (apoptosis) (*Kerr 1971; Wyllie et al. 1980*). Eukaryotic membrane vesicles are released as a response to different stimuli, i.e. heat (*Clayton et al. 2005; Martínez et al. 2005*). The EVs are involved in many cellular functions such as cell-cell communication or transmission of signal molecules for example of Acetyl choline between nerve cells, in angiogenesis and immune response (reviewed in (*Théry et al. 2002; Muñoz and Rudy 2014*)). They protect molecules from degradation through extracellular proteases or other chemicals, but are also required for removing undesirable components from the cell (reviewed in (*Pilzer et al. 2005; Stahl and Raposo 2019*)).

3.2.2 Outer membrane vesicles (OMVs) in Gram (-) bacteria

First descriptions of bacterial membrane vesicles date back to the later 1960's, when Chatterjee & Das (1967) described “membrane sacs” produced by *Vibrio cholerae* during normal growth (*Chatterjee and Das 1967*). Although known for over 50 years, membrane vesicles of bacteria and archaea are only recently coming more and more into focus of research with the improvement of microscopic techniques, mass spectrometry and DNA sequencing. In Gram (-) bacteria, they are defined as discrete, closed, outer membrane blebs produced by growing cells that occur spherical with a bilayer or double bilayer membrane in electron microscopy. The average diameter ranges between 20 to 250 nm. They contain bacterial lipids, outer membrane proteins, periplasmic content, as well as nucleic acids (*Beveridge 1999; Kuehn and Kesty 2005*). Bacteria constitutently produce OMVs when growing planktonic or in surface attached biofilms, under lab conditions, and, more importantly, in natural environments (*Kadurugamuwa and Beveridge 1995; Beveridge et al. 1997; Beveridge 1999*). Over the past decades OMVs were found in all gram-negative bacteria investigated so far for example in *E. coli*, *Helicobacter pylori*, *Acinetobacter* spp., *Pseudomonas aeruginosa*,

and many more (Hoekstra et al. 1976; Borneleit et al. 1990; Kadurugamuwa and Beveridge 1995; Fiocca et al. 1999; Nasarabadi et al. 2017). Membrane vesicles were also described in some Gram-positive bacteria such as *Bacillus anthracis*, *Staphylococcus aureus*, or *Rumnicoccus* spp. (Klieve et al. 2005; Lee et al. 2009; Rivera et al. 2010). Archaea, for example *Sulfolobus* spp., *Ingnicoccus* spp., or *Halorubrum lacusprofundi* R1S1 release membrane vesicles that contain lipids, membrane proteins, toxin, and/or DNA (Rachel et al. 2002; Ellen et al. 2008; Erdmann et al. 2017).

3.3 Vesiculation mechanisms

3.3.1 Bacterial membranes

For the understanding of how Gram (-) bacteria produce outer membrane vesicles it is crucial to understand the structure and function of the bacterial cell membrane. The Gram (-) envelope consists of two distinct bilayer membranes – the outer membrane (OM) and the cytosolic, inner membrane (IM) separated by the periplasmic space which contains a thin peptidoglycan (PG) layer (Silhavy et al. 2010) (see Figure 3).

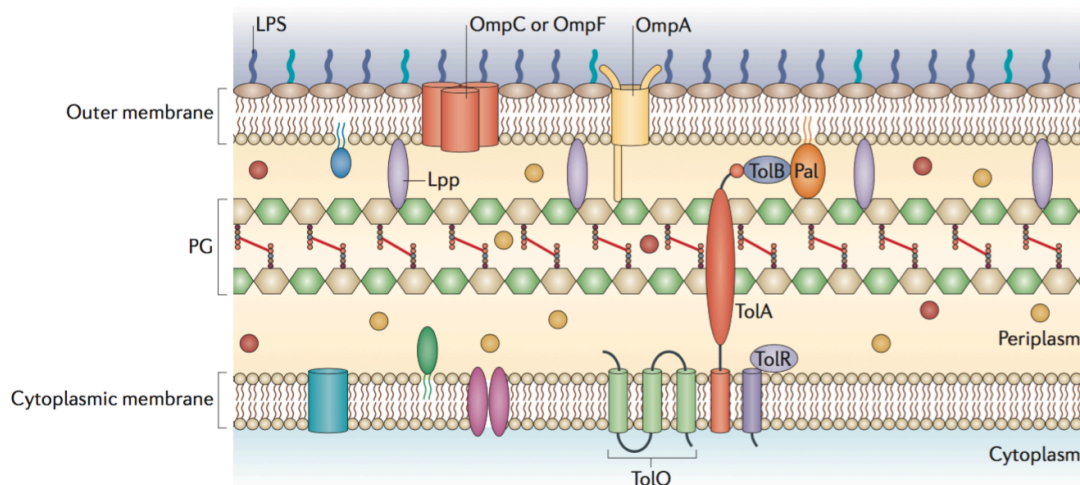


Figure 3 Schematic drawing of the Gram(-) cell wall by (Schwechheimer and Kuehn 2015). The cytosolic membrane consists of two leaflets of mainly phospholipids being almost non-permeable except for transmembrane proteins that can act as transporters (blue, purple). The outer membrane consists of an inner leaflet comprising of phospholipids and an outer leaflet that consists of lipidopolysaccharides. A peptidoglycan-layer provides stability and resistance against osmotic pressure changes and comprises typically of N-acetylglucosamine and N-acetylmuramic acid that are linked via 1,4-beta-glycosidic bindings and are crosslinked via tetrapeptides binding to a lactyl-residue at the disaccharides. The outer membrane is relatively permeable due to porins (OmpA, OmpC, OmpF). These porins are non-covalently linked to the PG layer, while the Tol-Pal complex and the periplasmic protein Lpp bind covalently to the peptidoglycans, all together providing more stability to the bacterial cell.

The inner membrane consists of phospholipids in both leaflets whereas the outer membrane's outer leaflet is dominated by lipopolysaccharides (LPS) and phospholipids only predominate in the inner leaflet (Silhavy et al. 2010). This asymmetry is provided due to the

action of ATP-binding cassette transporter proteins (ABC transporter) that translocate ATP-dependent LPS from its synthesis origin (cytoplasmic leaflet) to the outer leaflet of the cell membrane (Ward et al. 2007; Dong et al. 2017; Mi et al. 2017). Furthermore, divalent cations like Mg^{2+} or Ca^{2+} stabilize the surface charges of the outer membrane by forming salt bridges between negatively charged phosphates of neighboring LPS molecules (Tashiro et al. 2009; Tashiro et al. 2010).

The net-like peptidoglycan layer consists typically of the disaccharides N-acetylglucosamine, connected 1,4 beta-glycosidic to N-acetylmuramic acid and crosslinked by tetrapeptides (Fuchs 2007). It provides bacteria their shape and protection from shear stress or changes in the osmotic pressure (Vollmer and Bertsche 2008). Periplasmic tetrapeptides, such as the *E. coli* Lpp and its homologues in other species, crosslink the PG layer with the OM covalently, thereby maintaining the structural integrity (Braun and Rehn 1969; Braun 1975). Additional stability is provided on the one hand by the highly conserved Tol-Pal system, which is necessary for the OM invagination as part of the cell division machine and on the other hand on the OM porin OmpA, that contains a noncovalent PG interaction motif (Gerding et al. 2007; Smith et al. 2007; Park et al. 2011). These proteins are part of large secretions systems where some might span both membranes and are classified in at least eight families. They are important for translocating large molecules such as virulence factors or antimicrobial peptides with the help of ABC transporters from the cytoplasm to the environment (Green and Meccas 2016). In some bacteria, tripartite efflux pumps exist that also connect both membranes and are important for the export of toxic components, thus providing for example multidrug antibiotic resistance (Symmons et al. 2009; Du et al. 2014).

Intriguingly, the membrane of Gram(-) bacteria is highly dynamic as the bacterial cell needs its envelope to adapt to membrane biogenesis, cell division, and environmental changes and therefore rearrange it, while maintaining its integrity (Ernst et al. 2001; Silhavy et al. 2010; Egan and Vollmer 2013).

3.3.1.1 The cell envelope of cyanobacteria

Freshwater cyanobacteria exhibit some special characteristics in their cell envelopes. Although generally thought to have a cell envelope that places cyanobacteria to Gram (-) bacteria, they possess a thicker peptidoglycan layer, different lipid components and a higher degree of crosslinks between the PG-chains that are more similar to the cell wall of Gram (+) bacteria (Smarda et al. 1979; Hoiczky and Hansel 2000; Hahn and Schleiff 2014). However, some typical components of Gram (+) such as teichonic acid or L-lysine bacteria are not found in cyanobacteria (Hoiczky and Hansel 2000).

The lipid composition of cyanobacterial membranes is very different from that of proteobacteria, reflecting the adaption to a vast variety of environments. Yet, the protein composition of the outer membrane is comparable to that of other Gram (-) bacteria with

respect to the findings of outer membrane porins (Omps) and TolC-like proteins (*Hahn and Schleiff 2014*). However, the LPS composition is extremely different not only compared to enterobacteria, where those poly-saccharides are extensively studied, but also throughout cyanobacteria and even on strain level of bacteria from the same genus. The most obvious difference to the LPS of enterobacteria is the absence of a conserved heptose and the structural difference of Lipid A (*Weckesser et al. 1979; Hahn and Schleiff 2014*).

Often, cyanobacteria contain an additional layer - the surface- or S-layer with a variety of functions in maintaining the cell integrity, providing protection against the environment and influencing the cell-cell communication (*Engelhardt and Peters 1998; Šmarda et al. 2002*). This layer consists of a paracrystalline, mainly hexagonal array of proteinaceous material forming a layer between the outer membrane and the mucous sheath. However, this S-layer is not present in *Nostocales* and *Stigonematales* (*Liberton and Pakrasi 2008; Hahn and Schleiff 2014*).

A special characteristic of filamentous bacteria such as *Anabaena* sp. 7120, used in this study, is the observation that they have a thick PG-layer that is interrupted by septosomes, connecting adjacent cells, while arranged in a continuous outer membrane sack. This leads to the assumption of a continuous periplasmic space that facilitates transfer of products between vegetative cells and specialized N₂-fixing heterocysts (*Wilk et al. 2011; Hahn and Schleiff 2014*).

3.3.2 Bacteria produce OMVs by several mechanisms

The mechanisms underlying the production of OMVs by bacteria have been extensively studied over the past years and it becomes more and more evident that several mechanisms can lead to vesiculation, depending on the bacteria species (reviewed i.a. in (*Pathirana and Kaparakis-Liaskos 2016*)). In general, outer membrane vesicles are formed by bulging of the outer membrane and eventually fission, thereby enclosing periplasmic content (Figure 4).

Complex mechanisms are likely to be involved as not only the composition of the vesicle membrane but also the cargo differs from that of the cellular outer membrane and periplasm (*Bonnington and Kuehn 2014*). As described in 3.3.1, the outer membrane of bacteria is highly fluid/flexible and under constant change, thus forms bulges from which the vesicles can occur (*McBroom and Kuehn 2006; Schwechheimer et al. 2013*).

The actual bulging starts with a curvature of the outer membrane that can take place through different mechanisms such as the charge-to-charge repulsion between neighboring negatively charged LPS. In *Pseudomonas aeruginosa* for example, this can be enhanced by the quorum-sensing molecule PQS (*Pseudomonas* quinolone signal) probably through sequestering positive charges from LPS (*Mashburn-Warren and Whiteley 2006; Warren et al. 2008; Tashiro et al. 2009*).

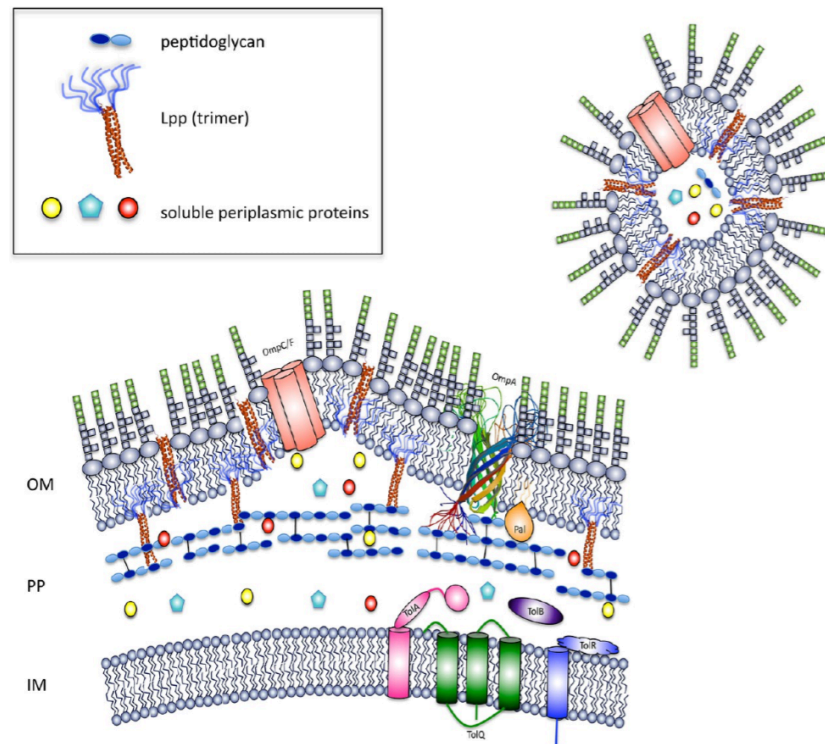


Figure 4 Schematic OMV production model of Gram (-) bacteria. The bacterial envelope consists of the cytosolic membrane with a phospholipid bilayer, a periplasmic space as well as its peptidoglycan (PG) net, and an outer membrane (OM). The OM consists of an inner leaflet with phospholipids and an outer leaflet with mainly lipopolysaccharides (LPS), and porin proteins such as OmpA or OmpC/F. The layers are interconnected by covalent (Lpp-PG) and non-covalent crosslinks (Tol-Pal, OmpA-PG). The bulging of vesicles can occur at sites of fewer crosslinks where cargo can accumulate and eventually fission takes place. Cartoon taken from (Schwechheimer et al. 2013).

A cellular mechanism for the biogenesis has been demonstrated for the highly-conserved phospholipid transporter VacJ/Yrb (ABC (ATP-binding cassette) transporter). Roier et al. (2016) reported that a decreased expression led to an outward membrane bulging and fission of OMVs due to an accumulation of curvature-inducing phospholipids (Roier et al. 2016). However, the bulging and fission of vesicles can only take place when the cross-link-system, such as Lpp, Tol-Pal or OmpA are weakened and eventually loosed. The cross-links occur in inconsistent intervals leading to a diverse spatial distribution over the outer membrane which promotes cross-link depleted regions more likely to become OMV sites due to localized breaking of bonds or down-regulation of the cross-linking proteins (Warren et al. 2008; Schwechheimer et al. 2013). On the other hand, outer membrane biogenesis might occur faster than the peptidoglycan layer synthesis at particular sites, leading to a more flexible outer membrane with less covalently bound crosslinks thereby providing an easy bulging zone (proposed by (Schwechheimer et al. 2013)). Recently, several genes, i.a. important for envelope stability (such as *ompA*), LPS/ PG synthesis and PG remodeling, were identified to modulate the biogenesis of OMVs in *Salmonella enterica* serovar Thyphi (Nevermann et al. 2019).

Additionally, autolysins are present in the periplasm of e.g. *E. coli* or *Porphyromonas gingivalis* that can cleave the peptidoglycan layer, hence promote OMV production and/or the uptake of the OMV's cargo (Zhou *et al.* 1998; Hayashi *et al.* 2002; Vollmer and Bertsche 2008).

The actual cargo might also play a role in the destabilization of the peptidoglycan-outer membrane crosslinks. As vesicles can act as cellular garbage cans or secrete special insoluble proteins (see 3.3.3 and 3.3.3.2), both could lead to an accumulation of cargo that either occurs at periplasmic regions without crosslinks or even prevent the formation of those, thus leading to bulging of the outer membrane to form vesicles (proposed by (Schwechheimer *et al.* 2013)).

The proposed non-exclusive models have in common that the natural flexibility of the outer membrane, where bulging is likely to occur, is accompanied by an increasing spatial distance of non-covalently bound proteins (Pal and OmpA) leading to weakened PG-OM cross-links (reviewed in more detail in (Schwechheimer *et al.* 2013; Schwechheimer and Kuehn 2015)). Additionally, it has been shown that the Lpp-PG connection is transient although no enzyme capable of cleaving that bond has been described so far. Yet, all above mentioned mechanisms share the fact that the vesicles are produced by living cells. However, in *Pseudomonas aeruginosa*, an additional vesiculation process has been observed. OMVs could form after a so called explosive cell lysis, where membrane fragments re-anneal into vesicles, entrapping surrounding components, for example free DNA. This mechanism was associated with phage-borne endolysin activity (Turnbull *et al.* 2016).

Taken together, different mechanisms, depending on bacterial strains or the actual function and content might lead to the formation of outer membrane vesicles. Studying not only the process of vesiculation itself, but the content of vesicles might provide a useful tool to derive potential mechanism of direct loading of cargo to the vesicles, to understand the biological significance of OMVs and whether the production is regulated or by-product of other cellular processes.

3.3.3 Biological function of OMVs

OMVs have been linked to numerous biological functions such as nutrient acquisition, inter- and intra-species communication, pathogenesis, biofilm formation, and phage-defense mechanism (see Table 1). In *Pseudomonas aeruginosa* for example, vesicles serve as predatory molecules by containing a strong lysin, thus facilitate nutrient acquisition. This bacteria's OMVs fuse to the outer membrane of other bacteria and release their strong lytic molecules, leading to a full lysis of the prey cell. Afterwards, *P. aeruginosa* can feed on the prey's cellular content (Li *et al.* 1996).

Table 1 Examples of outer membrane vesicle functions.

OMV function	Species
Biofilm formation/ maintenance/ communication	<i>Helicobacter pylori</i> (Grande et al. 2015), <i>P. aeruginosa</i> (Mashburn and Whiteley 2005; Schooling et al. 2009), <i>P. putida</i> , <i>Porphyromonas gingivalis</i> (Kamaguchi et al. 2003), <i>Stenotrophomonas maltophilia</i> (Devos et al. 2016), <i>H. influenza</i> (Schaar et al. 2013)
Defense against phages	<i>E. coli</i> (Manning and Kuehn 2011), Marine OMV samples (Biller et al. 2014), <i>Vibrio cholera</i> (Reyes-Robles et al. 2018)
Host-microbe interactions	<i>Algoriphagus machipononensis</i> (Alegado et al. 2012; Woznica et al. 2016; Woznica and King 2018), <i>Vibrio fischeri</i> (Aschtgen et al. 2015; Aschtgen, Lynch, et al. 2016), Mitochondria (Soubannier et al. 2012; Cook et al. 2014; Braschi et al.)
Nutrient acquisition	<i>Bacteroides</i> (Elhenawy et al. 2014; Rakoff-Nahoum et al. 2014), <i>P. aeruginosa</i> (Li et al. 1996), <i>Prochlorococcus</i> (Biller et al. 2014)
Pathogenesis	<i>Actinobacillus actinomycetemcomitans</i> (Meyer and Fives-Taylor 1993), <i>Bacteroides gingivalis</i> (Grenier and Mayrand 1987), <i>Chlamydia trachomatis</i> (Frohlich et al.), <i>E. coli</i> (Kolling and Matthews 1999; Wai et al. 2003), <i>Legionella pneumophila</i> (Fernández-Moreira et al. 2006), <i>Moraxella catarrhalis</i> (Vidakovics et al. 2010), <i>P. aeruginosa</i> (Ciofu et al. 2000), <i>Xylella fastidiosa</i> (Ionescu et al. 2014)
Phage receptor transmission	<i>Bacillus subtilis</i> (Tzipilevich et al. 2016)
Stress response	<i>E. coli</i> (McBroom and Kuehn 2006; McBroom et al. 2006), <i>P. aeruginosa</i> (Tashiro et al. 2009; Baumgarten et al. 2012)

Outer membrane vesicles have been found to possess several functions in the formation and maintenance of biofilms. For example, in *P. aeruginosa*, the affinity between OMVs and DNA can contribute to the biofilm structure (Schooling et al. 2009). Similar findings were made in *Helicobacter pylori* biofilms, where vesicle derived DNA might as well play a role in the formation of the biofilm matrix (Grande et al. 2015). The putative main function of DNA in vesicles, facilitating lateral gene transfer in a nuclease-protected environment, will be discussed in detail in 3.4.2.

Furthermore, OMVs contribute to the cell-cell communication, which is crucial for bacteria to synchronize large cell groups as in biofilms (Waters and Bassler 2005). Quorum sensing (QS) is a communication system, where bacteria use chemical signals to communicate and modulate expression of specific genes in response to these signals, thereby coordinating for instance their population density (Parsek and Greenberg 2000). However, these signaling molecules are often very hydrophobic which can negatively influence the diffusion between cells in the extracellular, mostly aqueous, milieu. *P. aeruginosa* circumvents this by packaging at least some of the QS molecules namely PQS into vesicles. Moreover, PQS is required for the actual formation of OMVs (see 3.3.2 and (Mashburn and Whiteley 2005)).

Ciofu et al. (2000) demonstrated that beta-lactamase is packaged in OMVs of *P. aeruginosa*, growing in biofilms on lung tissue of cystic fibrosis patients, leading to a high concentration of extracellular beta-lactamase. This inactivated the respective antibiotic, hence protecting deeper layers of bacteria and consequently promoting a high resistance of biofilm-associated cells to beta-lactam antibiotics (Ciofu et al. 2000). OMVs of the human oral cavity inhabiting *Porphyromonas gingivalis* facilitate a multispecies biofilm formation by trafficking quorum-sensing molecules, leading to the formation of bacterial aggregation of *Staphylococcus aureus*, *Streptococcus* spp., *Actinomyces* spp., and, also the opportunistic pathogenic fungi *Candida albicans* (Kamaguchi et al. 2003). Beta-lactamase containing vesicles derived from *H. influenzae* can also protect the often co-colonizing Gram (+) *Streptococcus pyogenes* from being destroyed by amoxicillin (Schaar et al. 2013). Similar findings are demonstrated for beta-lactamase containing OMVs from *Stenotrophomonas maltophilia* that can establish extracellular beta-lactam degradation and consequently increase the survival of the cohabitating species *P. aeruginosa* and *Burkholderia cenocepacia* (Devos et al. 2016).

Additionally, outer membrane vesicles may play a crucial role in communication with eukaryotic cells during infections, thereby promoting pathogenesis. One example is the inhibition of the maturation of phagosomes in macrophages by OMVs from *Legionella pneumophila*, that prevent the fusion of phagosomes and lysosomes in a mechanism independent from the type IV secretion (Fernández-Moreira et al. 2006).

In fact, promoting pathogenesis through several routes is one main function of outer membrane vesicles. In *Bacteroides gingivalis*, OMVs facilitate the attachments of the bacteria by a rather unspecific mechanism, which also promotes an additional attachment of otherwise non-co-aggregating bacteria and leads to increased pathogenicity (Grenier and Mayrand 1987). A similar mechanism has been described for *Actinobacillus actinomycetemcomitans* (Meyer and Fives-Taylor 1993). Another pathogenesis mechanism is the direct delivery of toxins such as the Shiga toxin via OMVs of *Escherichia coli* to epithelial cells that eventually damage them by inhibition of the 60S subunit of the ribosome, leading to an interruption of protein synthesis (Endo et al. 1988; Kolling and Matthews 1999). Since the toxins are protected from exoproteases, vesicles can facilitate and prolong infections (Kolling and Matthews 1999). ClyA, a toxin present in its monomeric, inactive form in the periplasm of *E. coli* cells becomes active (oligomeric) when packaged in OMVs. Wai and colleagues (2003) demonstrated that the toxin was eightfold more effective against eukaryotic cells when delivered via vesicles (Wai et al. 2003). Outer membrane vesicles can also act as decoys as indicated for the respiratory pathogen *Moraxella catarrhalis*, where vesicles redirect the immune response to produce antibodies that are inefficient in clearing the intact bacterial cell (Vidakovics et al. 2010).

Interestingly, even the intracellular, extremely small (0.5 μm) bacterium *Chlamydia trachomatis* produces vesicles that directly interacts with the host, promoting pathogenicity by delivering virulence factors and antigens (Frohlich et al. 2014).

The plant pathogen *Xyllela fastidiosa* produces OMVs that block the attachment to surfaces thereby modulating plant colonization and promoting two different life styles of the pathogen. Firstly, the bacterium is infective by adhering to insects and is therefore transmitted between plants and secondly, by the action of OMVs, the bacterium is no longer able to attach to several surfaces and consequently able to systemically infect the plant host (Ionescu et al. 2014).

3.3.3.1 OMVs are involved in species interactions

The above-mentioned functions of OMVs were mainly involved in promoting pathogenicity, thereby damaging the host. Yet, vesicles may also promote symbiotic interactions, thus support the maintenance of complex multispecies communities ((Warren et al. 2008) and reviewed in more detail in (Schooling and Beveridge 2006; Toyofuku et al. 2017)).

One symbiotic interaction may be providing nutrients in an otherwise dilute environment such as the Ocean which has been demonstrated by Biller et al (2014), where the cyanobacterium *Prochlorococcus* produces labile carbon containing vesicles that can be used by the heterotrophs *Alteromonas* and *Halomonas* (Biller et al. 2014). A similar mechanism is the distribution of enzymes such as hydrolases and polysaccharide lyases to the community by *Bacteroides*, enhancing the mutually break-down of complex polysaccharides in the gut (Elhenawy et al. 2014; Rakoff-Nahoum et al. 2014).

Several studies elucidated the importance of OMVs in the communication and development of bacteria and their eukaryotic hosts. The squid *Euprymna scolopes*' development is triggered by OMVs from the bioluminescent symbiont *Vibrio fischeri* through its LPS enriched cargo (Aschtgen, Wetzel, et al. 2016; Aschtgen, Lynch, et al. 2016). Another example is the phylogenetically basal choanoflagellate *Salpinx goecki* that switches its lifestyle upon a lipid based signaling molecule from *Algoriphagus machipononensis* from a unicellular swimmer to a multicellular rosette form. The bacterium releases highly hydrophobic, hence almost water insoluble sulfonolipids in outer membrane vesicles that directly fuse with the eukaryotic host (Alegado et al. 2012; Woznica et al. 2016; Woznica and King 2018; Lynch and Alegado).

Even mitochondria were shown to produce OMVs, promoting a Vps35 dependent vesicle transport between mitochondria and peroxisomes or multivesicular bodies (Soubannier et al. 2012; Cook et al. 2014; Braschi et al.). Actually, Gould and colleagues (2016) proposed that the eukaryotic endomembrane system, comprising of the nucleus, the endoplasmic reticulum, the Golgi apparatus, and phago-/ lysosomes, originated from bacterial outer

membrane vesicles. Those OMVs would have been released by the mitochondrial ancestor (most probably an alpha-proteobacterium) within the archaeal host at the origin of the eukaryotic cell (Gould *et al.* 2016).

A remarkable feature of OMVs is their contribution to phage defense mechanisms. Co-incubation of the T4-bacteriophage and outer membrane vesicles of *E. coli* results to a fast and irreversible binding, thereby leading to a significantly reduced infection efficiencies of T4 by the formation of phage-vesicle complexes (Manning and Kuehn 2011). A similar mechanism has been observed in OMVs derived by two pandemic *Vibrio cholera* strains that can partially inhibit and thereby neutralize three different phages (Reyes-Robles *et al.* 2018). Additionally, in marine OMV samples, phages were detected that clearly bound to OMVs (Biller *et al.* 2014). Yet, Tzipilevich *et al.* (2016) found an opposite mechanism where phages that infect *Bacillus subtilis*, fuse with membrane vesicles of this bacterium, thereby spreading phage receptors (Tzipilevich *et al.* 2016).

3.3.3.2 Outer membrane vesicles in stress response systems

Producing outer membrane vesicles might also be a regulated stress response independently from known stress-response systems in bacteria (see Box 2 for general stress responses). It complements other pathways for example by letting bacteria effectively remove undesirable membrane components that occur upon environmental stress such as temperature and exposure to toxic agents (McBroom and Kuehn 2006).

E. coli produces low amounts of OMVs under normal growth conditions, but the vesiculation is highly induced under stress conditions that lead to an accumulation of overexpressed or misfolded membrane components. McBroom and Kuehn (2006) showed that this mechanism is independent of other stress-response pathways such as σ^E , Cpx, Bae and the Psp response in *E. coli* although it might be sensed by σ^E (McBroom and Kuehn 2006). In *Pseudomonas aeruginosa*, Tashiro *et al.* (2009) demonstrated that vesicle production is enhanced when misfolded outer membrane proteins are present, supporting the important role of vesicles as a decoy of undesired products during stress response (Tashiro *et al.* 2009). The marine proteobacteria *Ahrensia kielensis* and *Pseudoalteromonas marina* react with an early onset of vesiculation after exposure to heat stress (Hagemann *et al.* 2013). Even biofilm formation can be enhanced after several stressors such as NaCl or heat as shown for *Pseudomonas putida* (Baumgarten *et al.* 2012).

Stress response systems in bacteria

Bacteria have the ability to react and adapt to a variety of fast occurring and strong stressors such as heat, salt, light, toxic agents or nutrient deprivation. These responses occur in several ways:

1. Specific, stress dependent **transcription factors** (sigma-factors) bind to promotor regions of operons that contain genes, necessary for the adaptation to the specific stressor (for example chaperons or other heat shock proteins) and **activate/enhance** their transcription (*Ron 2013*).
2. **Transcription factors** (e.g. HrcA in *Chlamydia*) act as **repressors** as they bind to an inverted repeat motif (CIRCE - "controlling inverted repeat of chaperone expression") in the promotor region of stress response genes such as *dnaK* or *groES* and *groEL*. These transcription factors are released upon sensing stressors and transcription of effector genes takes place (*Wilson and Tan 2002; Wilson and Tan 2004*).
3. **Small RNAs** might regulate stress response either negatively by facilitating the decay of effector genes mRNAs or positive by relaxing translation inhibiting RNA secondary structures amount of transcription factors in a cell and thus the transcription of stress effector genes (*Majdalani et al. 2001; Papenfort et al. 2006*).
4. **SOS response to DNA** damage controlled by conserved auto regulated proteases. While LexA acts as a repressor on genotoxic response genes, RecA binds to damaged DNA and subsequently cleaves LexA. Consequently, the effector genes can be transcribed (*Žgur-Bertok 2013*).
5. **Envelope stress response** by CpX and sigma E occurs in *E. coli*. These transcription factors are effective in complex signaling pathways after sensing damaged or misfolded outer membrane proteins (sigmaE) or misfolding and damage of the inner membrane or periplasmic components (two component system CpX). They regulate the transcription of several stress response genes such as SurA for OMP and LolA for lipoproteins (*Grabowicz and Silhavy 2017*).

3.4 Nucleic acids in outer membrane vesicles

Apart from containing proteins and lipids, OMVs can enclose DNA or RNA. This is a remarkable process as nucleic acids have to migrate from the cytoplasm through the plasma membrane into the periplasmic space. The vesicular DNA contributes to lateral gene transfer (see Box 3 for general mechanisms) in many species (for example (*Dorward et al. 1989; Yaron et al. 2000; Renelli et al. 2004; Fulsundar et al. 2014*), and recently reviewed in (*Domingues and Nielsen 2017*), and Table 2).

Box 3

Lateral gene transfer in bacteria

Prokaryotes have the ability to take up foreign DNA from the environment or neighboring cells (*Dickerson 1980; Sieber et al. 2017*) via a process, called lateral gene transfer (LGT). This process increase the bacteria's genetic diversity, thereby making LGT a key player in prokaryote genome evolution (*Popa and Dagan 2011*). Several mechanisms are known today.

Transformation: Bacteria take up free DNA form their environment and incorporate it to their genome (*Avery et al. 1944; Popa and Dagan 2011*). A requirement is the competence of bacteria to allow free DNA to pass the cell envelope via special competence-providing proteins (*Johnston et al. 2014*).

Conjugation: Bacteria transfer DNA by direct proteinaceous cell-to-cell junction (*Lederberg and Tatum 1946; Norman et al. 2009; Wozniak and Waldor 2010*). Mostly, self-transmissible or mobilizable plasmids are transferred (*Ochman et al. 2000*).

Transduction: DNA is taken up after the infection by a phage that has replicated in a bacterium and thereby taken up random DNA fragments (specialized tr.) or DNA adjacent to the attachment site of the phage (specialized tr.) (*Zinder and Lederberg 1952; Ochman et al. 2000; Thomas and Nielsen 2005*).

Gene transfer agents (GTA): GTAs are phage like particles with randomly packaged DNA from the producing bacteria, released to the environment and eventually taken up by recipient cells (*Marrs 1974; Lang et al. 2012*).

Nanotubes: Neighboring cells are bridged via tubular protrusions of membranous origin, transferring for example non-conjugative plasmids (*Dubey and Ben-Yehuda 2011*).

Cytoplasmic bridges: These were observed in the halophile archaeon *Halobacterium volcanii* that enables the cells to directly exchange DNA without mixing cytoplasmic content (*Rosenshine et al. 1989*).

3.4.1 RNA in outer membrane vesicles

Many studies focusing on the nucleic acid content mainly investigate the presence DNA (*Domingues and Nielsen 2017*). However, mRNA, rRNA, and tRNA have been found in several species, namely *E. coli*, *Neisseria gonorrhoeae*, *P. gingivalis*, and *Prochlorococcus*, although their functions remain to be elucidated (*Dorward et al. 1989; Biller et al. 2014; Ho et al. 2015; Blenkiron et al. 2016*). In *Pseudomonas aeruginosa*, several short RNAs (sRNAs) could be

found that are transferred to human airway cells, modulating the immune response by interacting with host mRNA and inducing of the pro-inflammatory cytokine IL-8 (Koeppen *et al.* 2016).

3.4.2 DNA in outer membrane vesicles

Already in 1982, Kahn *et al.* describe so called “transformasomes”, nowadays referred to as outer membrane vesicles, in *Haemophilus influenzae* that contain DNA which is protected from extracellular nucleases (Kahn *et al.* 1983). Over the past decades, nucleic acids have been found in the membrane vesicles of several Gram (-), as well as Gram (+) bacteria and even in membrane vesicles of Archaea. However, the mechanism of how nucleic acids are routed into vesicles is still under intensive research.

Over the last years, four main models have been proposed. The first model by Renelli *et al.* (2004) stated that vesicle can take up naked DNA from the environment by short term opening and closing of some vesicles or by a mechanism similar to transformation (Renelli *et al.* 2004). The authors, however admit that the actual amount of DNA found in the vesicles could not be explained by only that mechanism. A second model proposed the formation of vesicles during cell death where part of the chromosome might get trapped, a mechanism that have been shown in *P. aeruginosa* after a phage induced cell lysis (Berleman and Auer 2012; Turnbull *et al.* 2016). A third model considered the non-homogenously formed and therefore transient weakened peptidoglycan layer during formation of vesicles to be responsible that DNA can pass the inner membrane as well as the peptidoglycan layer by an unknown mechanism and consequently end up into new budding vesicles (Kadurugamuwa and Beveridge 1995). A fourth model claimed the formation of complex double bilayered OMVs by a yet unknown mechanism that comprise cytosolic proteins and consequently, DNA, thereby extending the model of Kadurugamuwa *et al.* (Kadurugamuwa and Beveridge 1995; Perez-Cruz *et al.* 2013; Pérez-Cruz *et al.* 2015). They found these complex vesicles in the pathogens *N. gonorrhoeae*, *P. aeruginosa*, *A. baumannii*, and in the Antarctic *Shewanella vesiculosa* M7^T in very low frequencies of 0.1 – 1.2% of total vesicles (Perez-Cruz *et al.* 2013; Pérez-Cruz *et al.* 2015). The DNA content of whole OMV samples ranges from 0.13 ng vDNA/ µg protein in *P. aeruginosa* up to 1.8 ng vDNA/ µg protein in *S. vesiculosa* M7^T (Perez-Cruz *et al.* 2013; Pérez-Cruz *et al.* 2015). Hagemann *et al.* (2013) described similar double-layered vesicles in *Ahrensia kielensis* and *Pseudoalteromonas marina* (Hagemann *et al.* 2013).

DNA in vesicles can be of different origin since plasmid- and phage associated genes were found as well as genes from the bacterial chromosome (Kolling and Matthews 1999; Yaron *et al.* 2000; Gaudin *et al.* 2014) (see Table 2).

The hyperthermophilic Archaeon *Thermococcus kodakaraensis* produces DNA bearing membrane vesicles similar to exosome formation in eukaryotes. Moreover, those

vesicles can transfer plasmid DNA between cells indicating that membrane vesicles might play a role in transformation of Archaea by protecting its cargo from thermo-degradation (Gaudin et al. 2013). In the Archaeon *Thermococcus nautilus*, a small rolling circle plasmid, pTN1 (3619 bp), bursts from NaCl treated purified vesicles while the theta replicating pTN2 (13,015 bp) does not. However, the large pTN3, a putative non-functional archaeal virus (18,345 bp) were also abundant inside the vesicles (Gaudin et al. 2014). In *Halorubrum lacusprofundi* R1S1, a predecessor of the mechanism, of how viruses transfer their genomic content has been proposed. The archaeal plasmid pR1SE is encapsulated in membrane vesicles, containing proteins encoded on that plasmid that are transferred to plasmid-free archaeal *Halorubrum lacusprofundi* R1S1 cells (Erdmann et al. 2017).

In Gram (+) bacteria, the ruminal cellulolytic *Rumnicoccus* spp. releases vesicles that contain longer stretches of <20 kb up to 90 kb linear DNA. Complementation of deletion mutants was possible with those membrane vesicles suggesting that the DNA-containing vesicles might play a role in lateral gene transfer in complex environments such as the rumen (Klieve et al. 2005).

Table 2 DNA containing membrane vesicles were studied in numerous organisms.

Species		DNA origin	References
<i>Acinetobacter baylyi</i>	Gram (-)	Linear DNA, plasmids	(Fulsundar et al. 2014)
<i>Acinetobacter baumannii</i>	Gram (-)	Plasmids	(Rumbo et al. 2011)
<i>Ahrensia kielensis</i>	Gram (-)	Linear DNA	(Hagemann et al. 2013)
<i>Escherichia coli</i> (various strains)	Gram (-)	Plasmids, bacteriophage related genes, chromosomal genes	i.a. (Kolling and Matthews 1999), (Yaron et al. 2000)
<i>Haemophilus influenzae/</i> <i>parainfluenzae</i>	Gram (-)	Linear DNA, Circular DNA	(Kahn et al. 1982; Kahn et al. 1983), (Sharpe et al. 2011)
<i>Neisseria gonorrhoeae</i>	Gram (-)	Linear DNA, plasmids	(Dorward and Garon 1989; Dorward et al. 1989)
<i>Phorphyromonas gingivalis</i>	Gram (-)	Chromosomal genes	(Ho et al. 2015)
<i>Prochlorococcus</i> spp.	Gram (-)	Linear DNA	(Biller et al. 2014; Biller et al. 2016)
<i>Pseudoalteromonas marina</i>	Gram (-)	Linear DNA	(Hagemann et al. 2013)
<i>Pseudomonas aeruginosa</i>	Gram (-)	Linear DNA, plasmids	(Kadurugamuwa and Beveridge 1995; Renelli et al. 2004; Bitto et al. 2017)
<i>Rumnicoccus</i> spp.	Gram (+)	Linear DNA	(Klieve et al. 2005)
<i>Thermococcus kodakaraensis</i>	Archaea	Linear DNA, plasmids	(Gaudin et al. 2013)
<i>Thermococcus nautilus</i>	Archaea	Plasmids, viral DNA	(Gaudin et al. 2014)
<i>Halorubrum lacusprofundi</i> R1S1	Archaea	Plasmids	(Erdmann et al. 2017)

The presence of DNA in Gram (-) bacteria has been studied in many species as shown in Table 2. Already in 1989, Dorward et al. (1989) demonstrated the presence of plasmids and chromosomal DNA in membrane vesicles of *Neisseria gonorrhoeae* and that those vesicles are able to deliver antibiotic resistance gene bearing plasmids but not chromosomal DNA to recipient cells (Dorward et al. 1989). Yaron et al. (2000) showed via electron microscopy that also OMVs of *Escherichia coli* contain intact plasmids as well as linear DNA fragments, most likely from the bacterial chromosome. Vesicle transformation experiments with OMVs containing the gene for GFP (green fluorescent protein) lead to a transformation efficiency of 10^3 transformants per μg of vesicle DNA. The authors additionally demonstrated that vesicles can mediate the transfer of active virulence genes between *E. coli* cells and to other enteric bacteria (here: *Salmonella* serovar Enteritidis). (Yaron et al. 2000). In a similar approach, Rumbo and colleagues (2011) demonstrated that *Acinetobacter baumannii*, a highly abundant pathogen in hospitals often being a huge reservoir of antibiotic resistances, is enriched with plasmids carrying carbapenem resistance genes that can be transferred between different *A. baumannii* strains (Rumbo et al. 2011). In a related bacterium, *Acinetobacter baylyi*, Fulsundar et al. (2014) showed that stress via antibiotics increased the gene transfer frequency significantly. In general, they estimated the transfer efficiency via OMVs depending on the input material up to 9×10^3 transformants per μg vesicle DNA which was ten times more efficient than natural transformation. Also, this group demonstrated the potential of OMVs to overcome species barriers. OMV derived from *A. baylyi*, containing a plasmid with the ampicillin-resistance gene could attach to *E. coli* cells and delivered their DNA-content (Fulsundar et al. 2014). Vesicles of the anaerobic pathogen *Porphyromonas gingivalis* have been shown to transfer antibiotic resistance genes with a transfer efficiency of 1.9×10^{-7} or less when vesicles were treated with DNase I prior transformation. However since the DNA content of vesicles was not quantified, this value is not directly comparable to the transformation efficiencies presented above (Ho et al. 2015).

Only few studies focus on the actual content of OMVs apart from intrinsic or foreign plasmids. Hagemann et al. (2013) studied the DNA content of OMVs derived from *P. marina* and *A. kielensis* by sequencing shotgun libraries. They found parts of the whole genome with complete genes in both species. All sequences were detected only once, thus the authors speculated that DNA is randomly packed into vesicles (Hagemann et al. 2013). On the other hand, Ho et al. (2015) amplified parts of the 16S genes as well as typical virulence genes in *Porphyromonas gingivalis* with different efficiencies leading to the assumption of higher abundances of at least some of the virulence genes in OMVs (Ho et al. 2015). In *P. aeruginosa*, sequencing revealed that vesicle DNA is of chromosomal origin with enrichments of specific

regions of around 1.09 kb and 4.16 kb in five replicates, containing mainly genes responsible for pathogenesis. (*Bitto et al. 2017*). The marine cyanobacteria *Prochlorococcus* spp. and *Synechococcus* spp. produced vesicles containing DNA heterogeneous in size with some at least 3 kbp long. Sequencing revealed over 50% of the chromosome was covered with a reproducible overabundance of reads at the terminus region (*Biller et al. 2014*).

However, all studies were conducted under one specific growth condition or were direct environmental samples. It remains an open question, whether parts of the chromosomal DNA or plasmids are specifically targeted to OMVs or whether this process is random. Additionally, species where vesicle DNA sequences are available, are mostly monoploid, while oligo- or polyploid bacteria might exhibit a different genetic content in their outer membrane vesicles.

4 Objectives

The aim of my PhD thesis was to characterize the genetic content of outer membrane vesicles in freshwater cyanobacterial organisms. My main research questions are as follows:

- I. Are outer membrane vesicles produced by unicellular and filamentous freshwater cyanobacteria?
- II. What is the effect of stress conditions on OMV production?
- III. What is the genetic content of cyanobacterial OMVs and is it affected under stress conditions?

5 Materials and Methods

5.1 General molecular biological methods

5.1.1 Isolation of genomic DNA of *Synechocystis* sp. PCC 6803

Isolation of genomic DNA was performed by a modified protocol of Hess et al. (2011) (Hess 2011). 50 ml of a culture in early stationary phase was centrifuged (15 min, 20°C, 12.000 rcf). The supernatant was discarded and the pellet resuspended in the left media and pooled in a 2 ml tube. The cells were pelleted again (12.000 rcf, room temperature, 5 min). Cells were frozen in liquid nitrogen and broken with mortar and pestle. Afterwards 200 µl 1x TE/ 0.15 M NaCl together with Lysozyme (final concentration 5 mg/ml) and RNase (0.1 mg/ml final concentration) was added and incubated for 30 min (37 °C, shaking). Proteins were degraded by incubation with Proteinase K to a final concentration of 100 µg/ml and SDS (final concentration of 2%) at 60 °C for 1,5 h (shaking). Precipitation of polysaccharides was achieved by incubating with 100 µl 5 M NaCl and 80 µl of 10% CTAB/0.7 M NaCl at 65 °C for 10 min (shaking). The extraction of DNA was then performed by adding 1 volume of phenol: chloroform: Isoamyl alcohol (25:24:1) and mixing it carefully. After separating the phases (5 min, 12.000 rcf, room temperature) the supernatant was pipetted into a clean tube. The separation was then repeated with 1 volume of chloroform: isoamyl alcohol (24:1) until the interface showed no white residues. The precipitation of the DNA was achieved by adding 0.6 vol of isopropanol, carefully mixing and incubation either at room temperature for 15 min or at -20°C overnight. DNA was collected by centrifugation for 40 min at 13 600 rpm, 4 °C and the supernatant was decanted carefully. The pellet was washed two times with 0.5 ml ice cold 75% Ethanol (10 min, max speed, 4 °C) and the pellet dried. The DNA was then hydrated with 50 to 100 µl of ddH₂O for 15 min at 37 °C and 600rpm (ThermoMixer, Eppendorf).

5.1.2 Whole genomic DNA isolation of stressed *Synechocystis* sp. PCC 6803 cells

Whole genome DNA of stressed *Synechocystis* sp. PCC 6803 from 5.2.4 was performed using the GeneJet Plant genomic DNA purification MiniKit (Thermo Fisher Scientific) according to manufactures guidelines apart from the cell lysis. This was done by pipetting the cells in 350 µl Lysis Buffer A and DTT (final concentration 40 mM) to Precellys tubes VK05 (0.5 mm glass beads; Precellys24). The cells were lysed in in the Precellys24 homogenizer with 3x 20 s, 4500 rpm and a 30 s rest on ice. Afterwards the manufactures guidelines were followed.

5.1.3 Isolation of RNA of *Synechocystis* sp. PCC 6803

For RNA isolation filtertips, special RNase-free tubes and RNase-free water with DEPC to a final concentration of 0.01% was used to prevent contamination with RNase. RNA isolation was performed applying the single-step isolation protocol from (Chomczynski and Sacchi

1987), using the RNA Plant Purification Reagent (Invitrogen) according to the manufactures guidelines with some modifications.

Cells from 30 to 50 ml culture were collected (5 min, 4500 rpm, room temperature) and resuspended in 500 µl cold Plant RNA Reagent (Invitrogen). The suspension was transferred to a tube with glass beads of different sizes (Soil grinding mix SK38; 200-300 µm, Precellys24) to break the cells with a homogenizer (Precellys24, 5 times, 30 s, 30 s chill on ice, max. speed). The mixture was then centrifuged (2 min, 12,000 rcf, room temperature) and the clarified supernatant transferred to a new tube, where 0.1 ml 5 M NaCl was added. To separate the RNA from other cell content, 0.3 ml Chloroform was added and the tube inverted to mix the liquids. After separating the phases (15 min, 12,000 rcf, 4°C), the aqueous phase was transferred into a new tube and the extraction step was repeated two times.

Precipitation of RNA was achieved by adding 1 volume of isopropanol and incubation at either room temperature for 10 min or at -20°C overnight. RNA was then pelleted (10 min, 12,000 rcf, 4°C) and the supernatant decanted. The pellet was washed three times with 75% ice cold Ethanol (centrifugation 1 min, 12,000 rcf, 4°C). After the last decanting step, the tubes were centrifuged again as described before to collect any residual liquid, which was removed with a pipette. The pellet was then dried and 30 µl of RNase-free DEPC water added to dissolve the RNA at 25°C, 300 rpm, 10 min in a shaker (ThermoMixer C; Eppendorf). Until further use, the RNA was stored at -80°C.

5.1.4 DNA- and RNA concentration determination

To determine the DNA- and RNA concentrations on genomic samples and plasmids, samples were applied to a spectrophotometer. The absorption at a wavelength of 260 and 280 nm were measured. At a 1 cm path length, an absorption of 1 equals 50 ng/µl dsDNA and 40 ng/µl RNA. To assess the purity of the nucleic acid solution, the 260/280 ratio was measured with ~1.8 as indicator for pure DNA and ~2 for pure RNA (*Barbas et al. 2007*).

5.1.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify cDNA, plasmid or genomic DNA templates and for amplification steps in the library preparation (see 5.3.8) (cycler: BioRad). All primers used in this study, apart from primers provided in kits, are listed in Table 3 and were synthesized by Eurofins. Phusion DNA polymerase (Thermo Scientific) was used together with its appropriate buffer, provided by the manufacturer. A typical reaction was:

25 µl: 2.5 µl 10x buffer
2.5 µl Forward primer
2.5 µl Reverse primer
0.1 - 1 µl DNA (between 10 – 50 ng)
to 25 µl ddH₂O

A typical program was:

98 °C	3 min	} x 30-40
98°C	10 s	
58 – 60 °C	10 s	
72°C	variable	
72°C	5 min	

The annealing temperature and elongation time were optimized for each PCR reaction.

Table 3 Primer list. All primers have melting temperatures between 59°C - 62°C and function well at 60°C. F = forward primer; R = reverse primer

Stock nr.	Primer name	Sequence (5' to 3')	Product size
37	rnpB_qPCR_F	GCCCTTTTGTAGAAAACAGCAACC	148
38	rnpB_qPCR_R	GGTTTACCGAGCCAGTACCTCTC	
64	6803_clpB1_F	GGCAACTTATTGAAACCCATGT	130
65	6803_clpB1_R	CCACTAAAACCTCCTGGAAACG	
66	6803_hspA_F	GATCGCCAGGATACCCATAG	108
67	6803_hspA_R	ATGTTTCATACCCGTTTCTTGG	
78	aphII_qPCR_F	TTATGCCTCTTCCGACCATC	176
79	aphII_qPCR_R	AACAGGAATCGAATGCAACC	
80	pCA2.4_qPCR_F	GGCGGAGGTCTCTTATGTGG	153
81	pCA2.4_qPCR_R	CTTGGTTCATGCCCGTGATG	
82	pCB2.4_qPCR_F	AGAAGTACAGGTAAGCGGGC	158
83	pCB2.4_qPCR_R	AAAGCGGGAACTACTTGCGA	

5.1.6 Gel electrophoresis and visualization

RNA and DNA was separated via gel electrophoresis to visualize the presence and correct size of desired fragments or whole genome (Johansson 1972; Sambrook et al. 1989). The separation was done on 1 – 3% Agarose-TAE gels, depending on the fragment size supplemented with 1 – 3 µl Midori Green (Nippon Genetics Europe GmbH). This dye stains nucleic acids and can be visualized under UV light. Samples were mixed with 10x DNA sample buffer and loaded on the gel. To estimate the fragment sizes 1 kb, low size and RNA ladder (Thermo Fisher Scientific) were used. Separation took place at 100 to 120 V for 25 to 40 min in TAE buffer. Afterwards the DNA/ RNA fragments were visualized using the ChemiDoc MP imaging system (Bio-Rad Laboratories) with the software Image Lab 5.0 (Bio-Rad Laboratories).

10x DNA sample buffer: 0.03% bromophenol blue

0.03% xylene cyanol FF

0.4% orange G

15% Ficoll® 400

10 mM Tris-HCl (pH 7.5)

50 mM EDTA

50 x TAE buffer 242 g tris base

57.1 ml acetic acid

100 ml 0,5M EDTA

to 1 l ddH₂O

5.1.7 Quantitative real time PCR (RT-qPCR)

Quantitative real time PCR (RT-qPCR) is a method to assess the amount of mRNA transcripts in a cell and thereby conclude on the activity of a gene or for the estimation of relative abundances of DNA fragments.

When using RNA as input material, digestion of any remaining DNA is crucial for precise results for RT-qPCR. The DNA in the RNA-samples was removed by using the DNA-free™ DNA removal kit (Thermo-Fischer) according to the manufactures guidelines. The remaining RNA was tested to be DNA free with qPCR as described below using 16S qPCR Primers (see Table 3). As a positive control, genomic DNA or cDNA from earlier experiments were used. Afterwards the RNA was transcribed to complementary DNA (cDNA), catalysed by reverse transcriptase. The iScript™ cDNA Synthesis Kit (Bio-Rad) was used for that purpose according to the manufacture's guidelines, transcribing 1 to 5 µg RNA. RT-qPCR was performed with the iTaq™ Universal SYBR green supermix (Bio-Rad) in a CFX Connect PCR-Cycler (Bio-Rad) in technical replicates. SYBR green is a fluorescent dye which intercalates with double stranded DNA. I calculated relative fold changes in the expression of the target gene compared to the reference gene *mpb* or *aphII*, respectively, using the $\Delta\Delta C_q$ method (Livak and Schmittgen 2001; Pinto et al. 2012).

Appropriate primers were designed by Custom Primers - OligoPerfect™ Designer (ThermoFisher Scientific, <https://tools.thermofisher.com/content.cfm?pageID=9716>) or Primer 3 (<http://primer3.ut.ee/>) with the desired gene as .fasta input, Application: PCR:Detection, and standard values for Primer size, Primer T_m, Primer &GC, Region of analysis, experimental conditions. The product size was set to 120bp.

The specificity of the primers was tested using Primer-BLAST (NCBI, https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome; Database nr, organism *Synechocystis* sp. PCC 6803 (taxid: 1148)) (Altschul et al. 1990; Ye et al. 2012). Primers are listed in Table 3 with their product sizes.

A typical reaction for RT-qPCR according to the manufacture's guideline was:

10 µl: 5 µl SYBR green Super Mix
1 µl cDNA (50 ng)
0.1 µl Primer Forward (10 µM)
0.1 µl Primer Reverse (10µM)
3.8 µl ddH₂O

The RT-qPCR program:

95°C 3 min

95°C 15 s

60°C 30 s

] x 40

melting curve (59 °C to 95 °C, raising the temperature by 5°C every 5 s)

A melting curve has been assessed in every run to ensure specificity of the reaction and the absence of by-products like primer-dimers.

5.1.8 Plasmid copy number determination

Whole genomic DNA samples from 5.1.2 were used to determine the plasmid copy number of pCA2.4 and pCB2.4 per chromosome in stressed and non-stressed *Synechocystis* sp. PCC 6803 (see 5.2.4) at the time point of outer membrane vesicle sampling similar to (Chen *et al.* 2016). The isolated DNA was diluted to a concentration of 40 ng/µl (10⁰) and a dilution series to 0.004 ng/µl (10⁻⁴) was prepared. All five dilutions were used in duplicates as input material for qPCR which was performed as described in 5.1.7. Primers specific to the regions on the respective plasmids and the chromosome (*rnpb*) (see Table 3) were used. PCN was calculated as described in (Škulj *et al.* 2008). In short, standard curves were assessed from the dilution series for all primer pairs and the amplification efficiency (E) for each primer pair and each input DNA was calculated with: $E = 10^{(-\frac{1}{slope})}$. The calculation for the actual PCN took the different E's into account: $PCN = E_{chr}^{Cq_{chr}} / E_{pl}^{Cq_{pl}}$, with chr = chromosomal, pl = plasmid primers and Cq is the quantitation cycle from the qPCR for the two amplicons (Škulj *et al.* 2008).

5.2 Microbiological methods

5.2.1 Strains and growth conditions

Synechocystis sp. PCC 6803 (containing a neomycin/spectinomycin resistance cassette in a neutral position; (Kunert *et al.* 2000)), *Anabaena* sp. PCC 7120 and *Chlorogloeopsis fritschii* PCC 6912 were cultured in standard BG11 media (Rippka *et al.* 1979). BG11 was

supplemented with 5 mM TES pH 8.0 (final concentration) to achieve a constant pH over the growth time. The media were prepared with demineralized water and autoclaved at 121°C and 1 bar for 20 min.

Cells were grown under a constant light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 30°C in cell culture flasks in a climate chamber (Binder). To protect against mechanical cell lysis and subsequent formation of debris-derived vesicles, the bacteria were cultured under relatively mild shaking or low bubbling with air (cultures >50 ml).

5.2.2 Long term storage

For long term storage, cells in mid exponential growth phase were pelleted and resuspended in 500 μl sterile 70% glycerol/ 30% BG11. The cells were stored at -80°C.

5.2.3 Growth curves

The growth curves of *Synechocystis* sp. PCC 6803 were acquired by measuring the optical density (OD) at a wavelength of 750 nm in cuvettes with BG11 as blank control in a spectrophotometer (Multiskan Go, Thermo Fisher Scientific). The growth rate and carrying capacity were determined with R using the package *Growthcurver* (Sprouffske and Wagner 2016). This program fits raw growth data to a standard form of the logistic equation that describes population size N_t at time t with:

$$N_t = \frac{K}{1 - \frac{K - N_0}{N_0} e^{-rt}}$$

N_0 is the population size at the beginning of the measurement, while K is the carrying capacity, defined as the maximum possible population size in a given environment. The growth rate r is the maximum growth rate that would exist, if there were no restrictions to the maximum population size.

5.2.4 Stress conditions

For the establishment of suitable stressors, several conditions were tested. For salt stress, BG11 medium was supplemented with different concentrations of NaCl (250, 500, 700 mM). Heat stress was applied by raising the growth temperature to 37°C and 40 °C. For light stress, the cells were grown under 3x higher light ($75 \mu\text{mol m}^{-2} \text{s}^{-1}$). 250 mM H_2O_2 was supplemented to the media to set *Synechocystis* sp. 6803 under oxidative stress while 500 mM sorbitol was added to achieve hyperosmotic stress. No stressors were added to control cultures. Growth curves were measured and analysed as described in 5.2.3.

5.3 Methods for studying outer membrane vesicles

5.3.1 Isolation of outer membrane vesicles

Cyanobacterial cultures were grown to the desired growth phase. The cells were pelleted and the supernatant was serial filtered through 0.8 μm , 0.45 μm and sterile filtrated with a 0.2 μm

filter (PES membranes). When needed, the supernatant was concentrated using a tangential flow filter (100 kDa cut-off; GE Healthcare) connected to a peristaltic pump (Whatman). The pressure was kept below 0.5 bar to avoid destroying of vesicles. The culture volume was reduced to about 70 ml and again sterile filtered. Afterwards the vesicles were pelleted in a Beckmann Ultracentrifuge (40.000 rpm, 4 h, 4°C), and resuspended in 200 µl to 2 ml 25mM HEPES. To ensure that no cells remain, 50 µl were plated on plates with BG11 and R2A agar (Carl Roth). OMV samples that showed bacterial growth (contaminations) on these plates were eliminated from further experiments. The vesicles were stored at -80°C until further investigations.

5.3.2 Quantitation of outer membrane vesicles

5.3.2.1 Protein measurement

Total amount of protein in vesicle preparations was measured with the Qubit system according to the manufacturer's guidelines (Invitrogen). The resulting protein concentrations were divided by OD₇₅₀ values at the time of the vesicle harvest to obtain protein/OD₇₅₀ ratios.

5.3.2.2 Lipid quantification

OMVs are outer membrane blebs that consists of a bilayer or double bilayer membrane. Therefore, the lipophilic fluorescent dye FM4-64 (Molecular probes) was used for quantitation that intercalates with lipid layers. The assay was adapted from (*Berleman et al. 2016*). In short, samples were mixed with the dye to a final volume of 100 µl and a final concentration of FM4-64 of 16 µM. All samples were tested in triplicates. Probe alone and vesicles alone were analysed as controls. The experiment was conducted in black 96 well plates and fluorescence was measured in a fluorescent plate reader (Tecan) by excitation at 530 nm and emission at 635 nm. The acquired relative fluorescent units (RFU) values without the dye were subtracted from the values with FM4-64 to remove any auto-fluorescence signals. The resulting RFU values were then divided by OD₇₅₀ values at the time of the vesicle harvest to obtain RFU/OD₇₅₀ ratios, adapted from (*Manning and Kuehn 2011; MacDonald and Kuehn 2013*).

5.3.3 DNase I treatment

To destroy any DNA, bound to the outside of vesicles or floating freely around the vesicles, the samples were treated with 2 U of DNase I for 30 min at 37°C according to the manufactures guidelines (Thermo Fisher Scientific). Another 2 U of DNase I was added and the incubation repeated as before. Afterwards the enzyme was inactivated at 70°C for 10 min.

5.3.4 SYBR® Gold staining

SYBR® Gold Nucleic Acid Gel Stain (10,000x) is a fluorescent dye to detect single and double stranded nucleic acids. A working solution of 25x in DMSO was prepared freshly before usage. The OMVs were digested with DNase I as described in 5.3.3 to remove any DNA surrounding

for bound to them. Afterwards, vesicles were fixed with glutaraldehyde to an end concentration of 2% (10 min incubation at room temperature). Following this SYBR[®] Gold was added to an end concentration of 2.5x. As a negative control, pure HEPES was stained as well. After 10 min incubation at room temperature in the dark, the vesicles were visualized as described in 5.3.5.

5.3.5 Light/ fluorescence microscopy

Bright field and real-time fluorescence microscopy was conducted with a microscope from Zeiss (Axio Imager M2m, Zeiss). SYBR[®] Gold as a detection agent for DNA (see 5.3.4) needs an excitation of 400 nm and emits at 537 nm. Bright field microscopy was used for visualizing the cyanobacteria. Images were taken by the AxioCam Mrm (Zeiss) and processed with the software Zen 2 pro (Zeiss).

5.3.6 Transmission electron microscopy (TEM)

For TEM, the vesicles were dropped on a copper grid and dried incubated 2 min. To stain the membranes, 1 to 2 drops of 1% uranyl acetate were applied and incubated for 1 to 2 minutes. With a small filter paper, superfluous liquid was removed. The grids were left to dry for 5 to 10 min before being viewed on a transmission electron microscope (Tecnai F30).

5.3.7 DNA isolation of vesicle DNA

The DNA of the vesicles was isolated as described in (*Biller et al. 2016*). After the DNase I treatment (5.3.3), vesicles were lysed using freshly prepared GES buffer in a 1:10 ratio on 100 µl vesicles. The mixture was incubated for 30 minutes at 37°C.

GES buffer (10x)	500 mM guanidine thiocyanate
	10 mM EDTA
	0.05% sarkosyl

The actual isolation was done with AMP pure beads (Beckmann Coulter) according to manufactures guidelines. DNA concentration was measured with the Qubit fluorometric quantitation system (Thermo Fisher; dsDNA HS Assay kit) according to manufactures guidelines. The protocol had a final elution step in a 50 µl volume. As this step led to highly diluted samples, a vacuum concentrator was used to concentrate the DNA to an approximately end volume of 20 µl.

5.3.8 Library preparation and sequencing of outer membrane vesicles

Library preparation was performed with the QIAseq FX Single Cell DNA Library Kit (Qiagen) according to manufactures guidelines. This method uses a Phi29 DNA polymerase and amplifies relatively bias free longer DNA stretches of 2 kb – 100 kb in an isothermal procedure.

One to 2 μ l of vesicle DNA (up to 300 pg DNA) were used as input material from step 5.2.4. As control, one library was prepared only with deionized water as input material. DNA shearing was conducted with the Covaris Sonicater and the program to yield 350 bp fragments (machine settings: Duty 10%, Int 5, Cycle Burst 200, time 45s). Concentrations of the whole genome amplification step and the libraries were assessed using the Qubit fluorometric quantitation system (Thermo Fisher; dsDNA HS Assay kit) according to manufactures guidelines. The libraries were sequenced on an Illumina MiSeq platform with 2x300 bp paired end, where all samples were on one lane For pilot sequencing of vDNA of *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and *Chlorogloeopsis fritschii* PCC 6912, library preparation was performed as described in (Maixner et al. 2016). In short, a modified Illumina multiplex sequencing protocol was used and a second amplification step was performed to improve library yield. Libraries were sequenced on an illumine MiSeq platform with 2x150 bp paired end.

5.3.9 Sequence handling

After sequencing, the samples were tested for outlier, which would need removal from the sample pool. To this end, kmer-counting (9 bp long fragments) was performed using jellyfish v2.2.9 (Marçais and Kingsford 2011) for each sample. The results were subjected to multidimensional scaling (MDS) and principal component analysis (PCA) and outlier were determined by assessing the total distance of each sample, to the rest of the conditional replicates. Samples were quality- and adapter trimmed using trimmomatic v0.36 (Bolger et al.), applying a sliding window of 4 bp, with a quality score ≥ 20 , removing adapters from 30 bps at the ends of the reads, and removing reads smaller than 36 bp after trimming. Quality control for the reads was performed applying fastqc suite v0.11.4 (Andrews 2010) to the samples. Trimmed reads were mapped against the reference genome (see Table 4 for accession numbers), including plasmids of the respective cyanobacteria.

To account for the circularity of the bacterial replicons, the full sequence of each replicon was pasted to the end of the respective sequence, resulting in a merge of the doubled sequence in the end and the beginning, prior mapping PE (paired-end) and SE (single-end) reads (which remained without a corresponding read after trimming) with bowtie2 v2.3.4.1 (Langmead and Salzberg 2012; Langmead et al. 2018) to this reference. The doubling was necessary, as reads spanning the beginning and end part of the sequence would otherwise be discarded by the aligner. Coverage for each base was calculated using the samtools v1.8 (Li et al. 2009; Li 2011) depth functionality, while read counts per replicon were determined using salmon v0.9.1 (Patro et al. 2017). To correct the coverage value, the reference sequences were split again at the merge point and the values of the second doublet was added to the values of the first half. The second half was discarded, resulting in the original reference sequence with correct coverage values for all parts of the sequence.

Table 4 Reference genomes used in this study.

species	chromosome/ plasmids	accession number	length (bp)	source
<i>Anabaena</i> sp. PCC 7120	Chromosome	NC_003272.1	6,413,771	(Kaneko et al. 2001)
	pAlpha	NC_003276.1	408,101	
	pBeta	NC_003240.1	186,614	
	pDelta	NC_003273.1	55,414	
	pEpsilon	NC_003270.1	40,340	
	pGamma	NC_003267.1	101,965	
	pZeta	NC_003241.1	5,584	
<i>Chlorogloeopsis fritschii</i> PCC 6912	whole genome shotgun sequence	NZ_AJLN00000000. 1	7,751,745	(Dagan et al. 2012)
<i>Synechocystis</i> sp. PCC 6803	Chromosome	NC_020286.1	3,569,561	(Trautmann et al. 2012)
	pCA2.4	NC_020289.1	2,378	
	pCB2.4	NC_020298.1	2,345	
	pCC5.2	NC_020290.1	5,214	
	pSYSA_M	NC_020287.1	100,749	
	pSYSG_M	NC_020288.1	44,343	
	pSYSM_M	NC_020296.1	118,712	
	pSYSX_M	NC_020297.1	106,004	

Based on the read counts per replicon, I calculated an equivalent to the plasmid copy number (PCN) per chromosome. That is: the ratio of sequence length corrected sum of read counts of the plasmid and the chromosome. Overrepresented genes were determined by a search for regions on each replicon and sample, where the local coverage deviated more than three times the MAD (median absolute deviation) from the median of the whole replicon in that sample. Only regions larger than 100 bp and with a median coverage of at least four in at least two replicates were considered as overrepresented. For annotation of possible genes within the overrepresented regions of *Chlorogloeopsis fritschii* PCC 6912 I used the sequences of the region to predict ORFs with the ExPASy tool (Artimo et al. 2012). Protein sequences were then used to blast (blastp) against the reference database for further annotation.

5.4 Statistics

The R software package v3.4.4 served for general scripting and statistic tasks (R Core Team 2018). A significance level $\alpha = 0.05$ was used. If applicable, a t-test was used, otherwise a Wilcoxon test was performed. For testing of variances, Levene's test was applied.

6 Results

6.1 Freshwater cyanobacteria produce outer membrane vesicles that contain nucleic acids

To test for the presence of outer membrane vesicles in different cyanobacterial species, cells were grown until stationary growth phase in an appropriate medium, the supernatant was sterile filtered to remove any cells and vesicles were enriched by ultracentrifugation. For visualization, transmission electron microscopy (TEM) and fluorescent microscopy was used. To investigate whether OMVs contain nucleic acids, I initially digested the vesicles twice with DNase I to remove any surrounding DNA that could be present in the buffer or attached to the OMVs. As a negative control, HEPES buffer alone was stained and showed unspecific or no binding of SYBR® Gold, which is recognizable by extremely fast fluorescence bleaching (Figure S 1A and B).

6.1.1 Outer membrane vesicles in the unicellular *Synechocystis* sp. PCC 6803

Looking for OMVs in *Synechocystis* sp. PCC 6803, I prepared the samples as described above and visualized the particles using TEM (Figure 5A). I found vesicles which were 20 – 150 nm in diameter and appeared empty (Figure 5A, black arrow) or with an electron dense luminal content (Figure 5A, white arrow). Further, SYBR® Gold staining revealed DNA content in at least some of the vesicles (Figure 5B). This indicates that *Synechocystis* sp. PCC 6803 is able to produce DNA containing OMVs. TEM imaging of whole bacterial cells revealed a freshly detached outer membrane vesicle with an electron dense membrane (Figure 5C, arrow) and the putative detaching zone on the cellular site (Figure 5C, arrow head). This observation supports the notion that OMVs are actively produced and shed by living bacteria.

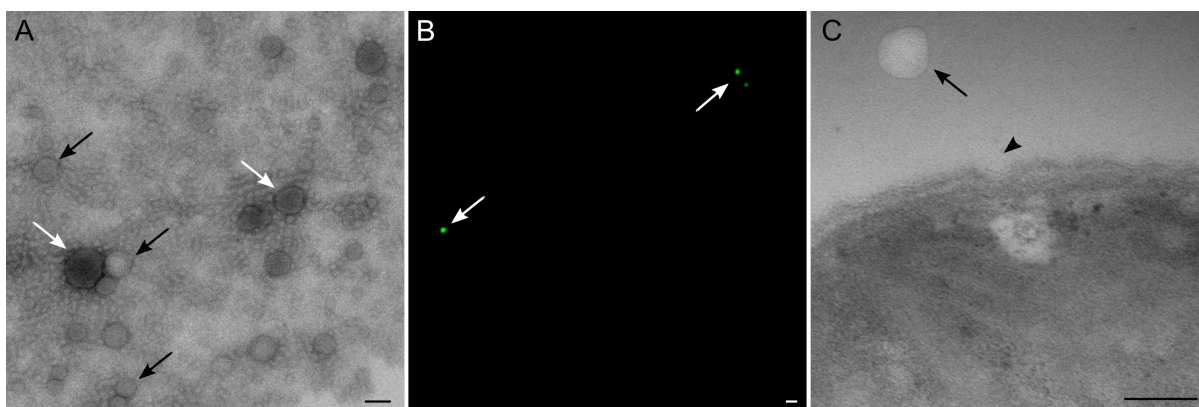


Figure 5 Outer membrane vesicles in *Synechocystis* sp. PCC 6803. (A) TEM image of outer membrane vesicles isolated from the cell free supernatant of *Synechocystis* sp. PCC 6803 of different sizes between 20 to 150 nm (black arrows). Some of the vesicles show an electron dense luminal content (white arrows). (B) DNase I digested and glutaraldehyde fixed OMVs stained with SYBR® Gold. Green dots are fluorescent nucleic acids in vesicles (white arrows). (C) TEM image of a freshly formed vesicle (black arrow) and the detachment point on the cellular site (black arrow head). Scale bars are 100 nm in (A; C) and 1 μ m in (B).

6.1.2 Filamentous, freshwater cyanobacteria from section IV produce outer membrane vesicles that contain nucleic acids

As examples for section IV filamentous growing cyanobacteria, I tested *Scytonema hofmanni* PCC 7110 and *Anabaena* sp. PCC 7120 for the production of outer membrane vesicles.

In *Scytonema hofmanni* PCC 7110, only few typical outer membrane vesicles could be detected with an estimated size of approximately 100 nm and an electron dense luminal content as shown in Figure 6A. However, the most abundant particles were vesicle like structures displaying a rod-shaped morphology with an unusual even size distribution of 80 nm (Figure 6B, black arrows). Additionally, most particles appear to have a septum in the center while others are approximately half the size (around 40 nm) without the septum (Figure 6 B, white arrows). Due to the very low abundance of typically formed OMVs and the presence of atypically small particles, I did not continue to work with this strain.

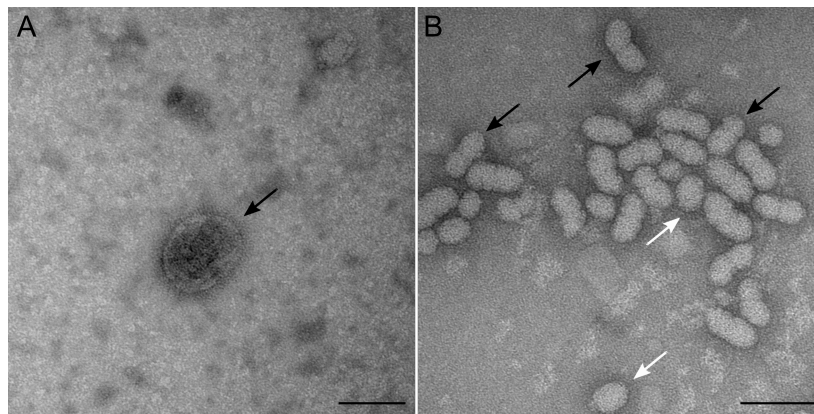


Figure 6 Outer membrane vesicles in the section IV cyanobacterium *Scytonema hofmanni* PCC 7110. (A) Negative stained TEM image of purified outer membrane vesicles. The black arrow shows an example of a typically shaped OMV with electron dense luminal content. (B) Vesicle like structures with an atypical rod-shaped morphology and a septum in the center (black arrows). Additionally, round particles, half the size of the rod-shaped entities could be detected (white arrows). Scale bars are 100 nm.

As a second example for section IV cyanobacteria, I investigated *Anabaena* sp. PCC 7120. In this strain, I also found outer membrane vesicles in the cell free supernatant. Vesicles occurred as round entities with a single layer (Figure 7A, white arrows) or bilayer (Figure 6B, black arrows) membrane. They appeared empty or with an electron dense luminal content and the diameter ranged from 15 to 120 nm. Testing OMVs with SYBR® Gold revealed nucleic acids in at least some of them (Figure 7B). Taken together, these results show that filamentous cyanobacteria of section IV produce OMVs which can contain nucleic acids.

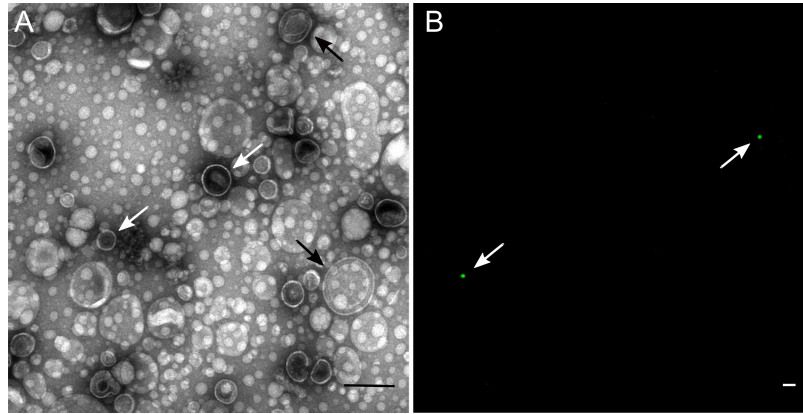


Figure 7 Outer membrane vesicles in the section IV cyanobacterium *Anabaena* sp. PCC 7120. (A) Negative stained TEM image of purified outer membrane vesicles of different sizes surrounded by a single membrane (white arrows) or a bilayer membrane (black arrows). (B) DNase I digested and glutaraldehyde fixed vesicles stained with SYBR® Gold. Green dots represent fluorescent DNA in vesicles. Scale bar in (A) is 100 nm and in (B) represents 1 μ m.

6.1.3 Particles in section V cyanobacteria

Cyanobacteria from section V are characterized by their multicellularity, forming differentiated cells for nitrogen fixation and their true branching. I investigated the cell-free supernatants of *Fischerella muscicola* PCC 7414, *Fischerella thermalis* PCC 7521, and *Chlorogloeopsis fritschii* PCC 6912 for the presence of OMVs.

In *Fischerella muscicola* PCC 7414 no typical outer membrane vesicles could be detected. However, I could detect very small rod-shaped particles sized around 10 nm (Figure 8A) that lack the typical shape and membrane which is generic for outer membrane vesicles. Nonetheless, I tested whether I could detect DNA bearing particles after DNase I digestion in the OMV extract and indeed found SYBR® Gold positive particles of around 50 – 200 nm. (Figure 8B). These results indicate that OMVs are either extremely rare and difficult to detect using electron microscopy, that the process of sample preparation for the TEM is eliminating the OMVs, or that the SYBR® Gold positive particles are of different origin.

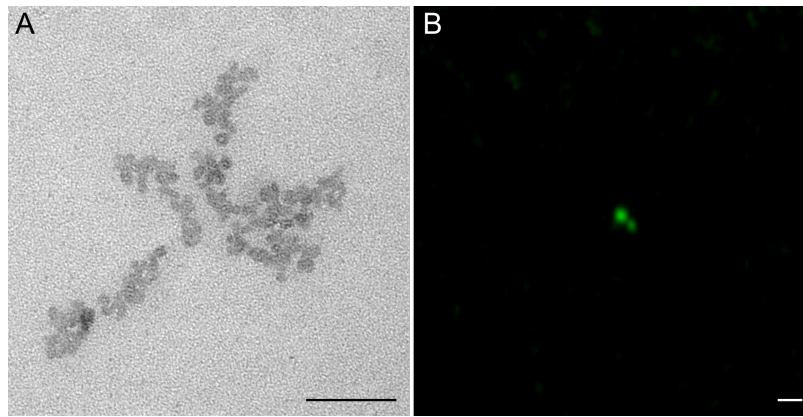


Figure 8 Particles in Fischerella muscicola PCC 7414. (A) No outer membrane vesicles could be detected in the section V cyanobacterium *Fischerella muscicola* PCC 7414 by transmission electron microscopy. However, some extremely small rod-shaped particles of approximately 10 nm could be detected. (B) SYBR® Gold staining revealed round particles containing nucleic acids (green dots). Scale bar in (A) represents 100 nm and in (B) 1 μ m.

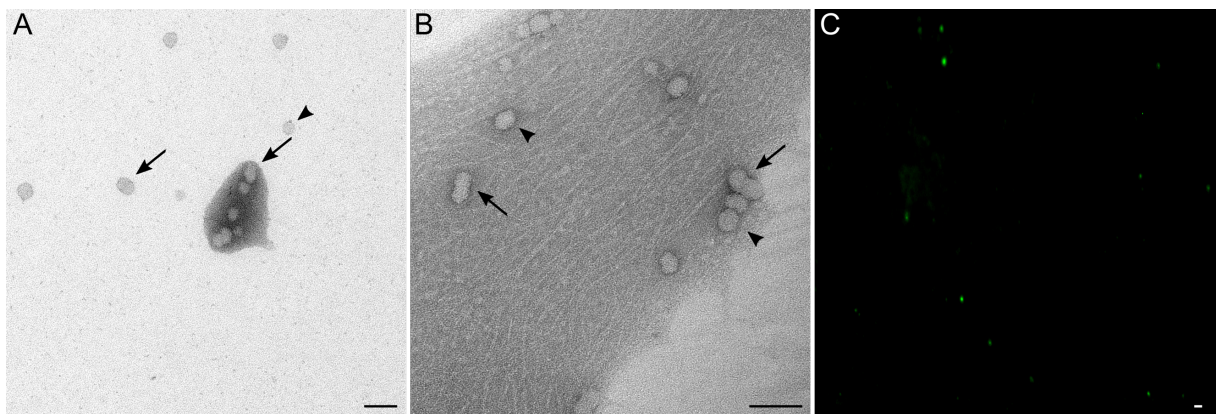


Figure 9 Particles in Fischerella thermalis PCC 7521. (A) Vesicle like structures with an atypical rod-shaped morphology and a septum in the center (arrows) in the supernatant of *Fischerella thermalis* PCC 7521 by transmission electron microscopy with a size of 80 to 100 nm. Additionally, round particles, half the size of the rod-shaped ones could be detected (arrow heads). (B) TEM image of rod-shaped particles of 80-100 nm with a septum (arrows) and round particles of approximately 40 nm (arrow heads) directly embedded in the exopolysaccharide layer of the bacteria. (C) DNase I digested and glutaraldehyde fixed vesicles stained with SYBR® Gold. Green dots represent fluorescent DNA in the particles. Scale bars in (A) and (B) represent 100 nm and in (C) 1 μ m.

TEM imaging of *Fischerella thermalis* PCC 7521 revealed the presence of atypical rod-shaped (~80 – 100 nm in diameter) particles with an electron dense membrane layer. Interestingly, I detected particles which appeared to be divided by a septum (Figure 9A, arrows). In addition, I found particles that were half the size (around 40 nm) and of rounder shape (Figure 9A, arrow heads). Section V cyanobacteria typically produce very thick exopolysaccharide layers, which appear as an electron dense mass consisting of short fibers (Figure 9B). In these layers, I could detect the same rod-shaped vesicles of approximately 80 nm with a septum (arrows) or round particles without a septum (arrow heads) of around

40 nm in diameter. Testing for DNA content in the particles with SYBR® Gold, I detected DNA positive particles (Figure 9C). Thus, *Fischerella thermalis* PCC 7521 produces atypical, rod-shaped, small, and presumably DNA containing particles and I did not include this strains into further analyses.

In *Chlorogloeopsis fritschii* PCC 6912, I also identified vesicle like structures in the supernatant. Those entities, however, showed a very homogenous size distribution with almost all entities having a diameter of 80 nm and only some were smaller or larger (Figure 10A, white arrows). Careful examination of the images revealed an octagonal form of most of the entities (Figure 10A & B, black arrows) and the appearance of tail-like structures (Figure 10A, black arrow head), which lead to the hypothesis, that this strain possesses a phage. Although the majority of the particles are comprised the putative phage, I could also identify OMVs that were round shaped with different sizes of 10 – 100 nm (Figure 10A: white arrows; B: black arrow heads).

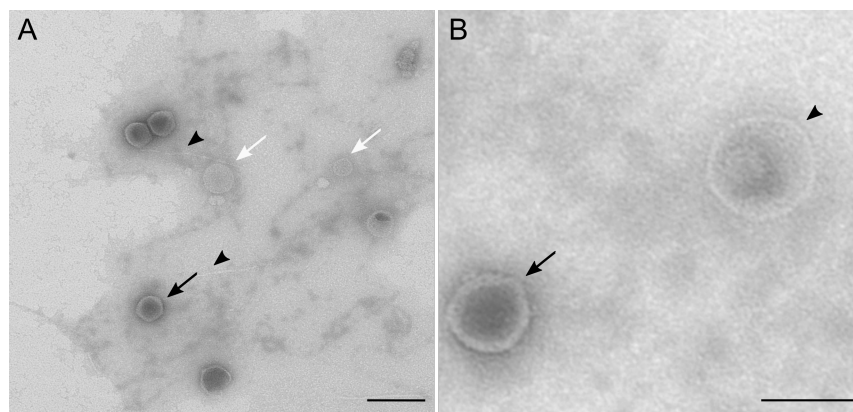


Figure 10 Outer membrane vesicles and putative phages in *Chlorogloeopsis fritschii* PCC 6912. (A) *Chlorogloeopsis fritschii* PCC 6912 shows few differently sized outer membrane vesicles (white arrows) and many phage heads with electron dense luminal content (black arrows) and tail structures (black arrow heads). (B) An enlarged TEM image of a phage head (black arrow). The arrow head hints to a putative OMV with electron dense luminal content. Scale bar (A) is 200 nm and (B) is 100 nm.

6.2 Sequencing of vesicle DNA from different strains: a pilot test

From the results above, I learned that at least some cyanobacterial species of different morphological complexities produce outer membrane vesicles that contain DNA. Next, I was interested in the actual genetic content of the OMVs, and whether the putative phage in *Chlorogloeopsis fritschii* PCC 6912 is indeed a phage. Therefore, I isolated vesicles from strains, where I clearly identified vesicles as described before, namely *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and additionally particles from *Chlorogloeopsis fritschii* PCC 6912. I digested them twice with DNase I to remove any DNA attached to the particles or floating in the supernatant, and afterwards isolated vesicle/particle DNA. The activity of DNase

I was tested in a preliminary test on genomic DNA samples with DNA concentrations comparable to those found in the vesicles up to ten times more DNA. The digestion of those samples was tested with quantitative PCR and no amplification could be observed nor a product was found when recording a melting curve (Table S 3). For every vesicle/particle DNA isolation run, I digested a genomic DNA sample as control and never observed amplification of a product indicating an active DNase I.

The vesicle DNA was purified and sequenced on a MySeq platform with 2x 150 bp. Read quality was assessed with fastQC v0.11.4 and the reads were quality trimmed using trimmomatic v0.36. Reads were mapped on the appropriate reference genome (see Table 4) with bowtie2 v2.3.4.1.

6.2.1 Sequencing results of vesicle DNA derived from *Synechocystis* sp. PCC 6803

Outer membrane vesicles from *Synechocystis* sp. PCC 6803 were sequenced in two batches (batch 1: Sy1; batch 2: Sy2, Sy3, Sy4). While the sequencing of Sy1 resulted in ~1.4 million reads; the sequencing of the second batch gave only 36,167 to 47,803 total reads before trimming (Table 5). In general, the quality of sequencing was low, indicated by high filtering rates of reads after trimming. In order to retain as much information as possible, all available reads after trimming were included into further analysis, regardless if paired or single end. Only 1.9% to 8.69% of the reads aligned to the reference genome.

Table 5 Total reads, reads after trimming and alignment rate

	Sy1	Sy2	Sy3	Sy4
total reads	1,374,596	47,803	37,160	36,167
both surviving	44,278	3,278	8,224	2,274
forward only	137,411	5,914	6,536	6,882
reverse only	7,174	1,283	1,794	681
filtered	1,185,733	37,328	20,606	26,330
alignment rate	6.86%	3.28%	1.90%	8.69%

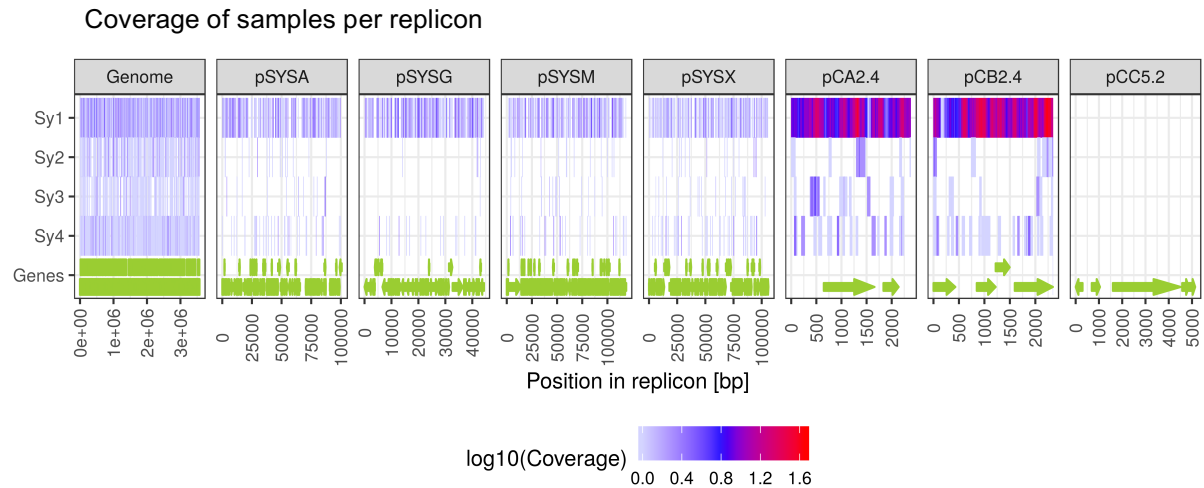


Figure 11 Coverage per base pair of vesicle DNA, isolated from four *Synechocystis* sp. PCC 6803 samples over all replicons presented as heatmap. Colors represent higher coverage in log₁₀ scale from blue (low) to red (high).

Figure 11 displays the coverage per base pair over all replicons of the four different replicates. I first noticed that the small plasmid pCC5.2 is not covered in any sample, thus it is either not present in vesicles or not present in the lab strain at all. In general, I found a fractionated coverage of all other replicons with very low coverages due to the low read numbers. In Sy1, the small plasmids pCA2.4 and pCB2.4 are completely covered with ten time higher medians compared to all other replicons (Table 6). Notably, the overall coverage fraction of both small plasmids was also highest in batch two, where it ranged from 24.11% (Sy2) to 54.26% (Sy4) in pCA2.4 and from 19.7% (Sy3) up to 56.39% (Sy4) in pCB2.4, which are more than ten times higher values than observed for the other replicons. An explanation could be that both plasmids are more than hundred times smaller than the larger fragments, thus it is more likely for them to be covered completely by only few reads. Taken together, apart from poor sequencing, the results hint to the conclusions that (I) parts of the chromosome as well as all plasmids are present in outer membrane vesicles and (II) both small plasmids might be enriched in outer membrane vesicles of *Synechocystis* sp. PCC 6803.

Table 6 Median, mean (in parenthesis), and overall coverage [%] of vDNA in four samples of *Synechocystis* sp. PCC 6803 OMVs over all replicons.

	Sy1	Sy2	Sy3	Sy4
chromosome	2	1	1	1
	(2.04)	(1.24)	(1.17)	(1.23)
	33.99%	1.15%	0.84%	2.43%
pSYSA	2	1	1	1
	2.17	(1.07)	(1.71)	(1.17)
	40.01%	2.05%	1.53%	3.68%
pSYSG	2	2	1	1
	(2.74)	(1.59)	(1)	(1.13)
	63.07%	0.93%	0.61%	3.29%
pSYSM	2	1	1	1
	(2.16)	(1.06)	(1.37)	(1.31)
	35.04%	1.65%	1.92%	1.96%
pSYSX	2	1	1	1
	(2.04)	(1.74)	(1.19)	(1.21)
	40.22%	1.41%	2.49%	4.31%
pCA2.4	17	2	2	1
	(17.67)	(1.67)	(1.93)	(1.46)
	100.00%	24.11%	37.54%	54.26%
pCB2.4	21	1	1	1
	(23.50)	(1.15)	(1.27)	(1.53)
	100.00%	33.42%	19.70%	56.39%
pCC5.2	0	0	0	0
	0	0	0	0
	0.00%	0.00%	0.00%	0.00%

6.2.2 Sequencing results of vesicle DNA from *Anabaena* sp. PCC 7120

For *Anabaena* sp. PCC 7120, I sequenced vDNA in two batches (first batch: An1; second batch: An2 & An3). While run 1 (sample An1) had around 14.7 million reads, batch 2 (An2 and An3) produced far less total reads with 47,844 and 261,304 respectively (see Table 7). After quality trimming, many reads with poor quality were filtered (25.7% in An1; 52.7% in An2, and 39.6% in An3). The overall alignment rate ranged between 63.2 to 92.5%.

Table 7 Total reads, reads after trimming and alignment rate

	An1	An2	An3
reads	14,699,672	47,844	261,304
both surviving	3,916,490	8,232	53,071
forward only	6,798,788	13,367	100,317
reverse only	185,041	1,013	4,504
dropped	3,799,353	25,232	103,412
alignment rate	92.5%	63.18%	88.96%

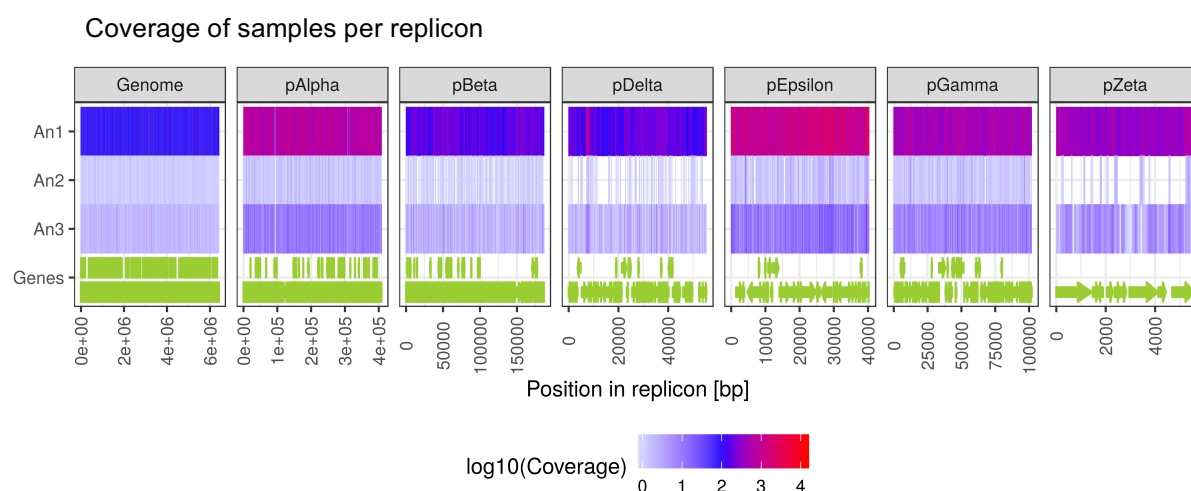


Figure 12 Coverage per base pair of vesicle DNA, isolated from three *Anabaena* sp. PCC 7120 samples over all replicons presented as heatmap. Colors represent higher coverage in log₁₀ scale from blue (low) to red (high).

Figure 12 displays a coverage plot over all replicons represented as a heatmap from low coverage (starting from 0 (white) over blue to high coverages (red) in log₁₀ scale. Vesicle DNA of *Anabaena* sp. PCC 7120 contains sequences from the chromosome as well as from all six intrinsic plasmids. The median/ mean coverages are highest for plasmid alpha, plasmid epsilon, and plasmid gamma in all three replicates despite of the overall sequencing quality (see Table 8). Additionally, the overall coverage was best in those plasmids. On plasmid delta, I detected three large peaks, present in An1 and An2 that have 3.5 – 7.5-fold higher median coverages (see Table 9. These peaks span up to 91% of 3 different transposase genes (WP_010999504.1, WP_084789056.1, and WP_010999798.1).

Taken together, the results indicate that parts of all replicons are present in OMVs of *Anabaena* sp. PCC 7120, that plasmid alpha, epsilon, and gamma might be enriched in vesicle DNA and that there might be overrepresented regions in vesicle DNA of *Anabaena* sp. PCC 7120.

Table 8 Median, mean (in parenthesis), and overall coverage [%] of vDNA in three samples of *Anabaena* sp. PCC 7120 OMVs over all replicons

Sample	chromosome	pAlpha	pBeta	pDelta	pEpsilon	pGamma	pZeta
An1	91	685	174	170	1157	472	365
	(100.78)	(751.29)	(190.56)	(203.26)	(1345.45)	(511.23)	(385.80)
	99.98%	100.00%	99.80%	100.00%	100.00%	100.00%	100.00%
An2	1	2	1	1	2	2	1
	(1.38)	(2.03)	(1.41)	(1.46)	(2.37)	(2.11)	(1.67)
	12.01%	56.51%	19.46%	17.01%	67.10%	59.11%	21.53%
An3	2	10	3	3	12	8	5
	(2.56)	(11.56)	(3.56)	(3.40)	(14.60)	(9.01)	(5.44)
	66.71%	98.72%	83.00%	78.81%	99.90%	99.50%	96.06%

Table 9 Prominent coverage peaks in vesicle DNA of *Anabaena* sp. PCC 7120.

Start (bp)	Stop (bp)	gene covered	max. median		replicon	gene	gene product
			coverage	change			
7108	8601	94.10%	7.5		pDelta	WP_010999504.1	Transposase
56511	57710	93.40%	6		pDelta	WP_084789056.1	Transposase
266191	267414	77%	3.4		pDelta	WP_010999798.1	Transposase

6.2.3 Sequencing results of particles from *Chlorogloeopsis fritschii* PCC 6912

Transmission electron microscopy images revealed putative phages next to outer membrane vesicles in *Chlorogloeopsis fritschii* PCC 6912. Next generation sequencing resulted in ~19.6 million reads from which around 3.17 million reads were filtered after trimming (Table 10). With the remaining reads, downstream analysis was conducted that led to an overall alignment rate of 97.02%.

Table 10 General statistics for sequencing of putative phages and OMVs in *Chlorogloeopsis fritschii* PCC 6912.

6912_1	
reads	19,634,930
both surviving	7,994,722
forward only	8,153,865
reverse only	319,307
dropped	3,167,036
alignment rate	97.02%
mean coverage	392.31
median coverage	2
overall coverage	70.08%

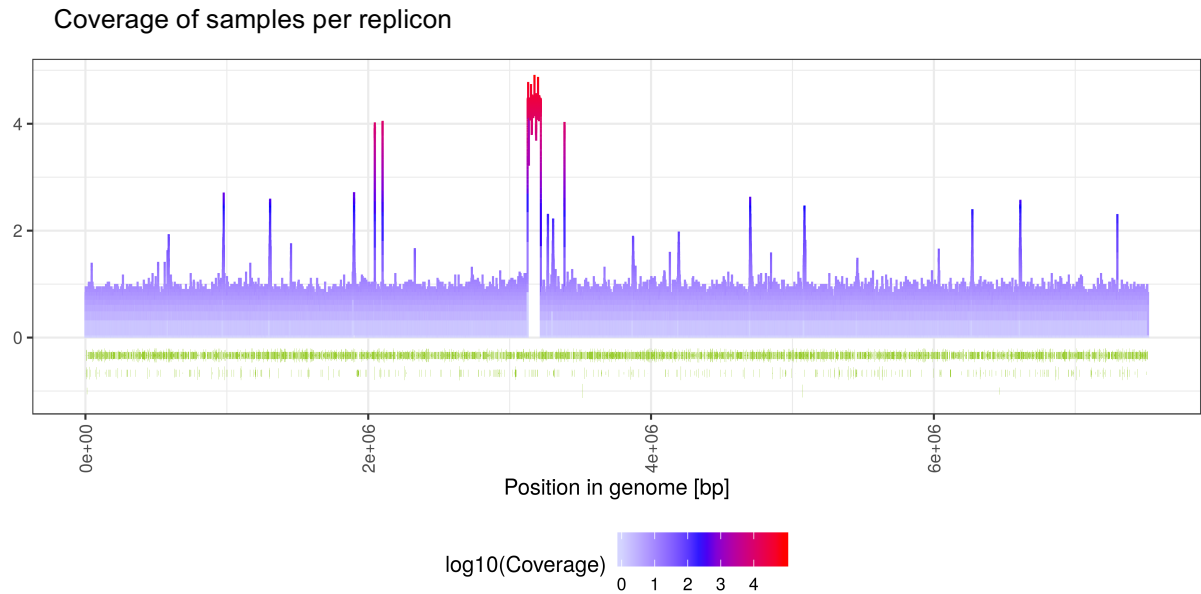


Figure 13 Coverage per base pair of particle DNA, isolated from particles of *Chlorogloeopsis fritschii* PCC 6912 over all replicons presented as heatmap. Colors represent higher coverage in log₁₀ scale from blue (low) to red (high).

Figure 13 shows the coverage of the whole chromosome with a 70% overall coverage of at least 1. Most parts have only very low coverage, however, 3 peaks with maximum coverages of around 10,000 and a region of around 90 kb in length with very high coverages up to 82,000 could be observed. The mean coverage over the whole chromosome is 392.31 while the median is 2, indicating an uneven distribution of coverages over the replicon. The 90 kb long region of high coverages resembles the region of the prophage in the genome of *Chlorogloeopsis fritschii* PCC 6912 (Dagan et al. 2012) with a mean coverage of 21,450, leading to the conclusion that most particles observed via TEM are indeed phages that can enter the lytic phase. The results indicate that the phage of *Chlorogloeopsis fritschii* PCC 6912 might be a temperate phage.

For three additional peaks with high coverages, I performed an ORF prediction and a BLASTp search. In the region of the three large peaks, the metabolic genes GTP-3,8 cyclase (identity 91% to *Scytonema millei*) and Glycerol- Acyl- transferase were identified (see Table 11). The fused response regulator/ thioredoxin-disulfide reductase is only partly covered, but with 100% identity. This predicted gene might play a role in signal transduction.

Table 11 The three most overrepresented regions apart from the phage genome in *Chlorogloeopsis fritschii* PCC 6912

Start (bp)	Stop (bp)	Length (bp)	Median coverage	Gene	Identity
2100277	2102211	1934	8969	GTP-3,8 cyclase	91%
3386972	3388413	1441	7403.5	fused response regulator/ thioredoxin-disulfide reductase	100%
2045557	2047095	1538	7259	Glycerol-Acyl-transferase	100%

Taken together, the results show that the DNA content of the phage/vesicle fraction is mostly of phage origin, but the genomic content of the outer membrane vesicles likely consists of parts of the whole genome where some genes might be more abundant than others. However, with methods currently available, it is not possible to separate phages from vesicles, hence, parts of the chromosomal DNA might also be part of the actual phage genome.

6.3 What is the genetic content of OMVs in *Synechocystis* sp. PCC 6803 under stress conditions?

The presence of DNA in OMVs is difficult to explain, since the vesiculation mechanisms proposed to date rule out the presence of any cytoplasmic content. Yet, recent models propose the possibility of forming complex double-bilayer vesicles that consequently comprise cytosolic plasmids and nucleic acids or as the formation of DNA containing vesicles as a by-product of explosive cell lysis (see 3.3.2). However, less focus has been given on the actual content of vesicle DNA and only few studies are available, that sequenced this DNA in mainly haploid species. However, *Synechocystis* sp. PCC 6803 is polyploid with 4-140 genome copies per cell, depending on substrain and growth rate (*Griese et al. 2011; Zerulla et al. 2016*), what could lead to a different DNA content in vesicles. On the other hand, although vesiculation is known for being a stress response (*McBroom and Kuehn 2006*), the influence of stress conditions on the actual genetic content of OMVs remains elusive. That is why, I chose *Synechocystis* sp. PCC 6803 as a model system to investigate the genetic content of OMVs under different growth conditions.

6.3.1 Finding suitable stress conditions for *Synechocystis* sp. PCC 6803

First, I aimed to find stress conditions that resemble natural occurring stresses and may affect the outer membrane integrity. Stress conditions tested comprise the addition of 250 mM hydrogen-peroxide, 500 mM sorbitol, or 700 mM sodium chloride to BG11 and otherwise standard conditions, as well as increased temperatures (37 °C and 40 °C).

Figure 14A presents the growth curves of the five different treatments. Cells that were grown under control conditions display a typical bacterial growth curve with a lag phase of approximately 24 h, an exponential growth until day 3.5, followed by the beginning of the

stationary phase. Raising the temperature to 37 °C did not alter the growth behavior at all. Thus, no stressed phenotype was observed at this condition. Applying oxidative stress with hydro-peroxide, however, killed the bacteria and no growth occurs. Under osmotic stress, achieved by cultivating *Synechocystis* sp. PCC 6803 in BG11 with 500 mM sorbitol, bacteria grew very slowly until day 4 to an OD₇₅₀ of 0.068 and already start dying at day 7 what can be seen by a decreased OD₇₅₀ of 0.046. These results indicate that both oxidative and hyper-osmotic growth conditions are too strong stressors for *Synechocystis* sp. PCC 6803 and were not applicable for further OMV experiments. Cells that were grown under BG11 with 700 mM sodium chloride or 40 °C showed an attenuate growth with lower end ODs₇₅₀.

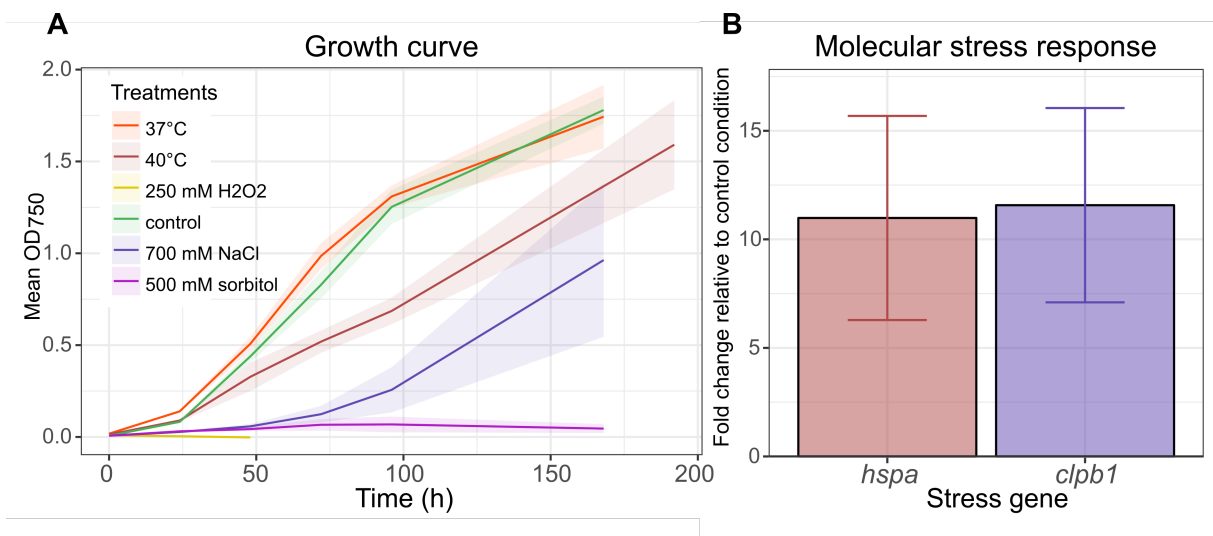


Figure 14 Different stressors attenuate growth and elicit stress response in *Synechocystis* sp. PCC 6803. (A) Growth curves of *Synechocystis* sp. PCC 6803 grown under different conditions reveal 700 mM NaCl and 40 °C growth temperature as two suitable stressors for further experiments Line = mean, shadow = standard deviation; $N \geq 3$. (B) Heat (40 °C) and high salt (700 mM NaCl) induce stress response genes *hspa* and *clpb1*, respectively ($N = 3$).

Additionally, I tested for a transcriptional upregulation of stress response genes for both conditions: *hspa* for heat (heat shock protein; (Los et al. 2008)) and *clpb1* for salt stress (chaperone protein *clpb1*; (Los et al. 2008)) via RT- qPCR. Figure 14B shows that under both conditions, the expression of stress response genes is around 11-fold upregulated relative to control condition, supporting the assumption that 40 °C and 700 mM, respectively. Thus, both conditions induce a stress response, both phenotypically as well as on the molecular level and I chose them as my treatments to test for differently vesicle production under non-optimal growth conditions compared to the normal growth condition.

Since previous studies reported that bacteria respond with higher vesiculation to stress, I hypothesized that also *Synechocystis* sp. PCC 6803 would react with an increased OMV production to stress (reviewed in (Orench-Rivera and Kuehn 2016)).

6.3.2 *Synechocystis* sp. PCC 6803 reacts with different growth patterns to stress

To test whether *Synechocystis* sp. PCC 6803 is response to stress conditions in terms of increased vesiculation and to investigate the genomic content of OMVs derived from these cells, I grew the bacteria under salt and heat stress as well as control growth conditions until the beginning of the stationary growth phase and subsequently isolated OMVs (Figure 15). The experiment was conducted twice with four replicates of each condition (8 replicates per condition in total). The position of culture flasks in the incubator was changed every day to avoid growth interference due to slightly different light exposure.

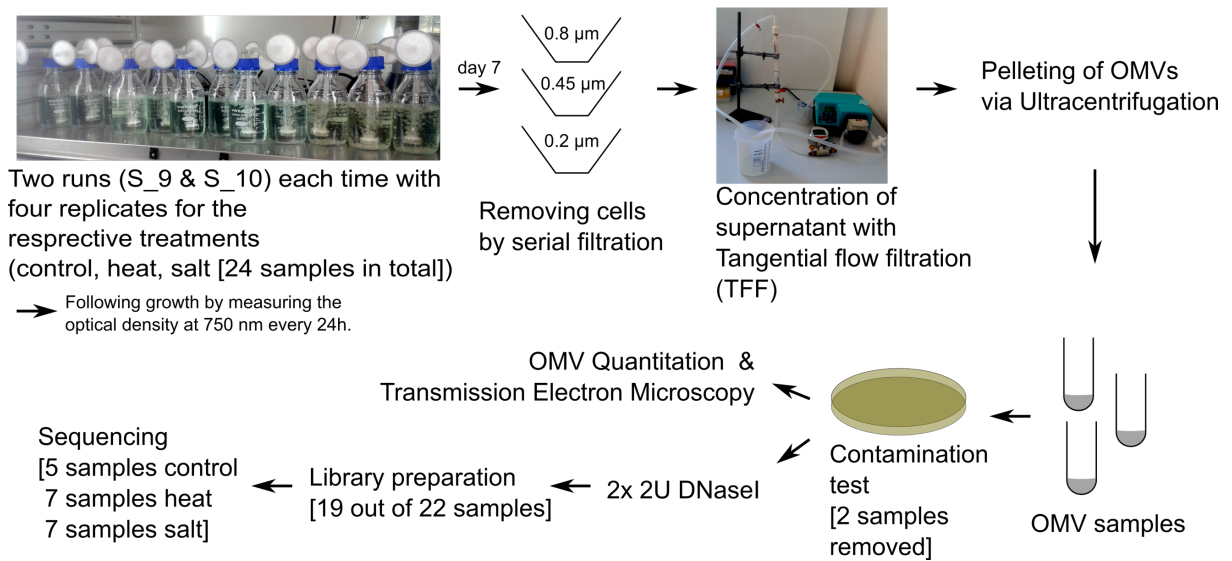


Figure 15 Schematic experimental setup

Growth characteristics measures were determined applying the growthcurver R package to calculate population growth characteristics. I observed different growth dynamics for the three conditions with good model fits (Figure 16A, Figure S 2A&B) as expected from my previous experiment (6.3.1). Non-treated *Synechocystis* sp. PCC 6803 exhibited a typical bacterial growth phase with a small lag phase of roughly 24 h, an exponential growth phase between day 2 to 5 and the beginning of the stationary phase from day 6 on with a final OD_{750} of 3.03 at day 7 (the end of this experiment, Figure 16A). On the other hand, salt stressed bacteria displayed a long lag phase until day 3.5, where the cells needed to adapt to salt (Figure 16A). Although the population growth rate (r) was significantly higher than that of control cells ($r_{\text{salt}} = 0.042$ vs. $r_{\text{control}} = 0.032$), the exponential growth phase is shortened, thereby leading to a significantly lower growth capacity (K) of 1.96 compared to the growth capacity of 3.45 under control conditions (Figure 16 B, C). Bacteria grown at 40 °C showed lag phase indistinguishable from the exponential phase, resulting in a significantly reduced the population growth rate compared to control conditions ($r_{\text{heat}} = 0.024$ vs. $r_{\text{control}} = 0.031$) (Figure 16A, B). Additionally, the carrying capacity was reduced in bacteria grown at 40 °C compared to control

conditions ($K_{\text{heat}} = 1.6$ in versus $K_{\text{control}} = 3.4$) (Figure 16 A, C). Further, a principal component analysis across all metrics clearly indicates three different growth patterns induced by three different growth conditions (Figure S 2C).

Taken together, these results indicate that salt and heat stress induces two different stress responses on growth behavior, suitable for OMV sampling in this experiment.

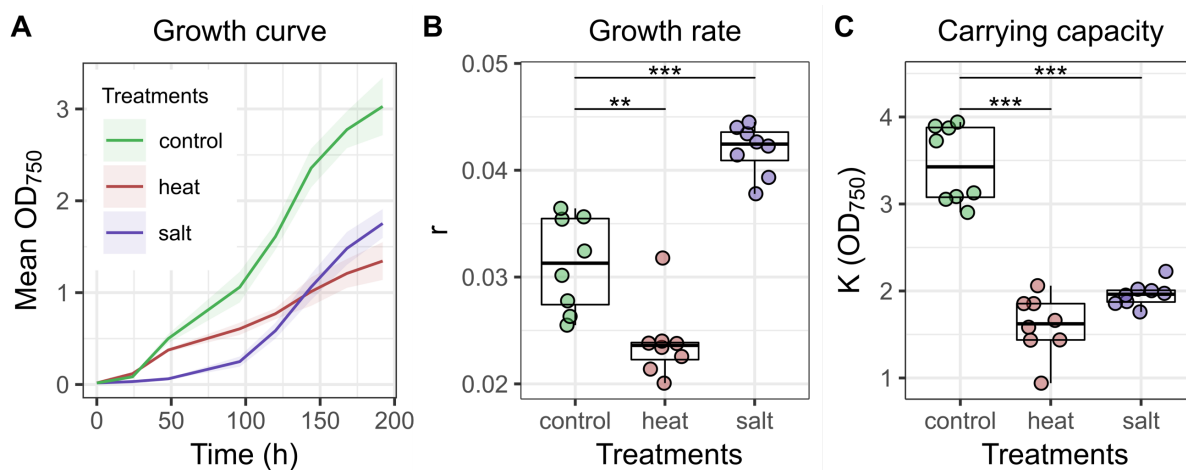


Figure 16 Growth curves and growth parameters of *Synechocystis sp. PCC 6803* under different stress conditions. (A) Growth curves under different conditions over time; line = mean, shadow = Standard deviation. (B) Intrinsic growth rate of the population r as boxplots. (C) Carrying capacity K as boxplots. Growth parameters were calculated using the R package “growthcurver” that applies the logistic equation. $N = 8$; Wilcoxon test: $p \leq 0.01$ (**); $p \leq 0.001$ (***).

6.3.3 *Synechocystis sp. PCC 6803* produces more OMVs under stress conditions

I sampled OMV containing supernatant at the end of the growth experiment to assess OMV production under stress conditions. The vesicles in the supernatant were concentrated by a tangential flow filtration system and pelleted via ultracentrifugation (Figure 15). Afterwards, vesicles were tested to be cell free on appropriate media plates. OMV samples, where any bacterial growth occurred on plates were eliminated from further experiments (2 in total, see Figure 15 and Table S 1).

The size distribution and appearances of the OMVs produced under stress conditions (Figure 17 B & C) resembles the OMVs of that of control condition (Figure 17A) as observed by TEM. Sizes of the OMVs ranged from 20 – 120 nm in diameter (Figure 17, arrows).

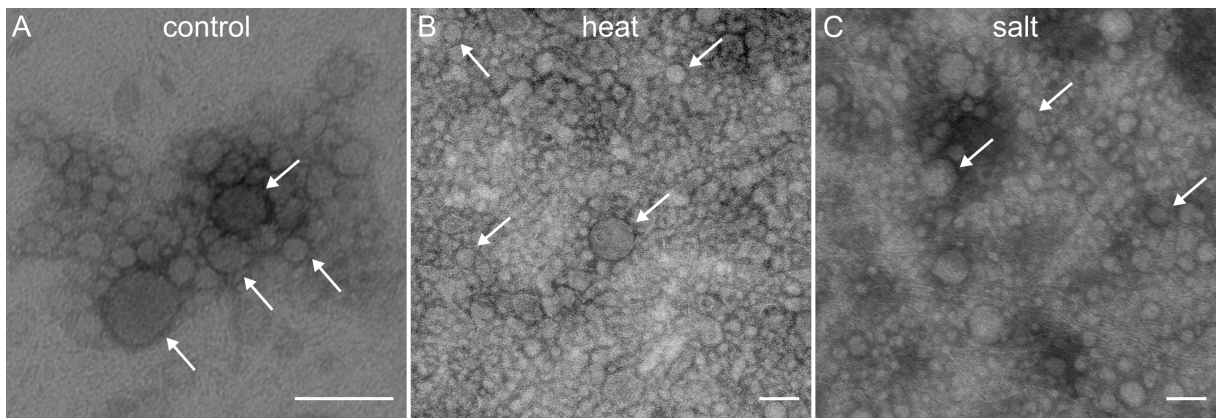


Figure 17 Outer membrane vesicles in *Synechocystis* sp. PCC 6803 under different stress conditions. Cell free outer membrane vesicles were negatively stained with uranyl-acetate and visualized with a TEM. OMVs produced under control (A), heat stress (B), or salt stress (C) appeared similarly in all conditions. The size of the particles ranged between 20 – 120 nm, with differently sized vesicles indicated by arrows. Scale bars represent 100 nm.

As mentioned above, I assumed an increased outer membrane vesicle production under stress conditions. Therefore, the relative amount of OMVs was determined by using two biochemically different approaches. The protein content of vesicles was measured with the Qubit protein assay, while lipid content was assessed with the dye FM4-64 that starts to fluoresces after incorporation into the lipid membranes of OMVs as described elsewhere (Aschtgen *et al.* 2015). The resulting values were normalized to the endpoint OD₇₅₀ to account for the distinct growth under different conditions. Salt treated bacteria produced moderate, yet significantly more outer membrane vesicles indicated by an increased protein and lipid content of 1.5-fold and 2.8-fold respectively, compared to control cells (Figure 18A, B). The vesiculation of bacteria grown under heat stress conditions increased higher by 4- fold and 17- fold in protein and lipid content, respectively compared to control conditions (Figure 18 A, B). Taken together, the quantitation of vesicles by protein- and lipid content demonstrate a significantly induced production of outer membrane vesicles under both tested stress conditions.

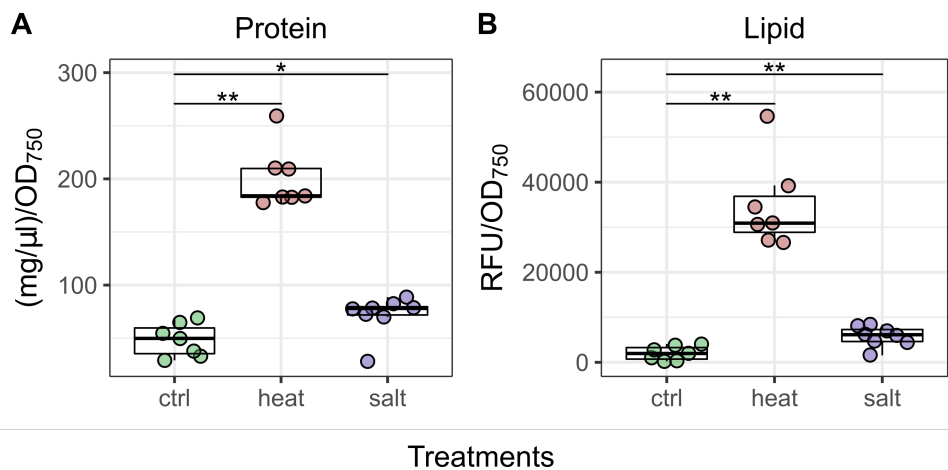


Figure 18 Quantitation of OMV content under different growth conditions. (A) The relative protein content of vesicles samples measured by Qubit protein assay relative to OD₇₅₀ increases significantly under both stress conditions (B) Significantly higher relative lipid contents of OMVs derived from stress cells compared to control conditions were measured with the fluorescent dye F4-64 relative to OD₇₅₀; $N \geq 5$, $p \leq 0.05$ (*), $p \leq 0.01$ (**) (Wilcoxon test).

6.3.4 OMV genomics- overview of sequencing results

As known from my previous experiments (section 6.3.3), vesicles from *Synechocystis* sp. PCC 6803 contain DNA that can be sequenced. To increase the DNA yield, I isolated vesicles from one liter supernatant of the three conditions in 8 replicates (Figure 15, Table S 1 & Table S 2). Two out of 24 OMV samples were found to be contaminated and I eliminated them from further analyses.

In order to isolate the DNA inside the vesicles, I digested any free or vesicle bound DNA from the OMVs with the DNase I treatment described in 6.2. The protocol for vesicle DNA isolation had an elution step in a final volume of 50 μ l. The DNA content ranged from 0.076 ng/ μ l to 0.296 ng/ μ l in vesicles from control and heat stressed bacteria, while the DNA concentration of vDNA in OMVs of salt stressed bacteria was much lower ranging from 6 pg/ μ l to 0.081 ng/ μ l (see Figure 19A, Table S 1).

Despite the low vDNA input material, I managed to produce libraries out of 5 control, 7 salt and 7 heat samples (19 in total) which included a multi displacement amplification (MDA) of the DNA and library concentrations ranged between 1.1 ng/ μ l up to 23.4 ng/ μ l with mean concentrations of 6.8 ng/ μ l for control, 8.4 ng/ μ l for heat, and 6.7 ng/ μ l for salt samples (Figure 19B). Next generation sequencing was carried out on a MiSeq platform with 2x 300 bp resulting in mean read numbers of 380,516 for control, 373,096 for salt treated samples, and 406,367 for heat stressed samples (Figure 19C). For reference, sequencing of genomic DNA from *Synechocystis* sp. PCC 6803 was performed, resulting in 438,942 reads. The read length distribution showed no biases between replicates and additionally also not to the genomic DNA sample (Figure S 3).

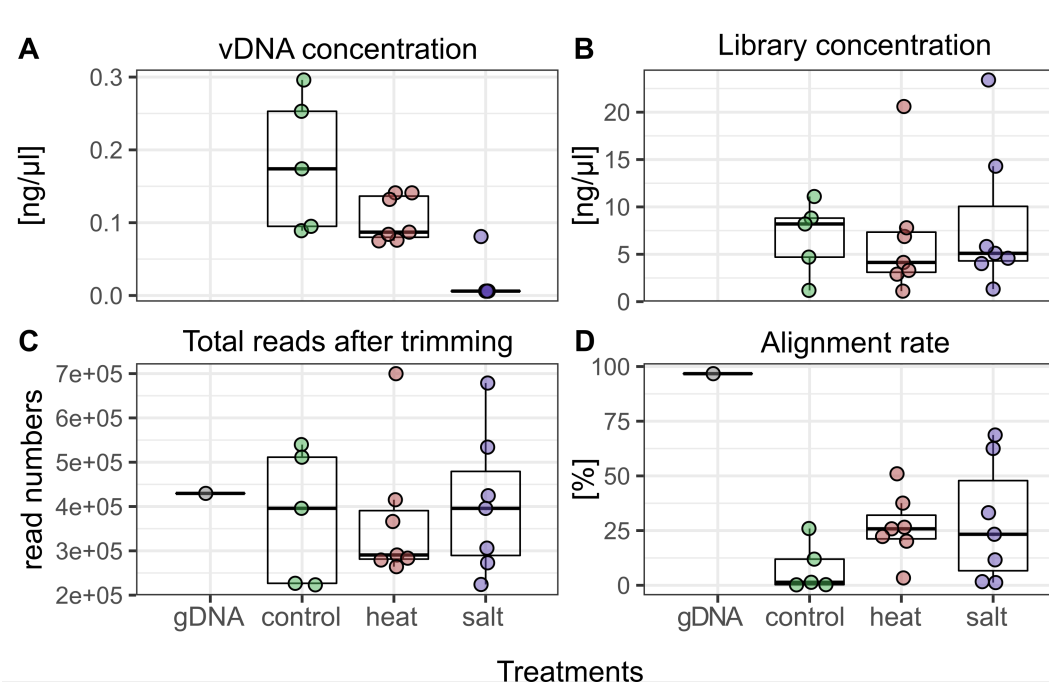


Figure 19 (A) Vesicle DNA content (no value for gDNA), (B) Library concentrations (no value for gDNA), (C) total read numbers, and (D) alignment rates of vesicle samples.

The reads were quality trimmed to remove low quality sequenced regions. Only a very minor portion of reads were removed completely (at most 1.03%; see Table S 1). After mapping to the reference genome, I noticed very diverse alignments rates ranging from only 0.21% up to 59.98% (Figure 19D). Low quality input material could lead to high amplification of random DNA during the MDA step of the library preparation and can explain these results. To check for outliers in the data set, I performed kmer (9 bp) counting of the samples and used logarithmic variances and distances to plot the samples in MDS and PCA plots respectively. I could neither identified clear outliers, nor groupings caused by systematic errors in the sample preparation or sequencing (Figure S 4A & B).

6.3.5 Vesicle DNA contains sequences from all replicons, especially from small plasmids

After quality control, I mapped the sequenced reads onto to the reference genome (containing the chromosome and all plasmids) and analyzed the overall coverage of the replicons (Figure 20). I noted that our lab strain does not possess the small plasmid pCC5.2 as no coverage has been found in the gDNA sample as suspected in the pilot tests (see 6.2.1). For the vesicle DNA, I found a general low and only fractionated coverage of the chromosome and the four large plasmids, but a complete and high coverage of the small plasmids in almost all samples similar to the findings from the pilot test (see Figure 20).

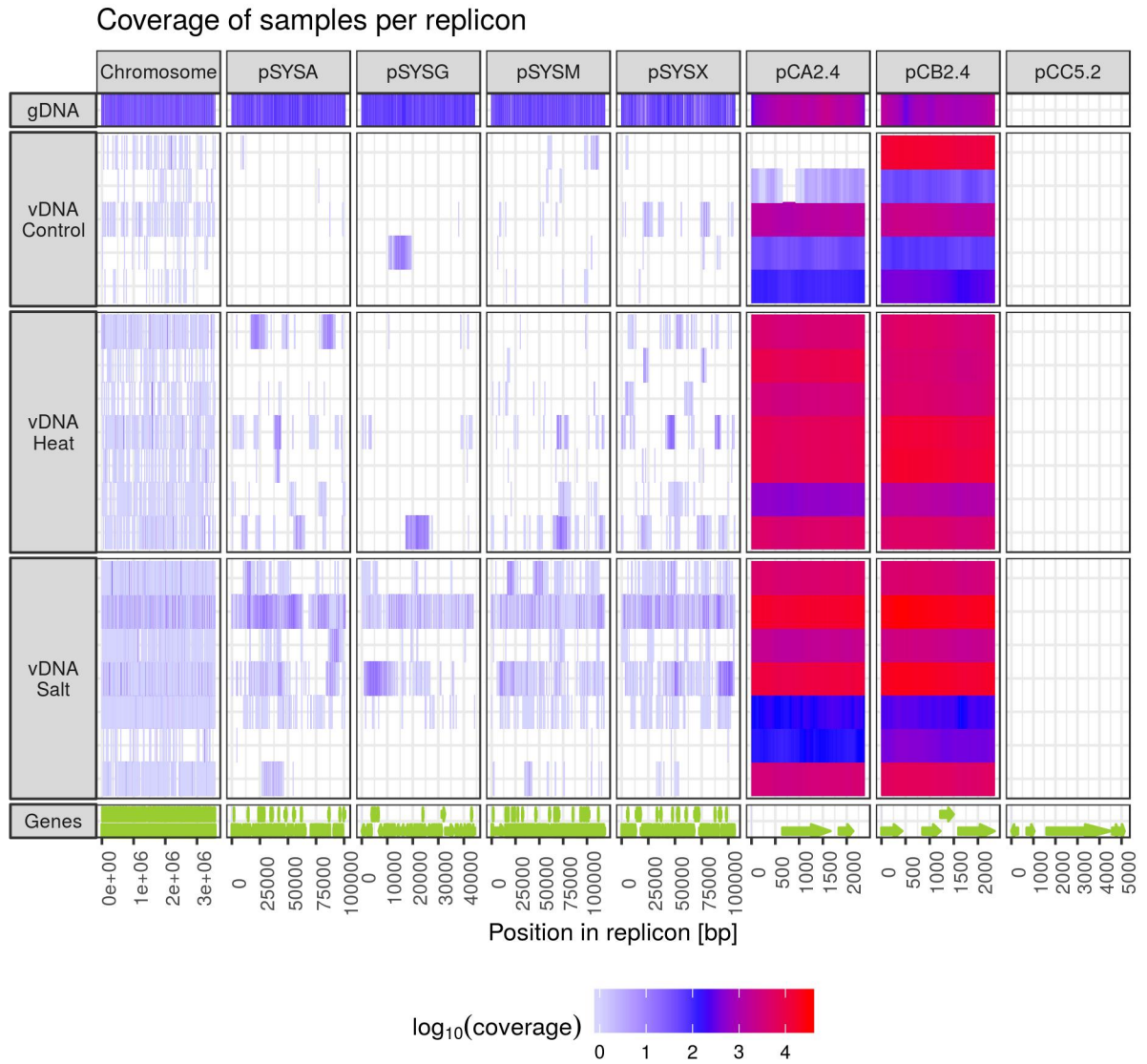


Figure 20 Coverage per base pair of vesicle DNA from different treatments over all replicons presented as heatmap. Colors represent higher coverage in \log_{10} scale from blue (low) to red (high).

While the median coverage of the chromosome in all vesicle samples ranged between 1 – 2 (Table 12; Figure S 5), the overall coverage differed highly, with fractions covered of 0.3 – 5.75% in control, 2.44 – 14.32% in heat and 2.04 – 77.18% in salt samples. Similar results in terms of median coverage and fraction of replicon covered were obtained for the large plasmids (pSYSA-X, see Table 12). For the small plasmids, I observed different results. Their coverage was highest in the gDNA sample compared to the other replicons with median coverage values of 1227.5 in pCA2.4 and 894 for pCB2.4, respectively, suggesting many plasmid copies in the cell. However, both small plasmids appeared to be highly enriched in the outer membrane vesicles, as under all conditions and in almost all replicates, depending on sequencing depth, the median coverage was much higher than the median coverages of all other replicons, with values up to 17,711.5 for pCA2.4 and 25,187 for pCB2.4 under salt conditions. Only two samples of control covered pCA2.4 only fractionated or not at all (see

Figure 20; Figure S 5). Taken together, these results indicate highly enriched small plasmids pCA2.4 and pCB2.4 in outer membrane vesicles.

Table 12 Range of median and mean (in parenthesis) coverages of the samples over the replicons. Additionally, the fraction covering the replicons is displayed as percentage.

	gDNA	control	heat	salt
chromosome	34	1 - 2	1 - 2	1 - 2
	(34.3)	(1.2 - 7.2)	(1.4 - 5.6)	(1.3 - 3.6)
	99.95%	0.30% - 5.75%	2.44% - 14.32%	2.04% - 77.18%
pSYSA	47	0 - 1	0 - 5	1 - 4
	(47.4)	(0.0 - 1.4)	(0.0 - 5.4)	(1.0 - 5.0)
	100%	0.00% - 0.76%	0.00% - 28.24%	0.27% - 84.29%
pSYSG	53	0 - 6	0 - 7	0 - 3
	(55)	(0.0 - 7.1)	(0.0 - 1.4)	(0.0 - 8.0)
	100%	0% - 19.36%	0.00% - 21.55%	0.00% - 66.23%
pSYSM	43	1 - 2	1 - 3	1 - 2
	(42.3)	(1.3 - 2.3)	(1.0 - 3.4)	(1.0 - 4.9)
	100%	0.57% - 9.00%	0.56% - 25.42%	0.15% - 74.52%
pSYSX	41	0 - 2	1 - 5	1 - 3
	(41.0)	(0.0 - 2.3)	(1.2 - 8.2)	(1.2 - 5.0)
	99.78%	0.00% - 18.48%	1.92% - 23.51%	0.54% - 85.80%
pCA2.4	1227.5	0 - 1409	563 - 8763	149 - 17,711.5
	(1188.1)	(0.0 - 3409.4)	(559.6 - 8625.5)	(151.1 - 17,551.7)
	100%	0.00% - 100.00%	100.00%	100.00%
pCB2.4	894	41 - 14479	1328 - 14,715	232 - 25,187
	(911.1)	(40.8 - 14349.3)	(1319.8 - 14,782.9)	(233.5 - 25,372.6)
	100%	100%	100.00%	100.00%
pCC5.2	0	0	0	0
	(0)	(0)	(0)	(0)
	0.00%	0.00%	0.00%	0.00%

6.3.6 Small plasmids pCA2.4 and pCB2.4 are highly enriched in OMVs

The sequencing results above indicate that the small plasmids pCA2.4 and pCB2.4 are overrepresented in the OMVs. To confirm these results, I assessed the plasmid copy number (PCN) in the cells, applying a RT-qPCR approach (see 5.1.8). Figure 21A and C displays the cellular plasmid copy numbers for pCA2.4 and pCB2.4, respectively. The median PCN for pCA2.4 ranges from 3.6 to 7.1 under all tested conditions and from 4.7 to 9.5 for pCB2.4 with no significant differences and resemble values from the literature (*Berla and Pakrasi 2012*).

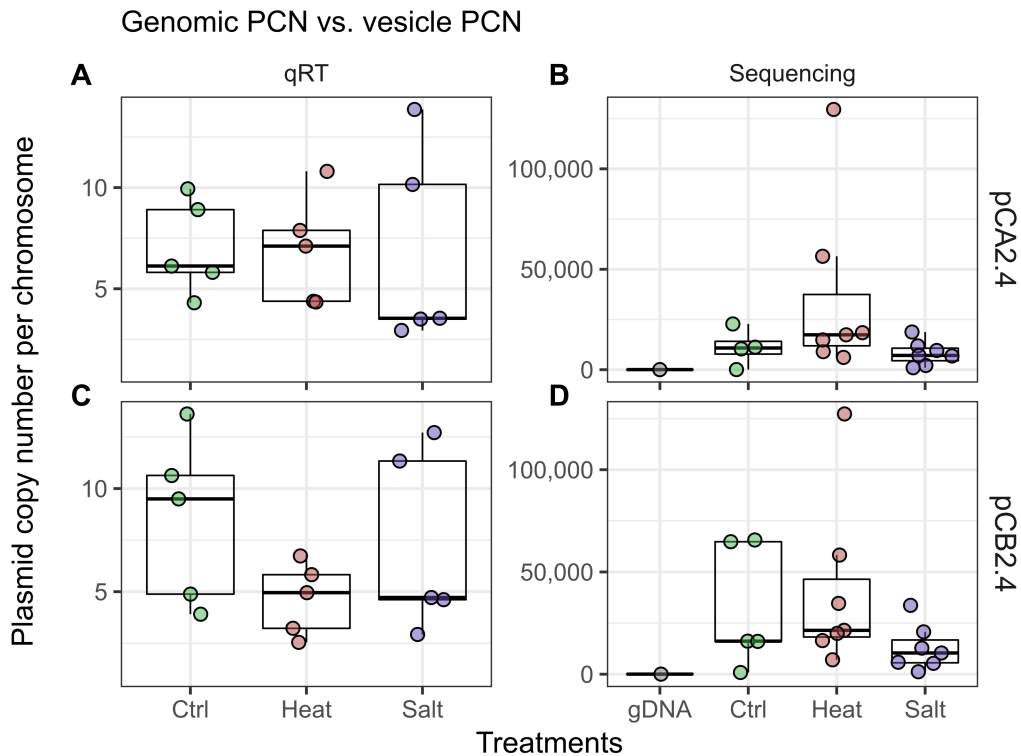


Figure 21 Plasmid copy numbers per chromosomes for pCA2.4 and pCB2.4 in cells of *Synechocystis sp. PCC 6803* (A and C) and for vesicles DNA (B and D). Plasmid copy number for cellular DNA was determined by isolating whole genomic DNA from bacterial cells. Quantitative real time PCR was performed on reference genes on the chromosomes and the plasmids, respectively. Wilcoxon test shows no significant differences between treated samples compared to control ($p \geq 0.05$, $N = 5$). Additionally, the variances also did not differ significantly between the treatments (Levene's test, $p \geq 0.05$, $N = 5$). PCN numbers for sequencing data were determined by calculating a ratio of plasmid and chromosome read counts adjusted to read length (in \log_{10} scale).

Due to the low DNA amount in the OMVs, I was unable to perform the same methodology to assess the PCNs in the vesicles. However, to get a measure of the level of enrichment of small plasmid DNA in the OMVs, I calculated a PCN equivalent on the bases of the sequencing results. To that end, I determined read counts of each replicon, which served as a measure for replicon abundance in the vesicles. I adjusted the raw read count number with the length of the replicon, as it is more likely to sequence a part of a large rather than of a smaller replicon. The ratio of plasmid and chromosome read counts was then used to estimate the PCN for the two small plasmids. The plasmid copy number of pCA2.4 reached a value of 34 in the cellular gDNA, while the PCN medians of vesicle-derived plasmids are several hundred times higher ranging from 7,098 in the salt treated samples up to 17,300 in heat treated samples (Figure 21B). Similar values were observed for plasmid pCB2.4 where the PCN in the genomic DNA sample was 27 while also this plasmid had much higher copy numbers in vesicle DNA ranged from 10,300 under salt conditions up to 21,400 in heat stressed samples (Figure 21D).

Taken together, these data indicate that both plasmids, pCA2.4 and pCB2.4, are highly enriched in vesicles independent from growth conditions.

6.3.7 Overrepresented regions in *Synechocystis* sp. PCC 6803 vesicles under different stress conditions

The coverage heatmap (Figure 20) and Table 12 indicate that only parts of the genome is covered in vesicle DNA samples. I was now interested, whether there are specific genes or regions that might be covered under all conditions or under specific stress conditions only and are thus enriched among the vesicle DNA. To that end, I calculated the median and the median absolute deviation (MAD) of all replicons. I defined an overrepresented region of each replicon as a region with a median coverage of at least four plus 3 times MAD to exclude very low covered regions/replicons from the analysis. Afterwards, I mapped the overrepresented regions to annotated genes which were at least covered by 75% of the region (approximately the top 25% of all overrepresented regions; see Figure S 6). For further analysis, I considered only genes which were covered by at least two overrepresented regions of two independent samples to account for the overall low coverages.

I found 65 genomic features (63 genes, 1 pseudogene, 1 direct repeat; see Table 13) overrepresented in all conditions. A Venn-diagram (Figure 22) reveals that four genes were overrepresented under control conditions, 31 under salt, and 30 genes could be found under heat conditions, hence none of the genes overrepresented were found in two or all three conditions simultaneously, which indicates a specific enrichment of genetic material in dependence of the growth conditions of the bacteria.

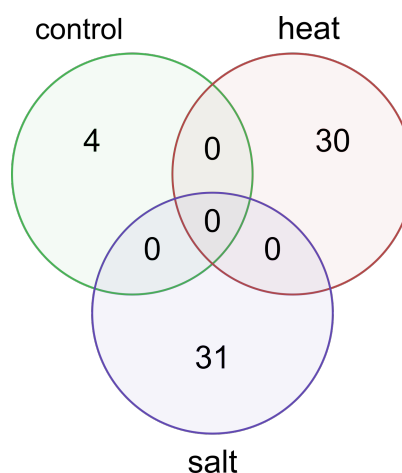


Figure 22 Venn diagram for overrepresented genes in vesicle DNA of *Synechocystis* sp. PCC 6803 under different conditions.

When analyzing the localization of the genes, I noticed that most genes occur in clusters of up to 11 genes (Figure 23) that are widely distributed over the replicons (Figure S 7). The majority of the overrepresented genes occurred on the chromosome. However, four

genes in heat and one in salt respectively, were found on plasmid pSYSA, and two genes occurred on plasmid pSYSX under salt conditions (Figure 23).

Next, I checked the annotated genes for their functional association (see Table 13). Seventeen out of the 65 genomic features are coding either for hypothetical proteins or for six DUF-domain containing proteins, all with no known functions. However, many other genes are annotated with functions mainly in metabolic pathways such as a 3-isopropylmalate dehydrogenase (MYO_RS07535) or a phytoltransferase (MYO_RS07545), having roles in amino acid metabolism and the biosynthesis of secondary metabolites. On the other hand, cluster 10 (cl_10; compare Figure S 8) comprises of 6 genes occurring in two replicates of salt stressed conditions as indicated by blue lines. Three of the genes are ABC transporters (see Table 13), that are active transport molecules, providing for example the asymmetry of the outer membrane (see Introduction). Additionally, two more genes (copper-translocating P-type ATPase [MYO_RS10660]; ammonium transporter [MYO_RS13870]) that are also involved in cellular transport functions were overrepresented under salt conditions.

Under heat stress, three genes (superoxide dismutase [Fe] [MYO_RS07530 [cl_11]]; diacylglycerol kinase [MYO_RS12050], type 1 glutamine amidotransferase [MYO_RS12055 [cl_16]]) were found that might play a role in signalling (see Table 13).

Taken together, I observed an over-representation of gene clusters dependent on the growth condition which might play functional roles in the stress response of *Synechocystis* sp. PCC 6803.

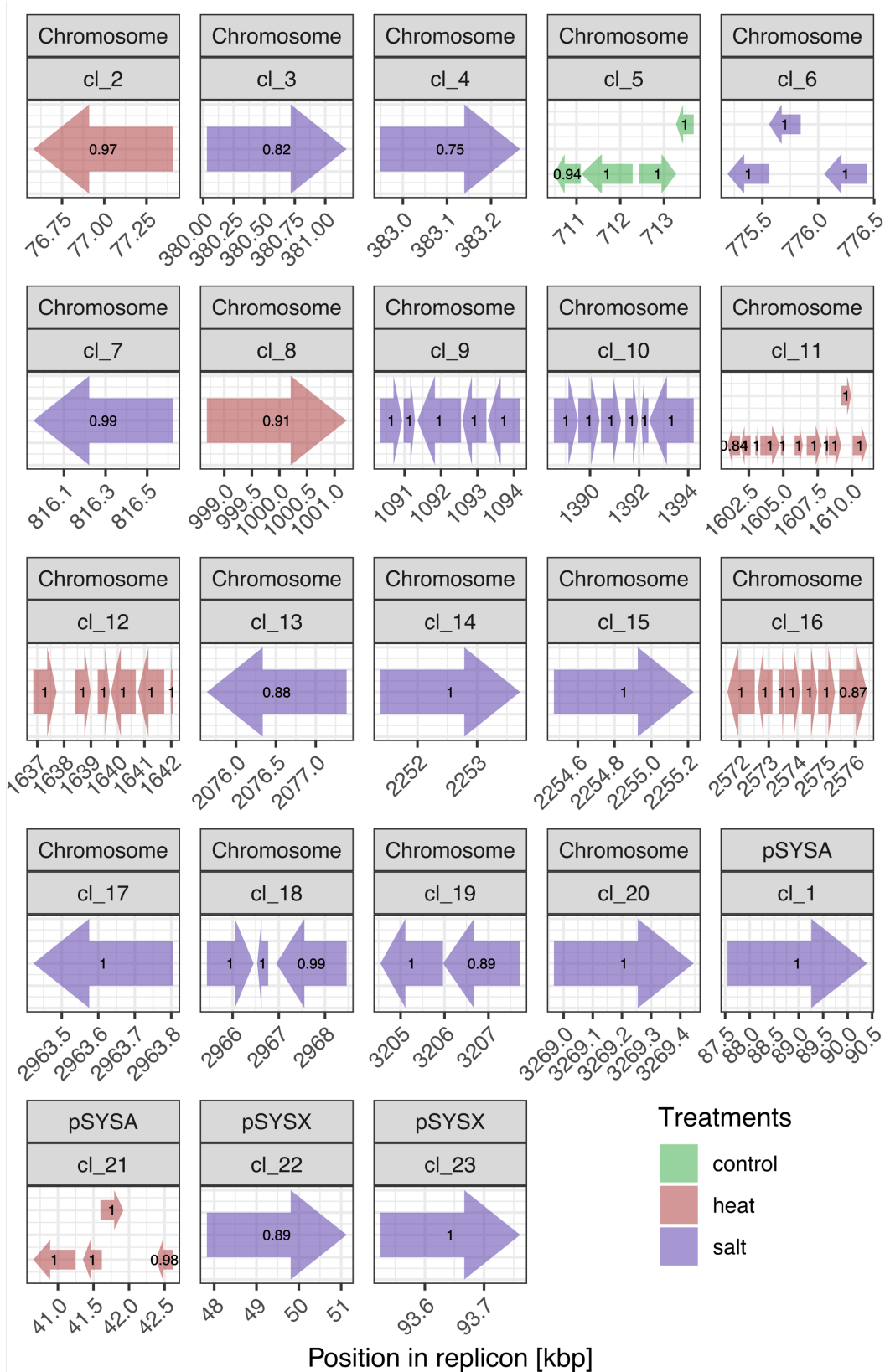


Figure 23 Overrepresented genes of vesicle DNA in different conditions. Neighboring genes are plotted together. Numbers in the arrows indicate the fraction covered by overrepresented regions. Cl_nr = Cluster.

Table 13 Overrepresented genes

Treatment	Name	Count	Median cov	Replicon	type	product
ctrl	MYO_RS03330	2	6.5	chr.	gene	serine esterase
ctrl	MYO_RS03335	2	6.5	chr.	gene	sporulation protein DUF1350 domain-containing
ctrl	MYO_RS03340	2	6.5	chr.	gene	protein
ctrl	MYO_RS03345	2	6.5	chr.	gene	ferredoxin
heat	MYO_RS00330	2	9	chr.	gene	4-hydroxy-tetrahydrodipicolinate reductase
heat	MYO_RS04700	2	12.5	chr.	gene	hypothetical protein
heat	ppnK	2	19	chr.	gene	NAD(+) kinase photosystem II biogenesis
heat	MYO_RS07510	2	19	chr.	gene	protein Psp29
heat	MYO_RS07515	2	19	chr.	gene	hypothetical protein putative bicarbonate
heat	MYO_RS07520	2	19	chr.	gene	transporter 2C IctB family DUF3288 domain-containing
heat	MYO_RS07525	2	19	chr.	gene	protein
heat	MYO_RS07530	2	19	chr.	gene	superoxide dismutase [Fe] 3-isopropylmalate
heat	MYO_RS07535	2	19	chr.	gene	dehydrogenase
heat	MYO_RS07540	2	19	chr.	gene	hypothetical protein 2-carboxy-1%2C4-
heat	MYO_RS07545	2	19	chr.	gene	naphthoquinone phytyltransferase pentapeptide repeat-
heat	MYO_RS07550	2	19	chr.	gene	containing protein
heat	MYO_RS07555	2	19	chr.	gene	oxidoreductase glycosyltransferase family 2
heat	MYO_RS07730	2	18.5	chr.	gene	protein
heat	MYO_RS07740	2	18.5	chr.	gene	hypothetical protein DUF29 domain-containing
heat	MYO_RS07745	2	18.5	chr.	gene	protein glycosyltransferase family 2
heat	MYO_RS07750	2	18.5	chr.	gene	protein
heat	MYO_RS07755	2	18.5	chr.	gene	porphobilinogen synthase
heat	MYO_RS07760	2	18.5	chr.	gene	cytochrome b6
heat	MYO_RS12030	2	8.5	chr.	gene	hypothetical protein acetolactate synthase small
heat	MYO_RS12035	2	8.5	chr.	gene	subunit DUF3285 domain-containing
heat	MYO_RS12040	2	8.5	chr.	gene	protein
heat	MYO_RS12045	2	8.5	chr.	gene	endoribonuclease YbeY
heat	MYO_RS12050	2	8.5	chr.	gene	diacylglycerol kinase type 1 glutamine
heat	MYO_RS12055	2	8.5	chr.	gene	amidotransferase

heat	MYO_RS12060	2	8.5	chr.	gene	chlorophyll synthase ChIG
heat	MYO_RS17415	2	6	pSYSA	gene	hypothetical protein
heat	MYO_RS17420	2	6	pSYSA	gene	hypothetical protein
heat	MYO_RS17425	2	6	pSYSA	gene	hypothetical protein
heat	MYO_RS17430	2	6	pSYSA	gene	hypothetical protein
salt	MYO_RS01825	2	9	chr.	gene	glycosyltransferase family 4 protein
salt	MYO_RS01845	2	6.5	chr.	gene	UDP-glucose 4-epimerase putative toxin-antitoxin system toxin component 2C PIN family
salt	MYO_RS03630	3	4	chr.	gene	hypothetical protein
salt	MYO_RS03635	2	6	chr.	gene	hypothetical protein
salt	MYO_RS03640	2	6	chr.	gene	hypothetical protein
salt	MYO_RS03795	2	7.5	chr.	gene	Uma2 family endonuclease pseudo
salt	MYO_RS18625	2	6	chr.	gene	hypothetical protein
salt	MYO_RS05100	2	6	chr.	gene	cytochrome b
salt	MYO_RS05105	2	6	chr.	gene	hypothetical protein
salt	MYO_RS05110	2	6.5	chr.	gene	hypothetical protein
salt	MYO_RS05115	2	6.5	chr.	gene	carotenoid oxygenase tRNA epoxyqueuosine(34) reductase QueG
salt	MYO_RS05120	2	6.5	chr.	gene	phosphate ABC transporter permease subunit PstC
salt	MYO_RS06540	2	11	chr.	gene	phosphate ABC transporter 2C permease protein PstA
salt	MYO_RS06545	2	11	chr.	gene	phosphate ABC transporter ATP-binding protein
salt	MYO_RS06550	2	11	chr.	gene	peptidyl-prolyl cis-trans isomerase
salt	MYO_RS06555	2	11	chr.	gene	4a-hydroxytetrahydrobiopterin dehydratase
salt	MYO_RS06560	2	11	chr.	gene	DUF389 domain-containing protein
salt	MYO_RS06565	2	11	chr.	gene	DNA repair protein RecN
salt	MYO_RS09860	2	8.5	chr.	gene	copper-translocating P-type ATPase
salt	MYO_RS10660	2	10	chr.	gene	YebC/PmpR family DNA-binding transcriptional regulator
salt	MYO_RS10670	2	11.5	chr.	gene	regulator
salt	MYO_RS13850	2	8	chr.	gene	chorismate mutase
salt	MYO_RS13860	2	10.5	chr.	gene	IS701 family transposase
salt	MYO_RS13865	3	6	chr.	gene	hypothetical protein
salt	MYO_RS13870	2	10	chr.	gene	ammonium transporter
salt	MYO_RS14890	2	14	chr.	gene	DUF697 domain-containing protein
salt	MYO_RS14895	2	10	chr.	gene	arginine-tRNA ligase

salt	MYO_RS15185	2	8	chr.	gene	cysteine desulfuration protein
					direct_r	SufE
salt	NA	2	4	pSYSA	epeat	
salt	MYO_RS18160	2	5.5	pSYSX	gene	hypothetical protein
salt	MYO_RS19165	2	7.5	pSYSX	gene	hypothetical protein

7 Discussion

The formation of outer membrane vesicles is an important mechanism in Gram (-) bacteria. OMVs provide a decoy for cargo, which is protected from extracellular enzymes such as proteases or nucleases or transmit a high local concentration for bioactive compounds. Hence, OMVs are involved in pathogenesis (promoting virulence and modulating host immune response), interspecies communication (cell-cell communication), biofilm establishment and maintenance, nutrient acquisition, and DNA-transfer (see Table 1 and reviewed i.a. in (Schatz and Vardi 2018)). The DNA content of vesicles is of particular interest for several reasons. Several studies demonstrated a close association of DNA to the inner and outer surfaces of OMVs and the transfer of OMV-associated DNA on an inter- and intraspecies level (Kahn et al. 1982; Schooling et al. 2009; Perez-Cruz et al. 2013; Fulsundar et al. 2014; Bitto et al. 2017). This puts OMVs forward as another mechanism for lateral gene transfer, with (compare Box 3) likely consequences for the evolution of bacteria, for example for the transmittance of antibiotic resistance genes (e.g. (Yaron et al. 2000; Fulsundar et al. 2014; Bitto et al. 2017)). However, it is challenging to explain how DNA can be packed into OMVs as it has to re-localize from the cytosolic site through the cytosolic membrane and the peptidoglycan layer. Additionally, it is unclear, whether there is an active packaging mechanism for specific DNA sequences or whether the loading of DNA to the vesicles occurs by purely stochastic means.

In my thesis, I investigated the DNA content of OMVs in the freshwater cyanobacteria *Anabaena* sp. 7120, *Chlorogloeopsis fritschii* sp. 6912, and in *Synechocystis* sp. PCC 6803, cultured under a variety of conditions. I found that the chromosomes and most plasmids are found with low frequencies, while small plasmids were enriched in OMVs from *Synechocystis* sp. PCC 6803, regardless of the growth condition. Furthermore, I found gene clusters to be overrepresented dependent on the respective stress condition in this strain.

7.1 Cyanobacterial OMVs resemble those of other organisms.

Transmission electron microscopy revealed the presence of outer membrane vesicles in cyanobacteria of different complexities. OMVs produced by *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and *Chlorogloeopsis fritschii* PCC 6912 were on average between 20 to 150 nm in diameter in size. They had a well-defined bilayer or double bilayer membrane, indicating their production by living cells rather than being artefacts of sample procession. These results are in line with findings from other bacteria (reviewed in (Toyofuku et al. 2019)) as well as from the marine cyanobacterium *Prochlorococcus* (Biller et al. 2014). The finding of vesicles in *Anabaena* sp. 7120 was recently supported by a study on the exoproteome of the same bacterium. The authors found OMVs that look phenotypically similar and display the same size range as vesicles described in this thesis (Oliveira et al. 2015). The appearance of OMVs in *Synechocystis* sp. PCC 6803 has also been confirmed by other studies in

transmission electron- as well as scanning electron microscopy experiments (*Pardo et al. 2015; Oliveira et al. 2016*).

However, the majority of particles, I found in *Chlorogloeopsis fritschii* PCC 6912 by transmission electron microscopy were not OMVs, but rather exhibited a phage-like morphology with equal size, octagonal shape, and electron dense luminal content. This finding was supported by sequencing of the OMV/phage fraction. Outer membrane vesicles can have different interactions with phages. On the one hand, phage receptors and phage/viral DNA might be transmitted by vesicles (*Hagemann et al. 2013; Tzipilevich et al. 2016*). On the other hand, several studies showed that OMVs might act as decoys for phages, thus acting as an extracellular phage defense system (*Manning and Kuehn 2011; Biller et al. 2014; Reyes-Robles et al. 2018*). However, I did not find any attachments between phages and OMVs in *Chlorogloeopsis fritschii* sp. PCC 6912. Future works needs to be done to explore whether vesicles might also act as a phage defense mechanism in the here described cyanobacterium. Especially, the ultracentrifugation of particles could be improved for detecting intact phages in transmission electron microscopy and for separating vesicle and phage fractions. Using for example a density gradient is (I) gentler and (II) could lead to a cleaner OMV/phage fraction, thus increasing the chances for finding intact phages and potential phage/vesicle complexes.

7.2 Environmental stress has an impact on *Synechocystis* sp. PCC 6803 vesiculation pattern

Formation of vesicles occurs continuously throughout the growth phase of bacteria although environmental stress has been demonstrated to affect the release rate (*MacDonald and Kuehn 2013*). Vesiculation might even function as an additional route for bacteria to react to stress. One important role could for example be the accumulation of misfolded outer membrane proteins in OMVs that are eventually removed from the cell as demonstrated in *P. aeruginosa* and *E. coli* (*McBroom and Kuehn 2006; Tashiro et al. 2009*). In my thesis, I also found higher vesiculation rates under both, salt and heat stress. However, a direct comparison of the quantity of OMVs between studies is hampered by the enormous variation of applied methods, ranging from directly counting particle numbers via FACS or microscopic techniques to measurements of protein and or lipid content of vesicle samples by a range of different biochemical approaches (reviewed in (*Orench-Rivera and Kuehn 2016*)). The quantification additionally depends on the purity of vesicle samples and the need for normalization to the number of bacteria in the pre-culture by counting colony forming units or using optical density. Based on this, it is reasonable to use a combination of quantitative techniques for a more realistic measurement (*Orench-Rivera and Kuehn 2016*). To that end, I used two different and independent quantification methods with which I assessed the protein and lipid content, respectively. Indeed, I found that the protein content in the stressed OMV samples increased by a factor of around 1.5 to 2.8, while the lipid measurement displayed an increase of factor

4 under salt stress up to factor 17 under heat stress as compared to normal growth conditions. Thus, *Synechocystis* sp. PCC 6803 reacts with different vesiculation patterns in terms of the actual protein and lipid content of OMVs to the applied stress conditions. For heat stress, very different vesiculation patterns were observed in other bacteria. A heat shock applied to *Pseudomonas putida* by raising the temperature to 55 °C lead to an increased vesiculation (Baumgarten et al. 2012). However, producing more OMVs under temperature stress is not an inherit response in all bacterial strains as no higher vesiculation was observed in *A. baylyi* (Fulsundar et al. 2014) or in *Pseudomonas aeruginosa* after raising the temperature from 25 °C to 37 °C or 39 °C (MacDonald and Kuehn 2013). On the contrary, *Serratia marescens* cells responded conversely to temperature, producing high amounts of OMVs at 22 °C – 30 °C and only a negligible amount of vesicles at 37 °C (McMahon et al. 2012).

Applying salt stress throughout the whole growth phase also led to an increased vesiculation in *Synechocystis* sp. PCC 6803, however the protein- and lipid contents did not increase as much as compared to heat conditions. This hints either to a distinct vesicle membrane content or to a specific vesicle cargo compared to OMVs shed by heat-treated cells. The pathogen *Pseudomonas putida* produces significantly more vesicles in response to a hyperosmotic shock (2 M NaCl) (Baumgarten et al. 2012). However, it should be taken into account that NaCl could lead to disruption of the outer membrane of the bacterium rather than higher vesiculation rates, although I did not observe open vesicles indicating extensive cellular debris. On the contrary, although *Synechocystis* sp. PCC 6803 is a fresh water cyanobacterium, it evolves a remarkable salt tolerance (Reed and Stewart 1985), which supports the assumption that the observed larger vesicle amounts are indeed a product of higher vesiculation rates. Further studies should investigate the actual protein- and lipid content of vesicles from cyanobacteria under different conditions to get further insight into the actual cargo and thus the function of OMVs under stress conditions in those bacteria. Taken together, the results of my thesis show that OMV production rates are highly variable and depend not only on the specific strains but also on growth conditions as demonstrated in other bacteria for temperature, exposure to antibiotics, and nutrient availability (Li et al. 1996; Kadurugamuwa and Beveridge 1998; Sabra et al. 2003; McBroom et al. 2006; MacDonald and Kuehn 2013; Devos et al. 2015).

7.3 Cyanobacterial outer membrane vesicles contain plasmid and chromosomal DNA

Outer membrane vesicles contain not only proteins and lipids, but also DNA, which has been demonstrated for a wide range of species (recently reviewed in (Domingues and Nielsen 2017)). Indeed, I also detected DNA in vesicles derived from freshwater cyanobacteria of different complexities by either staining vesicles with SYBR® Gold, a dye specific for nucleic acids and/or subsequently sequencing. However, it should be noted that it was not possible to

distinguish between vesicle DNA and phage DNA in *Chlorogloeopsis fritschii* PCC 6912, as discussed in more detail 6.1.3.

The actual presence of DNA in OMVs is difficult to explain as DNA would have to pass the cytosolic membrane and the peptidoglycan layer before being packed into vesicles. However, several mechanisms have been proposed: some DNA bearing vesicles might be the result of cell death and random uptake of naked DNA by opening and closing of vesicles or the result of explosive cell death, where cellular structures are dissolved and DNA can freely access the resulting vesicles (Renelli *et al.* 2004; Berleman and Auer 2012; Turnbull *et al.* 2016). As I only found occasionally open vesicular structures as a sign for cellular debris and no phage was present in our lab strains of *Anabaena* sp. PCC 7120 and *Synechocystis* sp. 6803, this mechanism unlikely explains the amounts of DNA, I observed. In contrast, other observations point to an active shedding of double bi-layered vesicles, where cytosolic components, including DNA, were detected in those vesicles (Perez-Cruz *et al.* 2013; Pérez-Cruz *et al.* 2015). Similar vesicles have for example been also demonstrated in *Ahrensia kielensis* and *Pseudoalteromonas marina* (Hagemann *et al.* 2013). During my experiments, I also rarely but consistently detected double bi-layered OMVs in both, *Synechocystis* sp. PCC 6803 and *Anabaena* sp. 7120 vesicle preparations. The small amount of double bi-layered OMVs is in concordance with recent studies, reflecting a mechanism where bacteria tolerate a short rupture of their peptidoglycan layer and cell membrane (Hagemann *et al.* 2013; Perez-Cruz *et al.* 2013; Pérez-Cruz *et al.* 2015). Moreover it has been demonstrated that DNA is not uniformly distributed in vesicles, showing that less than 1% of vesicles are fluorescent after staining with SYBR® Gold (Biller *et al.* 2016). Although this is a very conservative estimation as only vesicles containing more than 9 kb DNA are likely to be detected by this method. Notably, it supported well the numbers reported by Pérez-Cruz *et al.* (2013 & 2015), and the results in my study, where I only found few particles exhibiting a positive SYBR® Gold fluorescence signal (see Figure 5 and Figure 7) (Tomaru and Nagasaki 2007; Perez-Cruz *et al.* 2013; Pérez-Cruz *et al.* 2015). The results of my study add two cyanobacterial species to the model of shedding of double bi-layered OMVs.

7.4 The structure and high abundance of small plasmids might promote random packaging in OMVs

As outlined above, many studies detected DNA of plasmid- and phage associated genes as well as genes from the bacterial chromosome in OMVs (Kolling and Matthews 1999; Yaron *et al.* 2000; Gaudin *et al.* 2014). This renders OMVs a remarkable additional gene transfer mechanism as they provide a nuclease-protected decoy, where the genetic cargo is locally enriched. Indeed, lateral gene transfer of antibiotic resistance genes or GFP via OMVs has been demonstrated in mainly pathogenic species. These vesicles contained vectors as well as natural plasmids (Dorward *et al.* 1989; Yaron *et al.* 2000; Rumbo *et al.* 2011; Fulsundar *et*

al. 2014; Tran and Boedicker 2017; Tran and Boedicker 2019). Transfer rates in *Escherichia coli* for example, were comparable to that of transduction while in *Acinetobacter baumannii*, the transfer efficiency was up to ten times higher as compared to natural transformation and depended also on stress conditions, strengthening the hypothesis that vesicles might provide a protected decoy for LGT (Fulsundar et al. 2014; Tran and Boedicker 2017). However, no study so far actually investigated the content of OMVs with respect to naturally inherited plasmids and whether the loading process is actively controlled or whether the DNA content of OMVs is random.

When sequencing the DNA content of OMVs, I found in a pilot test that all replicons in the three cyanobacteria *Anabaena* sp. PCC 7120, *Chlorogloeopsis fritschii* PCC 6912, and *Synechocystis* sp. PCC 6803 are present in very low abundance. However, for *Synechocystis*, I also sequenced a cellular genomic DNA sample and I could show that the small plasmids pCA2.4 and pCB2.4 are highly enriched in the OMVs as compared to the cellular level, independently from the respective growth condition (see Figure 20 and Figure 21). Several reasons might cause this enrichment in vesicles. First, high copy numbers might lead to higher probability of ending up in OMVs. The small plasmids pCA2.4 and pCB2.4 have copy numbers of up to 10 copies per chromosome in *Synechocystis* sp. PCC 6803 (see Figure 21 and (Berla and Pakrasi 2012; Armshaw et al. 2015)). This bacterium might have 4 up to 140 chromosome copies in the cell, although this number is highly variable and at the beginning of the stationary growth phase the number is more likely to be lower (Griese et al. 2011; Zerulla et al. 2016). However, this still means that dozens to hundreds of small plasmids are present in a single cell, likely leading to a higher probability of being packed in vesicles. A study by Tran et al. (2019) investigated the influence of plasmid characteristics on vesicle mediated DNA transfer. They found that plasmids with higher PCNs are more likely to be incorporated into vesicles supporting the assumption of a random DNA uptake mechanism in vesicles (Tran and Boedicker 2019).

Additionally, both small plasmids in *Synechocystis* sp. PCC 6803 are rather small with roughly 2.4 kb and have no known function, thus are referred to as being cryptic (Yang and McFadden 1993; Yang and McFadden 1994). This small size is likely characterized by higher diffusion properties as those small, high copy plasmids are thought to diffuse freely and randomly throughout the cell (Durkacz and Sherratt 1973), reviewed in (Wang 2017). Another possible explanation might come from studies that found high copy plasmids to be located in dense clusters (Eliasson et al. 1992), for example at the midpoint of the cells or their quarter positions (the new midpoint of daughter cells after cell division) (Pogliano et al. 2001). Most likely, both mechanisms occur in cells, where plasmids might move freely except for the nucleoid region and thus might accumulate at distinct sides, e.g. at the cell poles (Reyes-Lamothe et al. 2014; Hsu and Chang 2019). The latter scenario, with both, clusters and

diffusing plasmids, would enhance the probability for plasmids to be packed into vesicles, hence again strengthen the hypothesis of a stochastic DNA uptake mechanism. Interestingly, also in *Neisseria gonorrhoeae*, a cryptic plasmid was present in vesicles (Dorward et al. 1989). As this study has been conducted prior single cell sequencing was available, it is likely that also this plasmid is highly abundant in OMVs as it would not have been identified, when its individual DNA concentration was too low. Furthermore, the Archaeon *Thermococcus nautilus* produces vesicles containing a small plasmid of around 3.6 kb (pTN1) while a larger plasmid (18.3 kb) was not found (Gaudin et al. 2014). Interestingly, the small plasmid pTN1 replicates by the rolling circle mechanism, while the larger plasmid does not. Replication by rolling circle mechanism leads to long stretches of DNA, is a very rapid replication and thereby causes high abundances of the plasmids (see Box 1). Thus, the actual replication model might play a role in a yet unknown fashion by increasing the likeliness for the plasmids of being loaded to the vesicles.

Although most likely a random process, the distribution of plasmids in vesicles might contribute to the long-term persistence of pCA2.4 and pCB2.4 in *Synechocystis* sp. PCC 6803 - a hypothesis under current intensive research.

7.5 Several genes are enriched under different stress conditions in *Synechocystis* sp. PCC 6803

Apart from the overabundance of the small plasmids, I found in general very low coverage of the other replicons with median coverages of only one to at most seven reads in *Synechocystis* sp. PCC 6803 under all conditions. However, the heatmap revealed that some clusters consisting of up to 11 genes exist, which were overrepresented in at least two replicates (see Figure 20 and Figure 23; overrepresented means: 75% of a gene is covered with at least median read count plus 3x median absolute deviation of the respective replicon). Notably, these gene clusters differed between the growth conditions. In general, most of the genes are involved in the cell metabolism such as amino acid metabolism (see Table 13). However, I also identified genes that are important for cellular transport functions in the OMVs harvested from salt stressed cells. This is different to the OMVs from heat stressed cells, which contained genes that are important for signaling. The only other study so far on vesicle DNA content in cyanobacteria, namely the marine species *Prochlorococcus* and *Synechococcus*, found a pattern, where the terminus region of the chromosome was overrepresented. Here the authors speculated that there might be a so far unknown link between the DNA presence in vesicles to the cell cycle (Biller et al. 2014). This pattern differs from my study, as I found a distribution of overrepresented genes over the whole replicon (see Figure S 7). However, although information on the main overabundant genes in the study by Biller et al. (2014) were mostly lacking, i.e. the genes were hypothetical, they also detected metabolic genes, such a citrate synthetase (Biller et al. 2014). Hagemann et al. (2013) discovered in marine proteobacteria,

namely in *Ahrensia kielensis* and *Pseudoalteromonas marina*, genes that are important for defense, e.g. a toxin-antitoxin component, as well as many metabolism associated genes (Hagemann et al. 2013). As the authors found all genes just once in their shotgun libraries, they hypothesized that the DNA uptake in vesicles occurs random. On the contrary, in the oral pathogen *P. gingivalis*, virulence genes were found to be overrepresented, letting the authors speculate that there might be a directed route of DNA into OMVs by a yet unknown mechanism (Ho et al. 2015). While Ho et al. (2015) only searched for known genes by semi-quantitative PCR, Bitto et al. (2017) found overrepresented virulence genes in *Pseudomonas aeruginosa* by sequencing the vesicle DNA content (Bitto et al. 2017).

Strikingly, all studies, except for Ho et al. (2015) as they only used specific primer pairs for DNA detection, found genes of the ABC transporter family to be either present (as in *P. marina* (Hagemann et al. 2013)) or even highly overabundant as in *Prochlorococcus* spp. (Biller et al. 2014) or *Pseudomonas aeruginosa* (Bitto et al. 2017). My thesis adds now a fourth species, where four ABC transporter associated genes (belonging to the family of PstC and PstA) were overrepresented, although only under salt stress conditions. ABC- transporters are a large super family of important proteins that facilitate the delivery and uptake of several molecules in an ATP-dependent manner ((Higgins et al. 1986), for a review see (Davidson and Chen 2004)), but some are also crucial for the turnover of membranes (reviewed in (Ford and Beis 2019)). They are also important factors for bacteria to cope with salt stress. For example, a PstS phosphate binding protein was highly abundant in proteomic studies of *Synechocystis* sp. PCC 6803 that were grown in a similar salt stress setting as this study (684 mM NaCl vs. 700 mM NaCl my study) (Huang et al. 2006). Another three ATP transporters are, for example, important for providing acid tolerance in *Synechocystis* sp. PCC 6803 (Uchiyama et al. 2018). Although ABC transporters are very diverse (over 50 ABC transporter-related genes are known in *Synechocystis* sp. PCC 6803 (Uchiyama et al. 2018)) and are thus not unlikely to find in genomic studies, their important role in stress responses and the fact that two ABC transporters from distinct related bacteria (VacJ/Yrb) play a crucial role in OMV formation by influencing the distribution of phospholipids in the outer leaflet of outer membranes (Roier et al. 2016), renders ABC transporters an interesting target for future studies as they might represent genes that are more likely to be packed into vesicles.

7.6 Several routes might enable the loading of specific DNA sequences to vesicles

One intriguing question is, how DNA fragments of the chromosomal DNA are produced and end up in vesicles while the integrity of both, the cell and the chromosome, is retained. As *Synechocystis* sp. PCC 6803 can be highly polyploid (up to 140 chromosome copies (Griese et al. 2011; Zerulla et al. 2016)), depending on the particular strain and the specific growth phase, one could hypothesize that parts of or whole excess chromosomes might end up in vesicles. Indeed, OMVs of different species have been demonstrated to be quite stable under

different conditions and the observed DNA sequences might come from vesicles, accumulated over the time of the experiment, thus including the complete growth phase (*Biller et al. 2014; Fulsundar et al. 2014*).

It is important to know, that the bacterial chromosome exists in the cell in a highly-condensed fashion in a protein-DNA complex, the nucleoid, which is very unlikely to be the source of the differently sized DNA fragments in vesicles. *Biller et al. (2014)* linked their finding of the overabundance reads at the chromosomal terminus region to a yet unknown mechanism in the cell cycle (*Biller et al. 2014*). During the cell cycle, DNA is present in a relaxed state, making the appearance of DNA fragments more likely through several speculative mechanisms that I discuss in the following paragraph. For example, topoisomerases play a crucial role in relaxing super helix structures of DNA before the proteins involved in DNA replication can bind to the chromosome. Several proteins belong to this replication machinery, such as the initiator protein DnaA, DnaC, the helicase DnaB, single strand binding proteins, primase DnaG, DNA polymerases, and DNA ligases. DNA replication starts at the origin of replication (ORI), from which this complex moves along one strand and synthesizes a new bacterial chromosome, while the other strand is replicated via one to two kb sized Okazaki-fragments and a different DNA polymerase. Both replication forks meet at the terminus sequence at the chromosome (explained in more detail in (*Fuchs 2007*)). In a model, where free DNA is taken up from lysed cells (*Renelli et al. 2004*), one can hypothesize that DNA might be protected from degradation by extracellular DNases while covered with the above described replication machinery. An alternative explanation is that incomplete or erroneous replication might lead to freely diffusing DNA fragments of different sizes, due to a yet unknown mechanism, that are thus more likely to end up in vesicles. Also, in *E. coli* for example, a new round of replication starts before the prior round ended, thus genes at the ORI are more abundant in a cell (replication-associated gene dosage effect) (*Cooper and Helmstetter 1968; Rocha 2008*). This mechanism might enhance the probability of those genes in ending up in vesicles. However, in my thesis, the overexpressed genes are distributed across the whole chromosome and do not accumulate at a particular site (see Figure S 7). Moreover, *Biller et al. (2014)*, found an overrepresentation of reads not at the ORI, rather at the terminus region of the *Prochlorococcus* genome (*Biller et al. 2014*).

An alternative option for explaining the observation might be that during transcription, the bacterial chromosome is also present in its relaxed state (due to topoisomerases). RNA polymerases and sigma factors perform the transcription of DNA into different RNA species (*Fuchs 2007*). Notably, free RNA polymerases are scarce in the cell as most of these enzymes are needed for the transcription of genes that encode for proteins important for translation while others might be attached to the chromosomal DNA without exhibiting transcriptional activity (*Browning and Busby 2004*). Bacteria can thus adjust their gene expression under

different environmental conditions by routing the remaining RNA polymerases to the required genes through various mechanisms, such as different sigma factors or transcription factors (*Browning and Busby 2004*). Hence, the ABC transporters described above might be more likely to be transcribed into RNA molecules under salt stress conditions, thus might also be covered by the transcription machinery and consequently be protected from degradation in a similar way as speculated before for DNA replication which would enhance the probability of being packed into OMVs. Interestingly, Dorward et al. (1989) found not otherwise specified DNA binding proteins in OMVs of *Neisseria gonorrhoea*, a finding that might hint to such a mechanism (*Dorward and Garon 1989*). Moreover, I found one gene (MYO_RS14895) that is directly linked to translation to be overrepresented under salt stress conditions (see Table 13) and also Biller et al. (2014) found at least six genes that might be involved in translational processes (*Biller et al. 2014*). These results could also support the mechanism described above as those genes are also more likely to be covered by the transcription machinery, as they are highly transcribed throughout the cell cycle (reviewed in (*Rocha 2008*)).

Alternatively, some DNA fragments might be more abundant in a bacterial cell under specific conditions due to a process called gene amplification. Many bacteria are capable of increasing the copy number of genes by a transient duplication under particular stressful growth conditions and thus control gene dosages on a genome level (e.g. (*Nicoloff et al. 2019*) and reviewed in (*Reams and Roth 2015*)). These DNA stretches might be more likely to end up in OMVs, independent of an active vesiculation process by producing DNA-containing OMVs or just by vesiculation after cell death. However, those gene amplifications are often highly instable and thus challenging to detect (*Nicoloff et al. 2019*).

All above speculative alternatives would increase the stochastic probability of specific DNA fragments to be loaded into vesicles and might function in all currently proposed models of how DNA can be included into OMVs (*Renelli et al. 2004; Perez-Cruz et al. 2013; Pérez-Cruz et al. 2015; Turnbull et al. 2016*).

Table 14 Overview of putative mechanisms for DNA enrichment in OMVs described in 7.4 & 7.6

DNA type	Hypothetic mechanism
Plasmids	High copy numbers of small plasmids
	Small plasmid size is characterized by high diffusion properties
	Plasmids might be localized in condensed clusters
	Unknown mechanism connected with the replication mechanism
Overrepresented regions/genes	Excess chromosomes
	Replication proteins might protect unwinded DNA from nucleases
	Incomplete/ erroneous replication might lead to DNA fragments
	Transcription proteins cover DNA stretches and might protect from nucleases
	Gene amplification under stressful conditions

7.7 Conclusions and outlook

Freshwater cyanobacteria live in a diluted environment, which is under constant circulation. Producing OMVs as protective decoys, containing different cargo such as proteins/lipids or DNA as demonstrated in my thesis for freshwater cyanobacteria such as *Anabaena* sp. PCC 7120, *Chlorogloeopsis fritschii* sp. PCC 6912, and *Synechocystis* sp. PCC 6803, would offer a selective targeting mechanism by which the necessary effector concentrations could be significantly reduced.

I hypothesize that DNA cargo ends up in OMVs via mainly two routes. First, through the production of bilayered OMVs as described by Pérez-Cruz et al (2013, 2015), and second by vesicles forming after cell death for example under membrane stress such as NaCl and heat, supporting the model of Renelli et al. (2004) and Turnbull et al. (2016) (Renelli et al. 2004; Perez-Cruz et al. 2013; Pérez-Cruz et al. 2015; Turnbull et al. 2016). Although the actual loading of DNA into vesicles is more likely to occur in a stochastic manner due to putative higher abundances of specific DNA sequences such as small plasmids or genes by several putative mechanisms as summarized in Table 14, this nuclease-protected vesicle DNA might provide an additional way of maintaining plasmids in a population and/or provide a decoy for genes that might be important for the population. Producing more vesicles under stressful growth conditions as shown here for *Synechocystis* sp. PCC 6803 will lead to a high availability of OMV-derived and –protected DNA. Consequently, this could lead to a higher probability for the DNA bearing OMVs to transfer their genetic cargo to other cells (Hagemann et al. 2013). To what extent OMV mediated DNA transformation occurs in cyanobacteria, how effective this is, and how OMVs influence plasmid maintenance in a population are key questions for future studies.

8 Supplemental figures

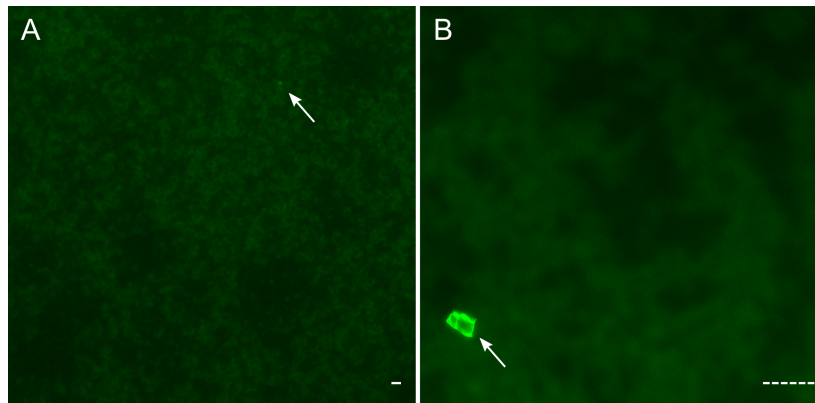


Figure S 1 SYBR® Gold staining from HEPES only. (A) shows an unspecific dot similar to OMVs. These unspecific dots were difficult to photograph since their fluorescence rapidly fades which was not the case for specific SYBR® Gold binding to nucleic acids. (B) displays a large particle with unspecific binding but clearly no OMVs or cells. Scale bars are 1 μm (A), 10 μm (B).

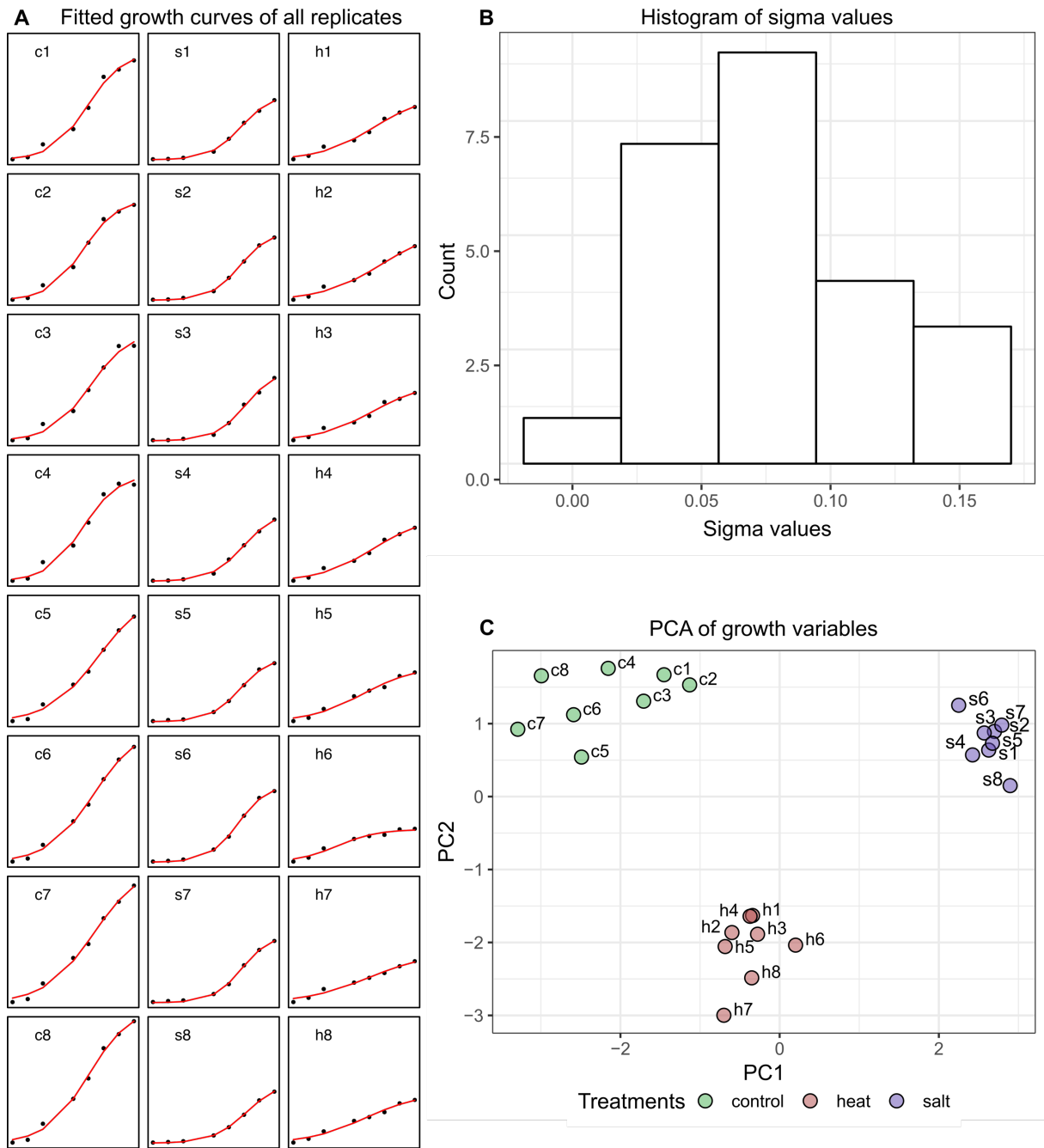


Figure S 2 Growth parameters and PCA analysis by R package “growthcurver”. (A) Fitted growth curves acquired by applying the logistic equation described in material & methods. (B) Histogram of sigma values. Sigma values are the residual sum of squares from the fit of the logistic fit. The sigma values here are small (meaning the fit is good). (C) Principal component analysis of all data, “growthcurver” calculates. It shows that replicates of the applied conditions cluster closely together, but the conditions themselves are grouped apart from each other.

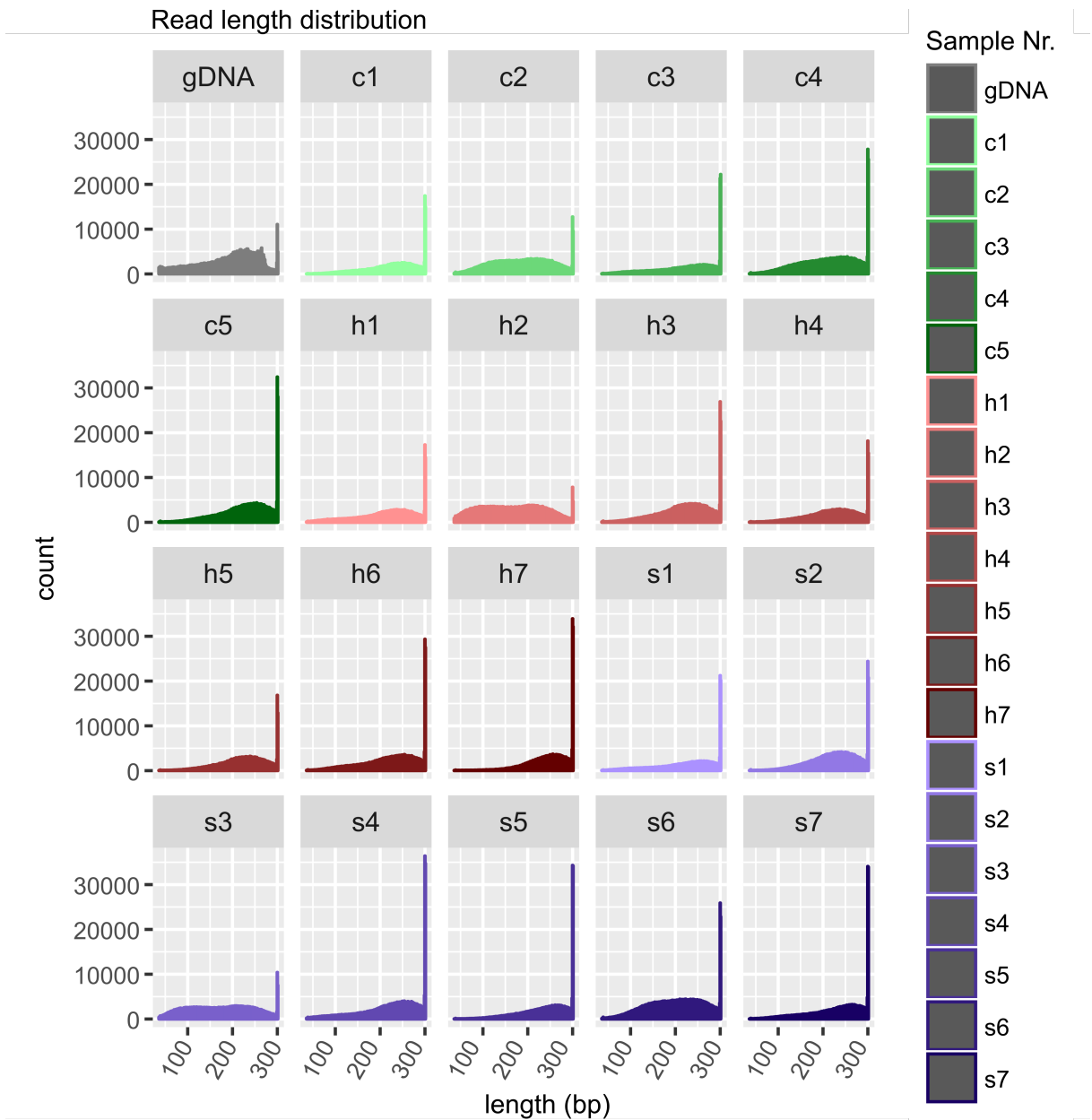


Figure S 3 Read length distribution. A histogram of the gDNA sample and all sequenced vesicle DNA samples from *Synechocystis sp. PCC 6803*. Most reads are the expected 300 bp long and only few replicates have a skewed length distribution (c2; h2; s3).

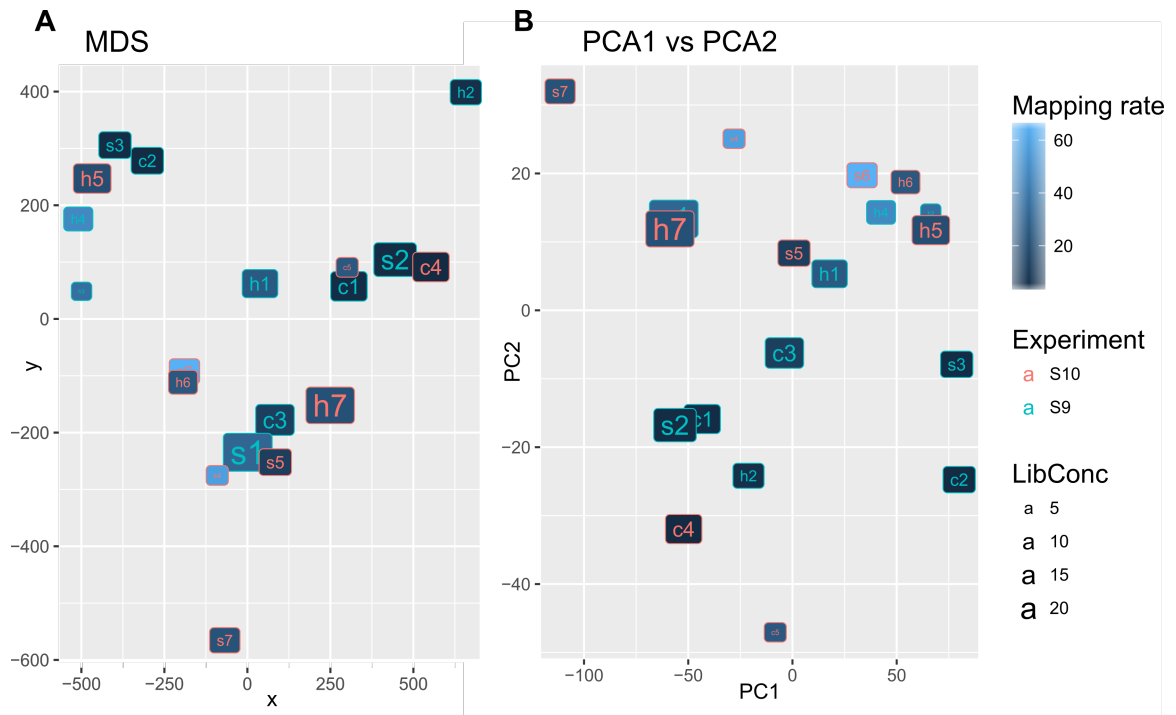


Figure S 4 Multidimensional scaling and principal component analysis on kmers of sequencing data from vesicle DNA from *Synechocystis* sp. PCC 6803, grown under stress conditions. To identify possible functional or systematic groupings of the samples, library concentration, isolation batch (experiment) and mapping rate against the *Synechocystis* sp. PCC 6803 genome was illustrated as size, label color, and fill color of the points. For sample abbreviations, refer to Table S 2.

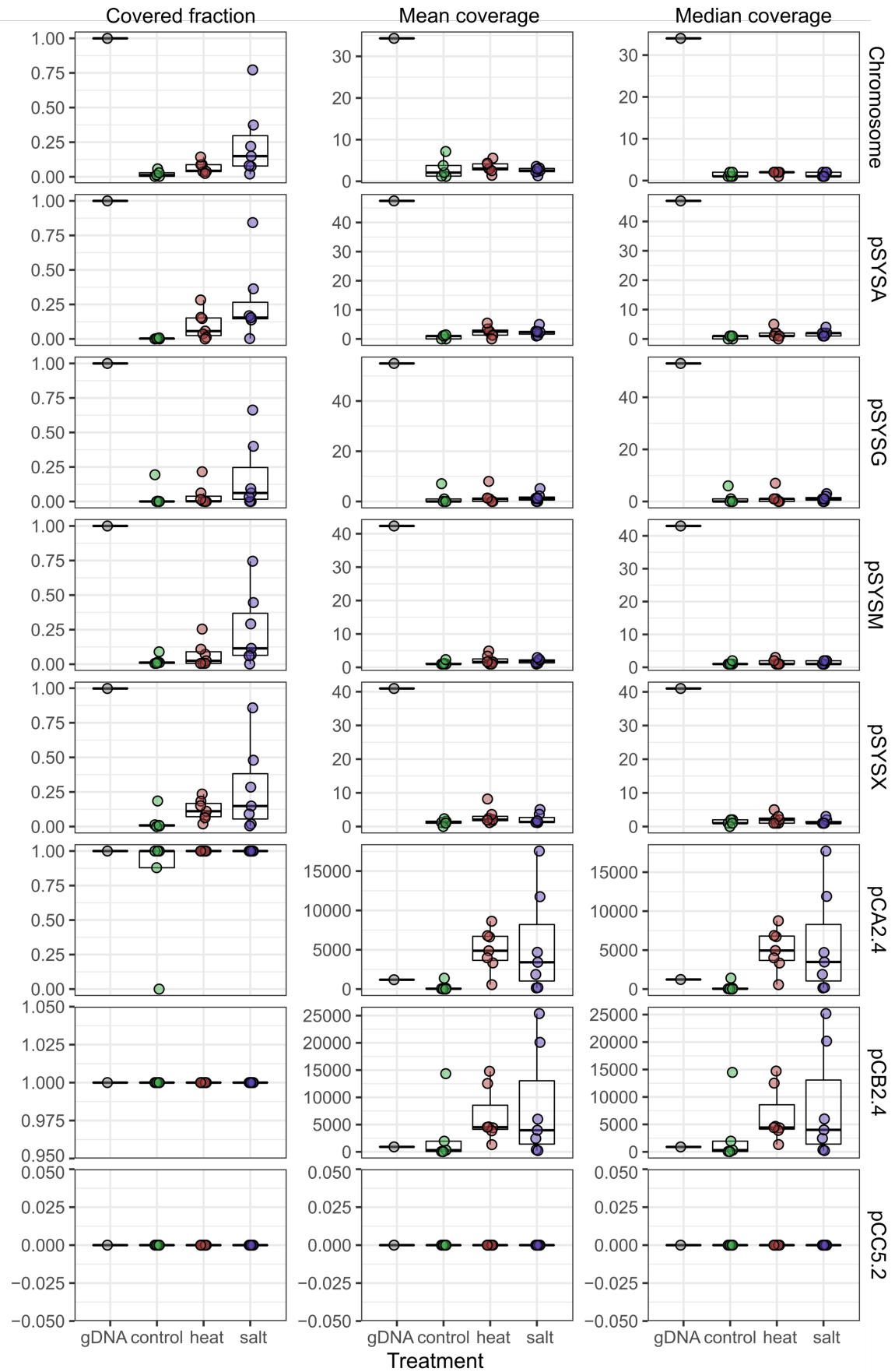


Figure S 5 General statistics for vesicle DNA of *Synechocystis* sp. PCC 6803 grown under different conditions and chromosomal DNA.

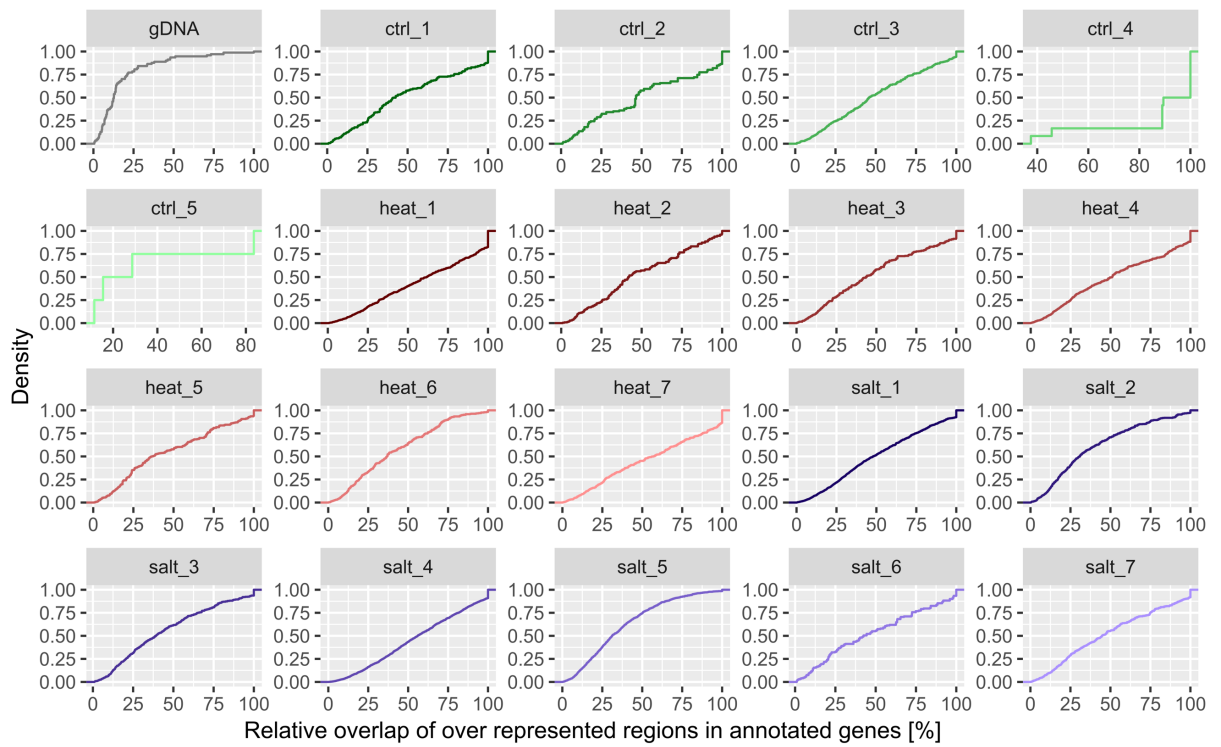


Figure S 6 Cumulative distribution function of relative overlap of the overrepresented regions on all annotated genes.

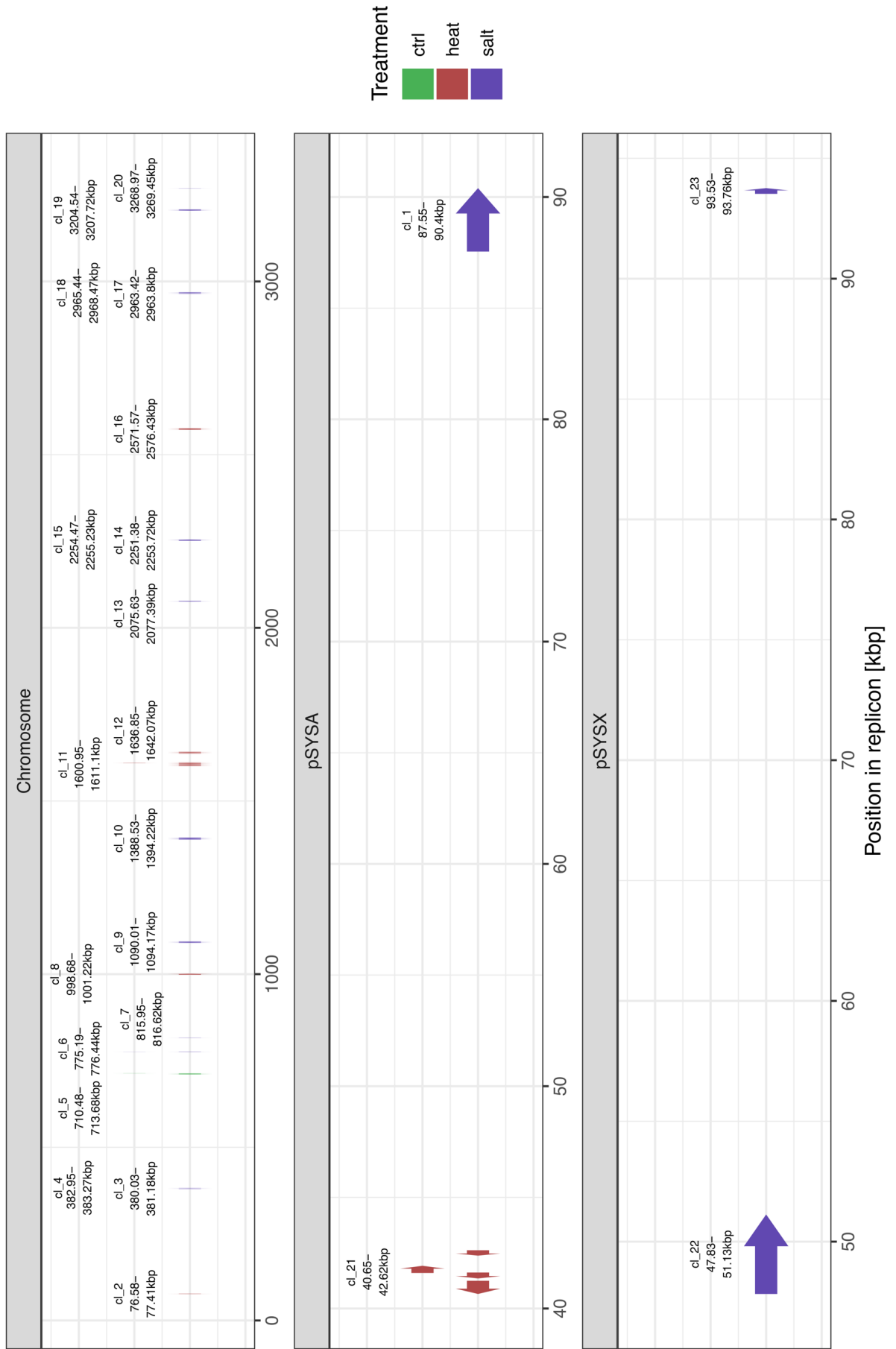


Figure S 7 Position of overrepresented genes on the chromosome, pSYSX, and pSYSY.

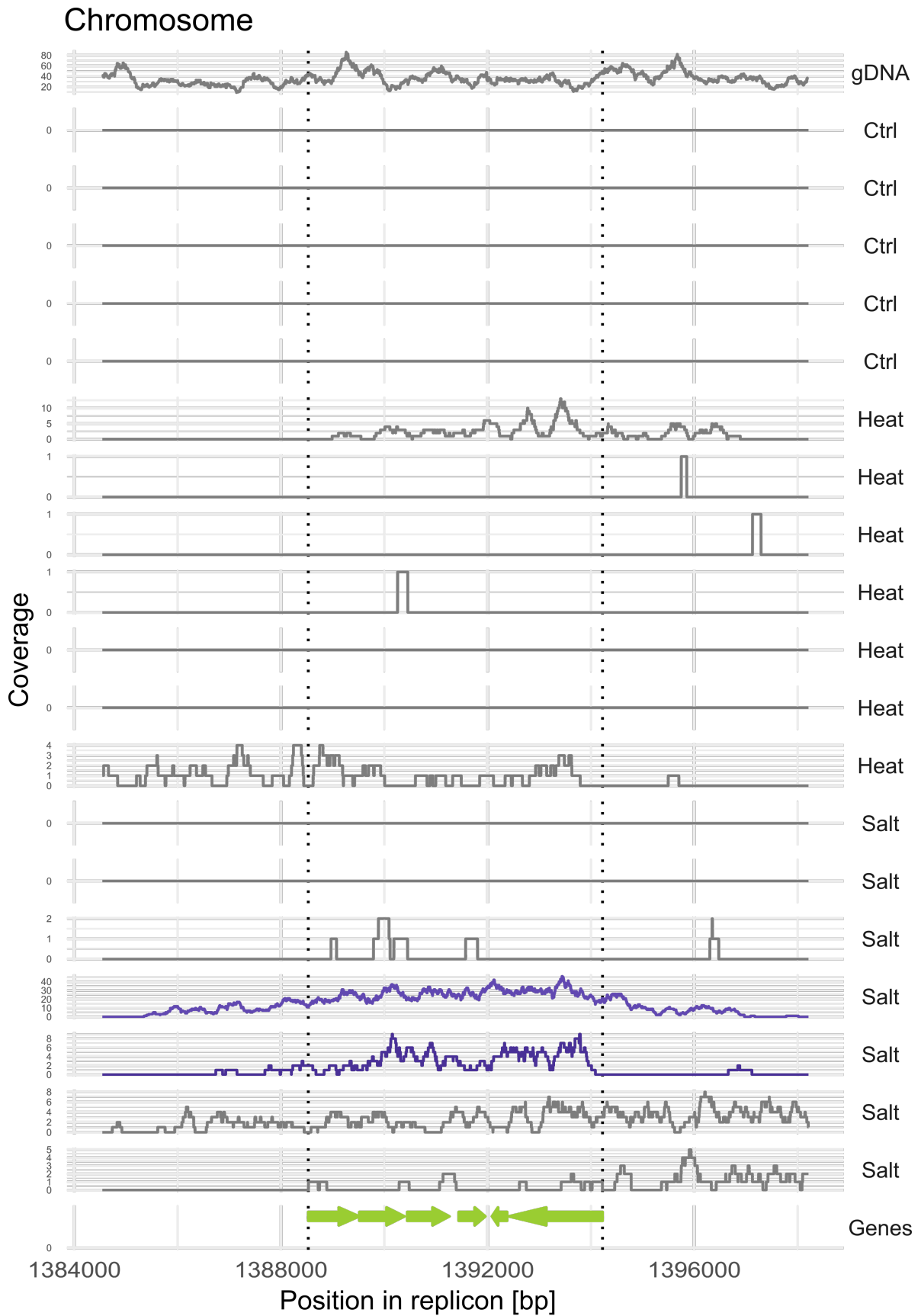


Figure S 8 Overrepresented cluster 10 plotted as line diagram. All sequenced samples at the position are shown. The y-axis displays the coverage of every sample. Blue color indicate that these two samples match the definition of overrepresented as explained in 6.3.7. Dotted vertical lines indicate the complete overrepresented cluster of 6 genes.

Table S 1 Summary of all processed OMV samples for stress conditions of *Synechocystis* sp. PCC 6803.

Treatment	Sequencing ID	Sample name	qubit DNA of OMVs (ng/μl) after conc.	library conc. (ng/μl)	total reads	reads after trimming (both)	forward only	reverse	Removed	Alignment rate (%)	Reads for analysis
control	G20145	S9_1	0.296	8.82	226957	106709	119900	88	260	1.37	226697
	G20146	S9_2	0.089	4.7	541607	84847	454822	117	1821	0.28	539786
	G20147	S9_4	0.095	11.1	223637	98424	124678	166	369	11.95	223268
	G20155	S10_1	0.174	8.2	512102	131761	379443	170	728	0.21	511374
	G20156	S10_3	0.253	1.19	398279	187828	207920	195	2336	25.95	395943
salt	G20148	S9_5	0.081	23.4	224483	98758	125248	85	392	33.17	224091
	G20149	S9_6	0.006	14.3	425027	133504	290796	122	605	1.23	424422
	G20150	S9_8	0.006	5.1	537227	74368	459583	88	3188	1.66	534039
	G20157	S10_5	0.006	1.33	397653	187405	208204	156	1888	62.54	395765
	G20158	S10_6	0.006	5.82	273216	142037	130724	126	329	11.63	272887
	G20159	S10_7	0.006	4.58	680416	122426	556173	133	1684	68.69	678732
	G20160	S10_8	0.006	4.02	306546	142772	163216	114	444	23.32	306102
heat	G20151	S9_9	0.076	6.88	291277	122135	168374	115	653	26.53	290624
	G20152	S9_10	0.075	4.14	707529	85401	614298	142	7688	3.35	699841
	G20153	S9_11	0.141	1.11	417622	181462	233642	191	2327	37.56	415295
	G20154	S9_12	0.132	2.9	279719	129873	149240	114	492	50.96	279227
	G20161	S10_9	0.141	7.8	283861	131165	152104	135	457	20.15	283404
	G20162	S10_10	0.084	3.3	367071	158891	207126	159	895	25.82	366176
	G20163	S10_12	0.087	20.6	264595	164772	99308	175	340	22.3	264255
genomic	gDNA				438942	333375	84908	11358	9301	96.73	429641

Non-sequenced samples

control	S9_3	OMVs contaminated
control	S10_2	Library preparation failed
control	S10_4	Library preparation failed
salt	S9_7	Library preparation failed
heat	S10_11	OMVs contaminated

Table S 2 Assignment from sample nr. to sample name

Sample name	Sample Nr.	Sample name	Sample Nr.	Sample name	Sample Nr.
S9_1	c1	S9_5	s1	S9_5	h1
S9_2	c2	S9_6	s2	S9_6	h2
S9_4	c3	S9_8	s3	S9_8	h3
S10_1	c4	S10_5	s4	S10_5	h4
S10_3	c5	S10_6	s5	S10_6	h5
		S10_7	s6	S10_7	h6
		S10_8	s7	S10_8	h7

Table S 3 DNase I activity test. Genomic DNA samples were either digested twice with 2U of DNase I for 30 min (+) or used directly for qPCR. The experiment was conducted with three genomic samples and qPCR was performed in duplicates. Primers for the small plasmids pCA2.4, pCB2.4, and the chromosomal gene rnpB were used to detect DNA in the samples (For primers see **Table 3**). Cq values over 35 are considered to be unspecific amplifications as no products were detected (N.d. = not detectable).

target region	gene/	DNA conc. [ng/μl]	DNase I	mean Cq value	melt temp. [°C]
pCA2.4		0.5	+	N.d.	N.d.
		5	+	39.5	N.d.
		0.5	-	15.9	81.5
		5	-	12.5	81.5
pCB2.4		0.5	+	38.1	N.d.
		5	+	39.0	N.d.
		0.5	-	15.9	83
		5	-	12.3	83
rnpB		0.5	+	39.4	N.d.
		5	+	N.d.	N.d.
		0.5	-	18.3	80
		5	-	14.9	80

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10 List of figures

10.1 Main figures

- Figure 1** Schematic drawing of cyanobacteria from Section (Sec.) I – IV with generic morphologies. a & d: heterocyst, b: hormogonium, c: akinets, e: trichome, f: necridia. Sec. V inspired by <https://www.spektrum.de/lexikon/biologie/fischerella/24614>. 6
- Figure 2** Light microscopic images of cyanobacteria used in this study. (A) *Synechocystis* sp. PCC 6803, (B) *Anabaena* sp. 7120, (C) *Chlorogloeopsis fritschii* 6912. Image credits B & C: Dr. Karina Stucken. 8
- Figure 3** Schematic drawing of the Gram(-) cell wall by (Schwechheimer and Kuehn 2015). The cytosolic membrane consists of two leaflets of mainly phospholipids being almost non-permable except for transmembrane proteins that can act as transporters (blue, purple). The outer membrane consists of an inner leaflet comprising of phospholipids and an outer leaflet that consists of lipidopolysaccharides. A peptidoglycan-layer provides stability and resistance against osmotic pressure changes and comprises typically of N-acetylglucosamine and N-acetylmuramic acid that are linked via 1,4-beta- glycosidic bindings and are crosslinked via tetrapeptides binding to a lactyl-residue at the disaccharides. The outer membrane is relatively permable due to porins (OmpA, OmpC, OmpF). These porins are non-covalently linked to the PG layer, while the Tol-Pal complex and the periplasmic protein Lpp bind covalently to the peptidoglycans, all together providing more stability to the bacterial cell. 11
- Figure 4** Schematic OMV production model of Gram (-) bacteria. The bacterial envelope consists of the cytosolic membrane with a phospholipid bilayer, a periplasmic space as well as its peptidoglycan (PG) net, and an outer membrane (OM). The OM consists of an inner leaflet with phospholipids and an outer leaflet with mainly lipopolysaccharides (LPS), and porin proteins such as OmpA or OmpC/F. The layers are interconnected by covalent (Lpp-PG) and non-covalent crosslinks (Tol-Pal, OmpA-PG). The bulging of vesicles can occur at sites of fewer crosslinks where cargo can accumulate and eventually fission takes place. Cartoon taken from (Schwechheimer et al. 2013). 14
- Figure 5 Outer membrane vesicles in *Synechocystis* sp. PCC 6803.** (A) TEM image of outer membrane vesicles isolated from the cell free supernatant of *Synechocystis* sp. PCC 6803 of different sizes between 20 to 150 nm (black arrows). Some of the vesicles show an electron dense luminal content (white arrows). (B) DNase I digested and glutaraldehyde fixed OMVs stained with SYBR® Gold. Green dots are fluorescent nucleic acids in vesicles (white arrows). (C) TEM image of a freshly formed vesicle (black arrow) and the detachment point on the cellular site (black arrow head. Scale bars are 100 nm in (A; C) and 1µm in (B). 37
- Figure 6 Outer membrane vesicles in the section IV cyanobacterium *Scytonema hofmanni* PCC 7110.** (A) Negative stained TEM image of purified outer membrane vesicles. The black arrow shows an example of a typically shaped OMV with electron dense luminal content. (B) Vesicle like structures with an atypical rod-shaped morphology and a septum in the center (black arrows). Additionally, round particles, half the size of the rod-shaped entities could be detected (white arrows). Scale bars are 100 nm. 38
- Figure 7 Outer membrane vesicles in the section IV cyanobacterium *Anabaena* sp. PCC 7120.** (A) Negative stained TEM image of purified outer membrane vesicles of different sizes surrounded by a single membrane (white arrows) or a bilayer membrane (black arrows). (B) DNase I digested and glutaraldehyde fixed vesicles stained with SYBR® Gold. Green dots represent fluorescent DNA in vesicles. Scale bar in (A) is 100 nm and in (B) represents 1µm. 39
- Figure 8 Particles in *Fischerella muscicola* PCC 7414.** (A) No outer membrane vesicles could be detected in the section V cyanobacterium *Fischerella muscicola* PCC 7414 by transmission electron microscopy. However, some extremely small rod-shaped particles of approximately 10 nm could be detected. (B) SYBR® Gold staining revealed round

particles containing nucleic acids (green dots). Scale bar in (A) represents 100 nm and in (B) 1 μ m. 40

Figure 9 Particles in *Fischerella thermalis* PCC 7521. (A) Vesicle like structures with an atypical rod-shaped morphology and a septum in the center (arrows) in the supernatant of *Fischerella thermalis* PCC 7521 by transmission electron microscopy with a size of 80 to 100 nm. Additionally, round particles, half the size of the rod-shaped ones could be detected (arrow heads). (B) TEM image of rod-shaped particles of 80-100 nm with a septum (arrows) and round particles of approximately 40 nm (arrow heads) directly embedded in the exopolysaccharide layer of the bacteria. (C) DNase I digested and glutaraldehyde fixed vesicles stained with SYBR® Gold. Green dots represent fluorescent DNA in the particles. Scale bars in (A) and (B) represent 100 nm and in (C) 1 μ m. 40

Figure 10 Outer membrane vesicles and putative phages in *Chlorogloeopsis fritschii* PCC 6912. (A) *Chlorogloeopsis fritschii* PCC 6912 shows few differently sized outer membrane vesicles (white arrows) and many phage heads with electron dense luminal content (black arrows) and tail structures (black arrow heads). (B) An enlarged TEM image of a phage head (black arrow). The arrow head hints to a putative OMV with electron dense luminal content. Scale bar (A) is 200 nm and (B) is 100 nm. 41

Figure 11 Coverage per base pair of vesicle DNA, isolated from four *Synechocystis* sp. PCC 6803 samples over all replicons presented as heatmap. Colors represent higher coverage in log₁₀ scale from blue (low) to red (high). 43

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12 Data repository

Raw data from this thesis can be found on a SD-card attached to this submission. The following files are included:

- DNaseI-Test qPCR raw data
- OMV quantification
 1. Lipidassay_s9_s10
 2. Proteinassay_s9_s10
- PCN
 1. Auswertung_PCN_S9_s10_gDNA
 2. Zusammenfassung_PCN_gDNA_S9_S10
- Sequencing
 1. 6803_pilot: raw sequences + coverage matrix from mapping on reference genome
 2. 6803_stress_conditions: raw sequences + coverage matrix from mapping on reference genome + overrepresented genomic regions
 3. 6912_pilot: raw sequences + coverage matrix from mapping on reference genome
 4. 7120_pilot: raw sequences + coverage matrix from mapping on reference genome
- stress conditions
 1. 2017-04-04_qpcr_Stress6,7,8_apHII_hspA_clpb1 (original qPCR files)
 2. 6803_dnaconc_libconc_reads_align.csv
 3. growth_s9_s10.csv
 4. growth_stress_pre
- TEM and SYBR images
 1. 2014-07_1 OMV 6803, 7120, 7414, 7521, 6912
 2. 2014-08 OMV 7110, 7120
 3. 2014-09 TEM 6803 whole cell AE TEC02607 H10
 4. 2015-05 OMVs 7120_5
 5. 2018-01_TEM-OMVs-S9_S10
 6. 6803_sybr-gold-staining
 7. C6803 D5.tif
 8. C6803 D5a.tif
 9. C6803 D5b.tif
 10. C6803 D5c.tif

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