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DOUTORAMENTO EM BIOLOGIA MOLECULAR E CELULAR

CyanoEV: characterizing Cyanobacterial Extracellular Vesicles, envisioning technological applications

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**CYANOEV: CHARACTERIZING CYANOBACTERIAL
EXTRACELLULAR VESICLES, ENVISIONING TECHNOLOGICAL
APPLICATIONS**

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“P.S. please if you get a chance put some flowers on Algernons grave in the bak yard.”

— Charlie

Flowers for Algernon, from Daniel Keyes

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List of Publications

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List of Abbreviations

AHLs	<i>N</i> -acyl homoserine lactones
ATP	Adenosine triphosphate
bp	Base pairs
CRISPRi	Clustered Regularly Interspaced Short Palindromic Repeats interference
DAP	diaminopimelic acid
DGDG	Diglycosyl diacylglycerol
DGU	Density gradient ultracentrifugation
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EOMVs	Explosive outer membrane vesicles
ETEC	Enterotoxigenic <i>E. coli</i>
EV	Extracellular vesicle
Ga	gigayears
GMOs	Genetically modified organisms
GTP	Guanosine triphosphate
HGT	Horizontal Gene Transfer
HME	Heavy metal efflux
IM	Inner membrane
ISEV	International Society for Extracellular Vesicles
Lpp	Lipoproteins
LPS	Lipopolysaccharides
ME	Membrana externa
MGDG	Monoglycosyl diacylglycerol
MISEV	Minimal Information for Studies of Extracellular Vesicles
MVBs	Multivesicular bodies
MVs	Membrane vesicles
MWCO	Molecular Weight Cut-Off
nt	nucleotides
NTA	Nanoparticle tracking analysis
OD	optical density
OIMVs	Outer-inner membrane vesicles
OM	Outer membrane
OmpA	Outer membrane protein A

OMVs Outer membrane vesicles
OprB Carbohydrate selective porin OprB
PAGE Polyacrylamide gel electrophoresis
PAMPs Pathogen-associated molecular patterns
PBS Phosphatebuffered saline
PCC Pasteur Culture collection of Cyanobacteria
PES polyethersulfone
PG Peptidoglycan
pMVs Primordial membrane vesicles
PNEV Portuguese Network on Extracellular Vesicles
PQS *Pseudomonas* quinolone signal
PRRs Pattern recognition receptors
PTE Phosphotriesterase
PUL polysaccharide utilization loci
RBS Ribosome binding site
RNA Ribonucleic acid
ROS Reactive oxygen species
rRNA Ribosomal ribonucleic acid
RT room temperature
RT-qPCR Reverse transcription quantitative polymerase chain reaction
RuBisCO Ribulose-1,5-bisphosphate carboxylase/oxygenase
SC SpyCatcher
SDS Sodium dodecyl sulfate
Sec General secretory
SEC Size-exclusion chromatography
sfGFP Super folder Green Fluorescent Protein
siRNA small interfering RNA
S-layer surface layer
SLH S-layer homology
sp. Species
spp. Species pluralis
SQDG Sulfoquinovosyl diacylglycerol
ST Spy-Tag
Tat Twin-arginine translocation
TEM Transmission electron microscopy
TFF tangential flow filtration
TLRs Toll-like receptors

tRNA Transfer ribonucleic acid

TSMs Tube-shaped membranous structures

UV ultraviolet

VEs Vesículas extracelulares

WT Wild type

Abstract

Extracellular vesicles (EVs) are non-replicable proteoliposome nanostructures. The release of EVs is a transversal, but still overlooked, mechanism of secretion. These nanostructures can traffic biologically active molecules to distant targets in a concentrated, protected and targeted manner. Cyanobacteria are a relevant group of Gram-negative prokaryotes, which remarkably pioneered oxygenic photosynthesis. Knowledge on cyanobacterial EVs lags far behind compared to that of other bacteria. The goal of this work was therefore to study cyanobacterial EVs, addressing fundamental aspects of their biogenesis, composition and biological roles. Therefore, a solid and reproducible protocol for isolating and characterizing cyanobacterial EVs was established, namely for *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). This well-characterized unicellular cyanobacterium, known as the “Green *E. coli*”, was the model organism used throughout this study.

Basic protein analysis of *Synechocystis* EVs revealed a group of outer membrane (OM) proteins. These are SLH/OprB porins that interact with the peptidoglycan, suggesting a link between this layer and the OM. *In silico* analysis confirmed a total of six genes encoding these porins in the genome of *Synechocystis*. Using a genetic-based approach, five single deletion mutants were successfully generated, whereas one gene was found to encode an essential protein (Slr1841), under the tested conditions. Among the remaining mutants, Δ slr1908, the mutant deleted in the gene encoding the second most abundant OM porin, produced more vesicles and presented alterations in iron uptake. Hence, in addition to their classical function as porins allowing inorganic ions diffusion, these proteins may play a role in OM structural stability and vesiculation.

Identification of a copper binding metallochaperone, CopM, in *Synechocystis* EVs released from a copper-sensitive hyper-vesiculating strain, Δ tolC, initiated the investigation of EVs as a novel and alternative copper-secretion mechanism. Metal homeostasis is crucial for optimal performance of cell metabolic pathways. When exposed to growth-challenging copper concentrations, Gram-negative bacteria quickly trigger copper-detoxification mechanisms. Along the activation of classical-detoxification systems, this study demonstrates that copper-stressed cells release EVs loaded with CopM. Analyses of Δ tolC EVs isolated from cells cultivated under standard conditions indicated the presence of copper therein, in significantly higher levels as compared to those from wild-type EVs. Remarkably, increased vesiculation upon exposure to high levels of copper occurs in bacteria inhabiting completely different ecological niches, as determined for *Escherichia coli* and *Pseudomonas aeruginosa*. Altogether, vesiculation appears to be a common bacterial

response mechanism in transporting copper out of the cell, playing a role in copper-homeostasis.

The first steps to tweak cyanobacteria to load a protein of interest into EVs were taken. As proof of concept, the super folder Green Fluorescent Protein (sfGFP) reporter was used. A construct was assembled holding a strong, heterologous, synthetic promoter, and a native signal peptide, which targeted sfGFP to the *Synechocystis* periplasm. Characterization of EVs isolated from fully-segregated mutants revealed the presence of sfGFP therein. Accordingly, the use of *Synechocystis* EVs as protein carriers systems was tested, namely for fish. *Synechocystis* EVs were shown to be biocompatible with zebrafish larvae, neither inducing mortality nor significant inflammatory responses. Remarkably, upon immersion treatment with sfGFP-packed *Synechocystis* EVs, an accumulation of fluorescent signal could be detected in the gastrointestinal tract of zebrafish larvae. This work cracks open the potential of *Synechocystis* EVs for carrying proteins/enzymes to fish, potentially modulating their nutritional status or stimulating specific adaptive immune responses.

Altogether, this thesis is a showcase of cyanobacterial EVs biology and biotechnology, paving the way for future work towards the clarification of other possible biological roles and as customized and targeted protein-carrier systems.

Keywords: Extracellular vesicles; Secretion; Cyanobacteria; *Synechocystis*; SLH/OprB porins; Metal homeostasis; Copper secretion; Protein carrier.

Resumo

Vesículas extracelulares (VEs) são nano-estruturas proteolipossômicas não replicáveis. A libertação de VEs representa um mecanismo de secreção transversal que ainda é negligenciado. Estas nano-estruturas podem transportar moléculas biologicamente activas para alvos distantes de forma concentrada, protegida e direcionada. As cianobactérias são um grupo de procariontes Gram-negativos relevantes, pioneiros da fotossíntese oxigénica. O conhecimento sobre VEs de cianobactérias fica muito aquém comparativamente a outras espécies bacterianas. O objetivo deste trabalho foi estudar VEs de cianobactérias, abordando aspetos fundamentais sobre a sua biogénese, composição e relevância fisiológica. Para o efeito, foi estabelecido um protocolo para isolar e caracterizar VEs de cianobactérias, nomeadamente para *Synechocystis* sp. PCC 6803 (daqui em diante *Synechocystis*). Esta é uma cianobactéria unicelular bem caracterizada, conhecida como a “*E. coli* verde”, e foi o organismo modelo utilizado ao longo deste estudo.

Análises proteicas básicas de VEs de *Synechocystis* revelaram um grupo de proteínas de membrana externa (ME). Essas proteínas são porinas SLH/OprB que interagem com o peptidoglicano, sugerindo uma ligação entre esta camada e a ME. A análise *in silico* confirmou um total de seis genes que codificam essas porinas no genoma de *Synechocystis*. Usando uma abordagem de base genética, cinco mutantes de deleção única foram gerados com sucesso, enquanto um destes genes codifica uma proteína essencial (Slr1841) para a célula, nas condições testadas. Entre os restantes mutantes, $\Delta\text{Slr1908}$, o mutante no qual se deletou o gene que codifica a segunda porina mais abundante da ME, produziu não só mais vesículas como apresentou alterações na captação de ferro. Assim, além da sua função clássica como porinas permitindo a difusão de iões inorgânicos, aqui relatamos que essas proteínas também podem desempenhar um papel na estabilidade estrutural da própria ME e na vesiculação.

A identificação de uma metalochaperona que se liga ao cobre, CopM, em VEs de *Synechocystis* produzidas por uma estirpe super-produtora de vesículas e sensível ao cobre, ΔtolC , iniciou a investigação das VEs como um mecanismo novo e alternativo para a secreção de cobre. A homeostasia de metais é crucial para o bom desempenho das vias metabólicas celulares. Quando expostas a concentrações de cobre que desafiam o seu crescimento, as bactérias Gram-negativas despoletam rapidamente mecanismos de desintoxicação de cobre. A par da activação de sistemas clássicos de desintoxicação, este estudo demonstra que células stressadas com cobre libertam VEs carregadas com CopM. Análises de VEs isoladas de células cultivadas em condições padrão revelaram a presença de cobre em vesículas da estirpe ΔtolC , em níveis significativamente superiores

comparando com VEs isoladas da estirpe selvagem. Notavelmente, o aumento da vesiculação após exposição a altos níveis de cobre ocorre em bactérias que habitam nichos ecológicos completamente diferentes, como *Escherichia coli* e *Pseudomonas aeruginosa*. Englobando tudo, sugere-se que a vesiculação é um mecanismo de resposta comum em bactérias para transportar cobre para fora da célula, desempenhando assim um papel na homeostasia do cobre.

Os primeiros passos para carregar uma proteína de interesse em VEs de cianobactérias foram efectuados. Como prova de conceito foi utilizada a proteína repórter sfGFP. Um constructo foi gerado possuindo um promotor forte, heterólogo, e um péptido sinal nativo, que direciona a sfGFP para o periplasma de *Synechocystis*. A caracterização de VEs isoladas de mutantes completamente segregados revelou a presença de sfGFP. Nesse sentido, foi abordada a utilização de VEs de *Synechocystis* como novos sistemas transportadores de proteínas, nomeadamente para peixes. Aqui, demonstra-se não só que as VEs são biocompatíveis com as larvas do peixe-zebra, assim como não induzem mortalidade ou respostas inflamatórias significativas. Notavelmente, após tratamento de imersão com VEs de *Synechocystis* carregadas com sfGFP, observa-se uma acumulação de vesículas no trato gastrointestinal de larvas de peixe-zebra. Este trabalho revela o potencial de VEs de *Synechocystis* para transportar proteínas/enzimas em peixes, potencialmente para modular o seu estado nutricional ou estimular respostas imunes adaptativas específicas.

Ao todo, esta tese é um mostruário sobre biologia e biotecnologia de VEs de cianobactérias, abrindo o caminho para futuros trabalhos para o esclarecimento de outros possíveis papéis biológicos e ao uso de VEs como sistemas de transporte personalizados e direcionados de proteínas.

Palavras-chave: Vesículas extracelulares; Secreção; Cianobactérias; *Synechocystis*; Porinas SLH/OprB; Homeostasia de metais; Secreção de cobre; Transporte de proteínas.

CHAPTER I – Introduction



1. Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are nanostructures actively secreted by cells from all domains of life (Deatherage & Cookson, 2012). Protected by a lipid bilayer and unable to replicate, EVs are implicated in a myriad of biological functions, as secretion and communication. Here, the topic of EVs is introduced and a broad overview of the field presented, emphasizing on bacterial EVs from Gram-negative bacteria and their potential for bioengineering.

1.1 EVs as fossils of the origin of life

What is life? How did it emerge and develop? Could the original formula that gave rise to life be replicated? Particularly since the *Age of Enlightenment*, these are undeniably among the most intriguing questions ever concocted by curiosity. Memorable scientists, from the French naturalist Buffon (1707–1788) to the geneticist Haldane (1892–1964) and biochemist Oparin (1894–1980), passing through Lamarck (1744–1829), Darwin (1809–1882) and Huxley (1825–1895), all devoted part of their attention to articulate plausible hypotheses to answer them (Tirard, 2010). Present-day researchers on the origin of life, armed with state-of-the-art technology, continue this quest. Here, a brief overview on life genesis is addressed, with a focus on the relevance of the membrane, as the ultimate frontier able to package life.

Albeit not decisive for research purposes, finding a unified definition for life was deemed beneficial. According to its working definition, life is “a self-sustaining chemical system capable of Darwinian evolution” (Joyce, 1994). Thus, molecules able to self-replicate were suggested to fulfil a key role for the origin of life. RNA is one of such molecules (Mizuuchi *et al.*, 2022). Indeed, under the RNA world hypothesis, RNA acts simultaneously as molecule of inheritance capable of evolution, and biocatalyst necessary for its replication and growth (Bernhardt, 2012). However, a cell membrane is still required to perform essential homeostatic, metabolic and motility functions (Cantine & Fournier, 2018). In agreement, a Lipid world theory was proposed in which protocells, developed in a prebiotic environment abundant in lipids, were shaped from natural occurring primordial membrane vesicles (pMV) (Segré *et al.*, 2001).

Fatty acids-rich pMV could have displayed the mandatory compartmentalization for nascent cells to concentrate key prebiotic chemical components, enabling natural selection to follow (Szostak *et al.*, 2001). Moreover, as lipid vesicles were shown to be stable between 0 and 100 degrees Celsius, these temperature variations were suggested to occur in pMV, supporting the occurrence of initially distinct cellular processes (Mansy & Szostak, 2008). For instance, RNA primitive replication could have benefited from these temperature

variations, performing strand separation at higher temperatures and a template-copying mechanism at lower temperatures (Mansy & Szostak, 2008). Also, improved nutrient uptake at elevated temperatures, due to increased pMV's fluidity, could have augmented the lumen concentration of relevant molecules, revealing an incipient membrane selectivity (Mansy & Szostak, 2008). How this equilibrium was maintained remains puzzling. In addition, under particular growing conditions, lipid vesicles exhibit fission behaviour, pointing towards a rudimentary self-sustaining capacity (Svetina, 2009). Theoretically, this mock self-reproducing mechanism allowed transference of compositional information to the sibling vesicles, as a similar membrane and lumen content are inherited from the parent vesicle. Therefore, pMV's were suggested to be tightly coordinated with the development of protocells and their gradually evolving metabolism. These pMV's are the best candidates as original vessels that concentrated and dramatically increased the chances for life to occur.

During the course of evolution, membrane vesicles (MV's) continued to be involved in relevant biological roles, such as cellular compartmentalization (e.g. organelles), storage (e.g. spatially limited accumulation of energetically favourable compounds) and molecular transit (e.g. endocytosis and exocytosis). Indeed, part of the intracellular (e.g. transport via lysosomes) and intercellular [e.g. secretion of extracellular vesicles (EV's)] trafficking are MV's-mediated processes (Fernández-Trillo *et al.*, 2017).

Interestingly, EV's release is ubiquitous to all domains of life (Deatherage & Cookson, 2012). They invariably present a similar architecture – a lipid membrane limiting a soluble lumen. EV's are regarded as a non-classical secretion route, by which organisms can exchange both soluble and insoluble material with their environment.

This chapter will now focus on a historical perspective of the EV's field as a way to position and lead the reader through this incredible journey that started more than 80 years ago. Afterwards, the spotlight will move on to bacterial EV's, mainly derived from Gram-negative bacteria. All paving the way to introduce a relevant evolutionary group of Gram-negative oxygenic photosynthetic prokaryotes – Cyanobacteria – and their EV's.

1.2 Storytelling overview of the field

Today, extracellular vesicles are commonly regarded as non-replicating nanostructures that are delimited by a lipid bilayer (Théry *et al.*, 2018). Nevertheless, this consensual and standard concept of EV's was challenging to define (Couch *et al.*, 2021). It is necessary to grasp the full history behind this designation to comprehend the present mentality in the field. To do so, we need to board on a past time travel, going beyond the bacterial EV's niche area.

The first unknowingly isolations of EVs were performed in the 1940s in the eukaryotic field, with studies of blood coagulation by the duo Chargaff and West (Chargaff, 1945; Chargaff & West, 1946). To evaluate the effect of high speed centrifugation on clotting time of human plasma, Chargaff enigmatically observed that increasing the centrifugation speed and adding the resultant pellet to the supernatant plasma shortened considerably the clotting time (Chargaff, 1945). Shortly after, they unveiled a 'particulate fraction', pelleted at 31,000 *g*, possessing high clotting potential. They went on suggesting that it included a myriad of sub-micron size "blood corpuscles" (Chargaff & West, 1946). However, these reports did not trigger the attention of the scientific community. Almost 20 years passed until Peter Wolf described a similar particulate material originated from platelets and similarly obtained by high-speed centrifugation – what is referred now to as the extracellular vesicle (EV) fraction (Wolf, 1967). Analysing it by transmission electron microscopy, Wolf labelled what he saw as "platelet dust" (Wolf, 1967). In fact, "EVs" as a catch-all term is frankly recent but follows the International Society for Extracellular Vesicles (ISEV) implemented guidelines (Théry *et al.*, 2018). As the field developed, various types of EVs were discovered, being clear the necessity for a precise nomenclature. These names are based on biogenesis pathways, being exosomes and microvesicles (or ectosomes) the most famous. Within the current Minimal Information for Studies of EVs (MISEV) recommendations, the term 'exosome' is used to describe vesicles released following the fusion of multivesicular bodies (MVBs) with the plasma membrane, while "microvesicle/ectosomes" refers to vesicles that are produced directly by outward budding at the plasma membrane.

Practically at the same time that Wolf was studying "platelet dust", bacterial EVs were, for the first time ever, documented (Knox *et al.*, 1966). It should be highlighted that, in earlier bacterial ultrastructural works, EVs were already distinguishable, but not clearly reported [e.g. (Bladen & Waters, 1963)]. The original observation undoubtedly pointing to bacterial EVs as differentiate "particles" were performed by Knox and colleagues in *Escherichia coli* (Knox *et al.*, 1966). In this work, transmission electron micrographs depicting the cell wall ultrastructure seemed to indicate detachment of nanospherical structures characterized by a single membrane surrounding an electron-dense centre. It was suggested that these particles participated in the secretion of lipopolysaccharides (LPS) and lipoproteins (Lpp). More observations quickly followed, namely in the oral bacterium *Veillonella parvula* (Mergenhagen *et al.*, 1966) and in the human pathogen *Vibrio cholera* (Chatterjee & Das, 1967). Interestingly, *V. cholera* released EVs possessing toxins, mainly during its log growth phase (Chatterjee & Das, 1967). Despite these promising observations, bacterial EVs were primarily disregarded, considered as mere artefacts of growth and deemed not relevant for

bacteria (Zavan *et al.*, 2020). It took almost ten years and the identification of “extracellular membranous material” in the spinal fluid of meningococcal patients (Devoe & Gilchrist, 1973, 1975) to ignite definitely the interest on bacterial EVs. Again, the hypothesis was that *Neisseria meningitidis* EVs were enriched in toxins, and produced in the log phase. At least in the context of pathogenesis, it seemed imperative to understand bacterial EVs’ formation processes, their functions within the host and how they benefited bacteria. Today, EVs are accepted as part of the normal bacterial functioning, as a continuous and fundamental homeostatic secretory mechanism, and are crucial in various physiologically relevant settings (Zavan *et al.*, 2020).

Bacterial EVs nomenclature is equally controversial. It is worthwhile mentioning that, in 1976, as these nanostructures seemed to derive exclusively from the outer membrane (OM) of the Gram-negative cell wall, they were proposed to be named outer membrane vesicles (OMVs) (Hoekstra *et al.*, 1976). The term OMVs was coined then and it prevailed, as most of the bacterial field advanced for many years on top of that terminology. Nevertheless, and like their eukaryotic counterparts, recent findings unveiled new types of bacterial EVs based on their formation routes (Toyofuku *et al.*, 2019) (further discussed in section 1.3 *EVs in Gram-negative bacteria – 1.3.1 Biogenesis*). “Bacterial EVs” is a fits-all term. Several types of EVs can be simultaneously released through different biogenesis mechanisms. Those EVs can share, or not, defining characteristics, such as size and buoyant density. Hence, it is challenging to clearly discriminate between them using the implemented isolation “bulk” methods (Couch *et al.*, 2021). Implementing further purification steps can positively increase the purity of the vesicle sample, for instance by removing soluble extracellular proteins (Klimentová & Stulík, 2015). Nevertheless, if not performed properly, purification could bring an unintentional bias towards particular types of EVs by enriching them in the final vesicle fraction. Importantly, in a single vesicle population, not all cargoes may be contained in the same vesicle, and different types of EVs with distinct cargo profiles may result from diverse production pathways (McMillan & Kuehn, 2021). Moreover, bacterial EVs derived from Gram-positive bacteria were also discovered and called membrane vesicles (MVs), as bacteria lack an outer membrane. Although far behind in terms of accumulated knowledge in comparison with Gram-negative bacteria, they are taking advantage of related research already performed (Palacios *et al.*, 2020). Strikingly, although fundamental differences between the architecture of their cell envelope exist, mounting evidence show similarities on cell wall remodelling processes governing bacterial EVs formation (McMillan & Kuehn, 2021). Altogether, the term “bacterial EVs” is used here to refer to the whole population of vesicles released by cells at a specific phase of growth, irrespective of their formation pathway.

Since the early years of the 21st century, the interest on EVs skyrocketed, as mirrored by number of publications and related grants, patents and emerging companies (Bazzan *et al.*, 2021). The foundation of this interest is the capacity of vesicles to safely and specifically transport multiplex biological cargoes to distant cells. Yet, this is also a consequence of a massive organization of the EVs' field community, which culminated with the formation, in 2011, of the ISEV. Mainly, ISEV plays a key role on the standardization of the field, which is best exemplified by “position papers”, such as the previously mentioned MISEV guidelines, out in 2014, updated in 2018 (Lötvall *et al.*, 2014; Théry *et al.*, 2018), being the next revision planned to 2022. On top of that, they weave international collaborations by organizing meetings and workshops, and propelled the formation of numerous national local networks. Those, in their turn, conduct their own local meetings and support EV research within their own countries (Couch *et al.*, 2021). In Portugal, the first Portuguese Network on Extracellular Vesicles (PNEV) meeting happened in 2021, leaving as prospect the formation of an official national entity. Nevertheless, it should be highlighted that ISEV, and nascent national entities, are unintentionally pushed to research eukaryote derived EVs. Even though a considerable recent effort to balance this “eukaryotic vs prokaryotic” EVs attention was implemented, this movement imperatively needs to speed up.

The field of EVs is now well established. However, its fast escalation promoted some inconsistencies, namely in best practices for isolation and analytical methods, which complicates comparisons between studies. To report findings properly and consistently is the key factor, as no universal agreement on these topics exists. All protocols to isolate and characterize EVs have their pros and cons, being the driving compass to choose one particular method that will enable a certain level of homogeneity for an intended downstream application (Gardiner *et al.*, 2016).

Bacterial EVs as a distinct and regulated secretion pathway is now openly accepted. This notion comes from rising evidence regarding their mechanisms of release, composition and their implication in a myriad of biological roles. But, how are bacterial EVs formed? What are they made of? Why do bacteria vesiculate? Do they have any biotechnological applicability? These questions have catalysed different works, whose results are summarized below.

1.3 EVs in Gram-negative bacteria

Bacterial EVs are resistant and discrete spherical lipid bilayered nanostructures, ranging between 20 and 500 nm in diameter, derived from the cell envelope and unable to replicate (McMillan & Kuehn, 2021). Discovered more than 50 years ago, it was initially postulated

that they were mere artefact remnants of bacterial growth, originated from dead cell debris. Nonetheless, further investigations demonstrated that vesicles production was not solely a consequence of cell lysis, or even loss of membrane integrity (McBroom *et al.*, 2006; Schertzer & Whiteley, 2012). Detection of bacterial vesicles in laboratorial and, above all, natural settings, aided in the validation of this secretion system (Biller *et al.*, 2014). Evidence of their involvement in triggering genetic regulation (Roier *et al.*, 2016) and in stress response pathways also contributed to this notion (Kulp *et al.*, 2015). Nowadays, studies regarding their compositional aspect and biogenesis pathways continue, and are crucial to understand bacterial EVs biological roles.

1.3.1 Biogenesis

How are Gram-negative bacterial EVs formed? Biogenesis studies mainly address the classical concept of OMVs, and so, most of the models describing vesicle formation and release derive from this perspective. However, new types of vesicles have been discovered and characterized, namely outer–inner-membrane vesicles (OIMVs), explosive outer-membrane vesicles (EOMVs) and tube-shaped membranous structures (TSMs) (Toyofuku *et al.*, 2019). As research advances, due to better-suited technology, it would not be a surprise to discover in the future even more types/subtypes of bacterial EVs.

EVs originate from the cell envelope. A detailed understanding of the bacterial cell wall composition and architecture is key to analyse the mechanisms involved in vesicle formation. Gram-negative cell envelope (Figure 1) subdivides into three main structures: the inner membrane (IM), the outer membrane (OM), and the peptidoglycan (PG) layer, found in the periplasmic space (Silhavy *et al.*, 2010). While the IM is a typical lipidic bilayer that acts as an electrochemical barrier, the OM is composed of two different leaflets, being the innermost commonly composed of lipids and the outermost of lipopolysaccharides (LPS) (Silhavy *et al.*, 2010). LPS consist of an interior hydrophobic lipid A portion, a core oligosaccharide and an exterior hydrophilic O-antigen, and are also known as endotoxins (Schwechheimer & Kuehn, 2015). The periplasm is an oxidative environment that allows protein folding, but it is deprived of nucleotide sources of energy, such as ATP or GTP (Silhavy *et al.*, 2010). The thin-net-like PG layer found in the periplasm gives bacteria their shape and protection from osmotic and sheer stresses. Moreover, envelope proteins can be soluble (found in the periplasmic space), partially anchored to the leaflet of either membrane via covalently attached bridges [e.g. Braun's lipoproteins (Lpp)], or transmembrane proteins. Envelope stability derives from various crosslinks, namely the Braun's Lpp connecting the OM to the PG, the non-covalent interactions between the PG and the porin outer-membrane protein A (OmpA), and the non-covalent interactions

between the PG and the Tol–Pal complex that spans from the IM across the periplasm to the OM (Schwechheimer & Kuehn, 2015).

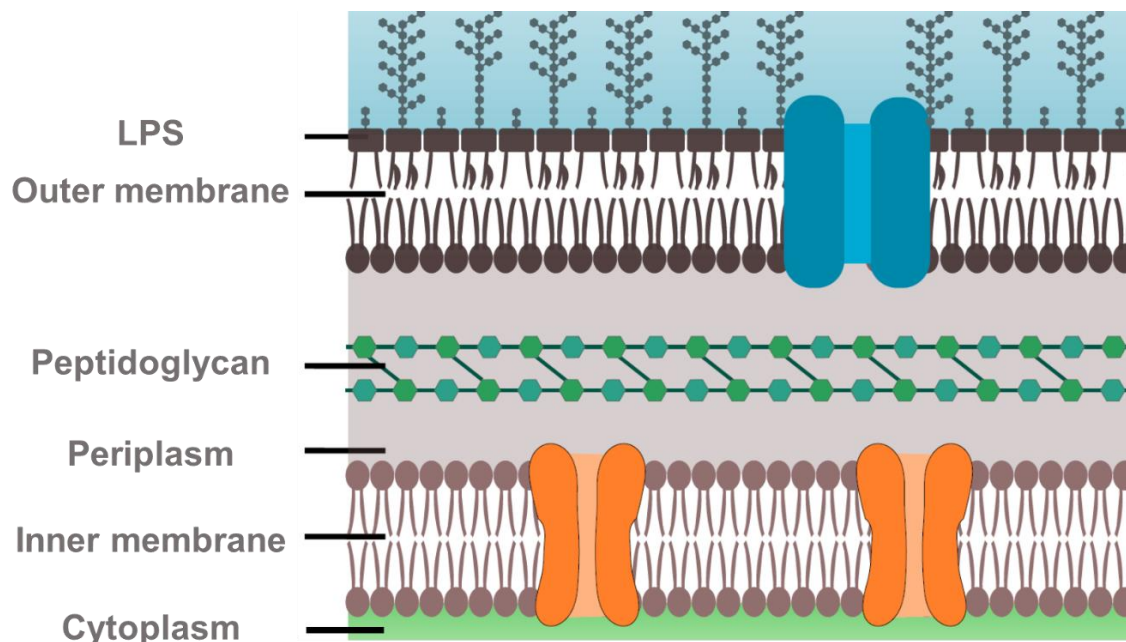


Figure 1. Representative schematic of the Gram-negative cell envelope. The cell envelope is subdivided into three main structures: inner membrane, outer membrane (OM), and peptidoglycan layer, found in the periplasm. Lipopolysaccharides (LPS) constitute the outer leaflet of the OM. Adapted from (Matinha-Cardoso *et al.*, 2022).

A selective permeable cell membrane is the ultimate frontier against an often-harsh extracellular milieu, shielding it from physical and chemical stresses. However, bacterial cells are not static entities. They are constantly integrating and reacting to a myriad of environmental stimuli. Hence, cell walls are firm barriers showing an essential degree of plasticity, allowing EVs generation and release. Accordingly, various not mutually exclusive vesicles formation pathways have been proposed (Figure 2). They are based essentially on: i) the modulation of OM-peptidoglycan links, ii) the existence of nanoterritories and iii) lipid microdomains.

Regarding the modulation of outer membrane-peptidoglycan links one can give the examples of porin OmpA and Braun's Lpp, which are distributed over the cell envelope, and have PG binding sites (McMillan & Kuehn, 2021; Schwechheimer & Kuehn, 2015). When these connections are locally broken, either by a temporary decrease in overall abundance, or a localized displacement of linkages, small OM portions bulge away, resulting in vesiculation. In agreement, several reports show hypervesiculation phenotypes in OmpA-deficient mutants (Deatherage *et al.*, 2009; Park *et al.*, 2012) and when the abundance of

Braun's Lpp is reduced (Deatherage *et al.*, 2009; Schwechheimer & Kuehn, 2013). Interestingly, the inverse is also true, i.e., increasing the number of linkages results in a reduction of vesicle production (Schwechheimer *et al.*, 2014; Schwechheimer *et al.*, 2015). Some argue that the absence of these major players directly affect membrane stability, and so generate vesicles that otherwise would not be produced. Nevertheless, one can claim that tweaking their abundance is a potential form to modulate vesiculation.

Another vesiculation model involves periplasmic nanoterritories, areas of the cell envelope with distinct biophysical properties, in which misfolded proteins and other affected cellular components accumulate, like PG fragments and LPS (McMillan & Kuehn, 2021; Schwechheimer & Kuehn, 2015). Following this abnormal confined build-up of biomolecules in the periplasm, envelope integrity decreases locally, resulting in the blebbing of the OM loaded with lumen content, which eventually pinches off, and a vesicle is released. Thus, this model serves to relieve membrane pressure, and is generally coincident with stress response pathways (McBroom & Kuehn, 2007; Schwechheimer *et al.*, 2014).

Another hypothesis has been proposed in which changes in the chemical features in lipids of the OM may promote vesiculation. Local integration of specific LPS or phospholipids, and/or specific LPS-associated molecules, leads to a fluidity and flexibility alteration in membrane microdomains (Schwechheimer & Kuehn, 2015). Selective export of hydrophobic molecules via EVs is used by bacteria to rapidly and bulkily detach lipid molecular forms, allowing cell envelope remodelling (McMillan & Kuehn, 2021). For instance, vesicle release linked to temperature alterations was shown in various bacterial species (Frias *et al.*, 2010; McBroom & Kuehn, 2007). However, *Pseudomonas aeruginosa* does not display EVs production variations in response to temperature (Macdonald & Kuehn, 2013). Indeed, responses to environmental shifts, like temperature variations, are affected by bacterial cell envelope particularities, which differ between species. In addition, specific extracellular LPS-associated molecules can promote vesiculation (Toyofuku, 2019). After binding, and due to their chemical nature, they force the membrane to curve and the outer-leaflet of the outer membrane to expand. This creates a disequilibrium that facilitates vesicle budding and release. For example, *P. aeruginosa* vesiculation is promoted by the quorum-sensing molecule *Pseudomonas quinolone signal* (PQS), following this route (Mashburn & Whiteley, 2005), which is independent of stress responses (more about PQS below, in section 1.3.3 *Biological roles*).

Other vesicle biogenesis mechanisms have been described (Toyofuku *et al.*, 2019). One is particularly important to explain the presence of cytoplasmic material in bacterial EVs. Outer-inner membrane vesicles (OIMVs) originate from a localized complete cell envelope

detachment, which involves both the IM and the OM (Pérez-Cruz *et al.*, 2015; Reimer *et al.*, 2021). At the base of their formation is an *in situ* perturbation of the PG layer performed by autolysins, which enable the invasion of the periplasm by the IM. This creates a local pressure to pinch-off an OIMV, whose envelope is similar to that of the producing cell. Despite putting forward an explanation for the presence of nucleic acids and cytoplasmic proteins in bacterial EVs, potential mechanisms of OIMV selective packaging are unknown. In addition, vesicle production can result from explosive cell lysis in *P. aeruginosa* (Turnbull *et al.*, 2016). Explosive outer-membrane vesicles (EOMVs) result from a general endolysins attack on the PG layer that trigger cell lysis. Afterwards, there is a recognized lipid self-aggregation phenomenon of membrane cell debris into EOMVs. As they randomly capture buoyant cellular material, their composition may vary greatly from other EVs types. Tube-shaped membranous structures (TSMs) are considered a different type of EVs. These include nanotubes, nanowires and nanopods. TSMs are biological structures found between cells, and can be encountered in *Myxococcus xanthus* (Remis *et al.*, 2014). As they connect the periplasm of neighbouring cells and can exchange biological material, TSMs seem relevant in biofilms (Zingl *et al.*, 2020). Notably, EVs have also been observed to be released from nanotubes in the opportunistic pathogen *Vibrio vulnificus* (Hampton *et al.*, 2017).

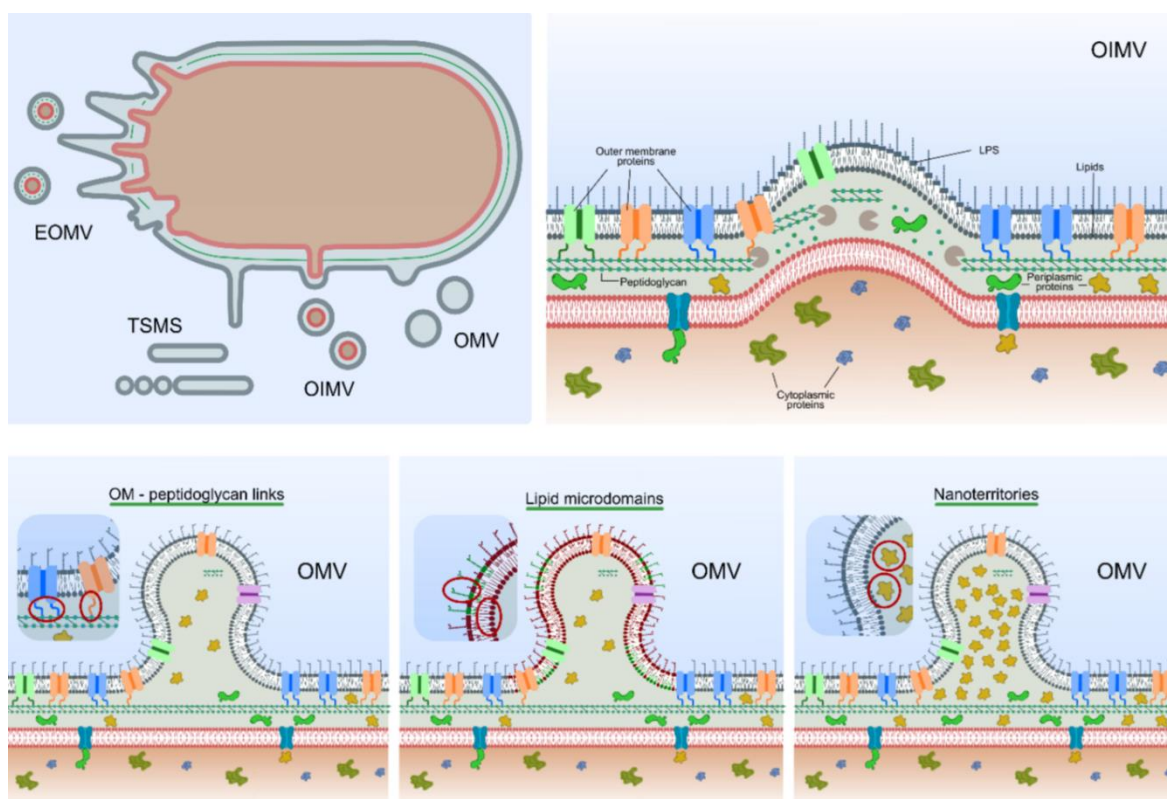


Figure 2. Gram-negative extracellular vesicles biogenesis models. Different types of EVs (upper left panel) have been described: the classical outer-membrane vesicles (OMVs), outer-inner-membrane vesicles (OIMVs), explosive outer-membrane vesicles (EOMVs) and tube-shaped membranous structures (TSMSs). OIMVs (upper right panel) have been proposed to form as a result of the action of peptidoglycan hydrolases in specific regions of the cell envelope, promoting the protrusion of the inner membrane into the periplasm and forcing the outer membrane to bulge. Different hypotheses have been formulated to explain the formation of OMVs (lower panels), including modulation of the outer membrane–peptidoglycan links (left lower panel), lipid/LPS differential composition in specific regions of the outer membrane, termed lipid microdomains (middle lower panel), and accumulation of misfolded periplasmic proteins or envelope components in nanoterritories (right lower panel). Inset in each panel highlights different key components putatively involved in OMV formation, as per the respective model. Retrieved from (Lima *et al.*, 2020).

Taken all together, no single and universal bacterial EVs' biogenesis route exists. Instead, several pathways have been described, some pointing to a general and independent stress response pathway (McMillan & Kuehn, 2021). Yet, all these mechanisms of vesicle production and release can occur simultaneously. Accordingly, the absence of EVs null-mutants could be due to the impossibility to suppress all EV production

mechanisms at the same time (Coelho & Casadevall, 2019). Abolishment of a mere subpopulation of vesicles would go unnoticed. Most protocols to isolate bacterial EVs are “bulk” methods, which mean that the isolated vesicle fraction is highly heterogeneous (Couch *et al.*, 2021). This highlights the complexity and careful evaluation that a vesicle fraction analysis requires, and anticipates an intricate genetic background. Moreover, the physiological state of the cells at the moment of harvest is another key parameter. These are all crucial factors to consider when analysing the molecular composition of bacterial EVs.

1.3.2 Composition

What are Gram-negative bacterial EVs made of? EVs composition is directly related to their biogenesis process. In agreement, these spherical nanostructures are enriched in OM components, namely LPS and OM proteins. In addition, vesicles have also been shown to contain periplasmic proteins, PG fragments, metabolites, and even cytoplasmic proteins and nucleic acids (DNA and RNA) (Figure 3) (McMillan & Kuehn, 2021; Schwechheimer & Kuehn, 2015; Zingl *et al.*, 2020). Procedures to isolate, purify and characterize vesicles are now constantly improving, refining compositional analyses. Several examples show EVs' selective cargo enrichment comparing to the producing cell. Hence, compositional data contributed drastically to the view of EVs as a bona fide secretory process.

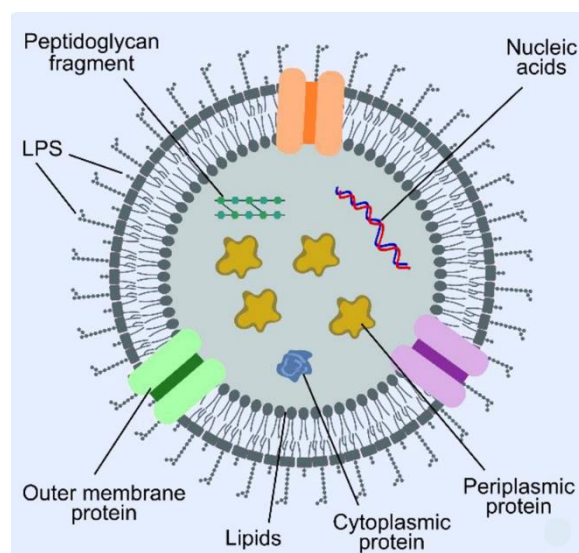


Figure 3. General composition of EVs from Gram-negative bacteria. Vesicles are composed mainly of outer-membrane components (lipopolysaccharides (LPS); outer-membrane proteins; and lipids), periplasmic content (proteins; peptidoglycan fragments; metabolites), and, in some instances, even cytoplasmic proteins and nucleic acids (DNA and/or RNA). Retrieved from (Lima *et al.*, 2020).

Packaging EVs with proteins upon environmentally stressful conditions contributes ultimately to bacterial viability. For instance, in response to oxidative stress, it was demonstrated a preferential allocation of oxidized proteins in *E. coli*'s vesicles (Orench-Rivera & Kuehn, 2021). This could be regarded as a mechanism to avoid deleterious effects caused by the damaged proteins, if not actively secreted. Moreover, selective export of lipids through EVs can also occur. The pathogen *Salmonella typhimurium*, the principal culprit of food poisoning in Western countries (Chimalizeni *et al.*, 2010), was subjected to biochemical conditions that replicated the transition from an extracellular to an intracellular host setting (Bonnington & Kuehn, 2016). Interestingly, when shifted to "intracellular conditions", cells secreted EVs with less-modified LPS species. Consequently, *S. typhimurium* cell envelope retained preferentially more-modified LPS species, helping in the adaptation towards new challenging conditions. Still in the pathogenesis context, various reports show the traffic of virulence factors, like toxins, by bacterial EVs. Perpetrator of most cases of traveler's diarrhoea and children's diarrhoea worldwide, the enterotoxigenic *E. coli* (ETEC) secretes the heat-labile enterotoxin (LT), both by classical (mediated through protein complexes integrated in the cell envelope) and non-classical (EVs-mediated) secretion mechanisms (Horstman *et al.*, 2004; Horstman & Kuehn, 2000). In summary, soluble LT and LT-EVs trigger different responses and act through different mechanisms (Chutkan & Kuehn, 2011). Bacterial EVs contain molecules, such as LPS, known to trigger host immune responses (Wang *et al.*, 2019). Thus, it is not surprising that vesicle associated toxins elicit distinct reactions as compared when available in their free form, determining the type and magnitude of host response. Besides LT, other toxins from different pathogens are enriched in their EVs, as the major outer membrane porin (PorB) from *Neisseria gonorrhoeae* (Deo *et al.*, 2018), the cholera toxin from *Vibrio cholera* (Zingl *et al.*, 2021) and the vacuolating toxin (VacA) from *Helicobacter pylori* (Chew *et al.*, 2021), just to mention a few. Nucleic acids have also been detected in bacterial EVs, both in laboratorial and environmental settings (Biller *et al.*, 2017; Biller *et al.*, 2014; Pérez-Cruz *et al.*, 2015). DNA, which can be on the surface and in the lumen of EVs, have been linked to bacterial and bacteria-host cell interactions. Notably, in *P. aeruginosa*, complete DNA fragments encompassing regions that encode proteins involved in virulence, stress response, antibiotic resistance and metabolism were detected in EVs (Bitto *et al.*, 2017). Interestingly, bacterial DNA associated with vesicles was able to enter eukaryotic cells (Bitto *et al.*, 2017). Regarding RNA, all classes were already detected in bacterial EVs (Lécrivain & Beckmann, 2020). Using a new modified RNA-sequencing protocol in *E. coli* O6:K15:H31 536 (UPEC), three EV-RNA libraries were generated: small - 15–50 nucleotides (nt), medium - 50-200 nt and large - >200 nt. While rRNA was present in all of them, 27% of the RNA found in the small library was tRNA (Blenkiron *et al.*, 2016). In any case, it remains unclear how these

nucleic acids are packed into EVs, and after entrance into the host or neighbouring bacteria, even if these fragments can be transcribed and/or translated.

To sum up, all major macromolecules were detected in Gram-negative bacterial EVs. Notably, bacterial cells can, when necessary, regulate vesiculation to secrete selected compounds. Particular vesicle composition found in different bacterial species derives essentially from their cell envelope characteristics and how they react to distinct environmental settings. As these nano-vessels recreate within their lumen a cell-like microenvironment, safer and distant transport of their cargoes can occur. Moreover, most of the studies addressing the topic of EV composition focus on analysing the effects of relatively pure EVs samples, recognized today to be mainly a mixture of subtypes of vesicles (Couch *et al.*, 2021). Allied to the advance of isolation techniques, it is expected that different subpopulations of vesicles have differential composition and specific roles. However, one should not forget that, in the true environmental stage, bacteria secrete simultaneously a variety of EVs, as well as soluble biomolecules, which can in their turn interact with vesicles. Therefore, even if a solid and independent investigation of all these individual characters is required, using the entire complex mixture could bring novelties and reveal the full scene about their physiological relevance.

1.3.3 Biological roles

Why do bacteria vesiculate? This is a fundamental question. Today, vesicle release is a well-accepted secretory process. Evidence of distinct formation mechanisms and differential packaging of EVs support this view. Obviously, these topics are not yet completely comprehended, as many open questions persist about their mechanisms of action, and whether they are fully shared across bacterial species. Yet, the beneficial or detrimental aspect of vesiculation to bacteria is a pressing topic (McMillan & Kuehn, 2021). One should not forget that releasing EVs is energetically demanding for the bacterial cell. Also, EVs trigger host immune responses (Johnston *et al.*, 2020), ultimately aiming to decrease bacterial survival. This creates a paradox towards the benefits of vesiculation, which deserves further comment. Why do bacteria would invest time and energy in something that could turn into a disadvantage? McMillan and Kuehn have recently reviewed this question from the “bacterial point of view” (McMillan & Kuehn, 2021). Ultimately, from a Darwinian perspective and as transversal mechanism, the price of releasing EVs should outweigh their costs. Here, an overview of the pros and cons inherent to bacterial EVs production is presented, emphasizing their physiological relevance for fulfilling various biological roles (Figure 4).

One of the main advantages is to allow a swift and sustainable membrane remodelling capacity. In this regard, EVs are a way of removing in bulk unwanted lipid species. To adapt to an ever-changing environment, bacteria need to regulate the composition of the cell envelope. These include LPS biochemical alterations – lipid A decoration, core modifications and/or O-antigen length - that render the membrane more suitable to specific environmental settings (Bonnington & Kuehn, 2016; Simpson & Trent, 2019). However, these are all cytoplasmic occurrences and there are no reported degradation and recycling processes for LPS (McMillan & Kuehn, 2021). Accordingly, cells require an efficient “*in situ*-outer-membrane” LPS discarding mechanism, to incorporate cytoplasmic newcomers and better suited molecules. To date, the release of EVs represents the only known process capable of this feat (McMillan & Kuehn, 2021). However, limitations of this strategy is the considerable amount of energy dispended. Assuming a positive energetic balance, EVs release can allow specific and mass removal of undesirable lipids/LPS. As they ease key envelope restructuration under ambient shifts, vesicles have a protective role.

Similar reasoning applies to export selected compounds, being some of them noxious when accumulated intracellularly, as misfolded proteins, antimicrobials and virulence factors. In the particular case of pathogenesis, as mentioned earlier, EVs loaded with virulence factors benefit bacteria by augmenting the chances of successful host invasion (Nadeem *et al.*, 2020). Instead of being promptly diffused, these cargoes exit the cell protected within an organic shield against environmentally and host lytic mediated degradation. They are safely trafficked in a concentrated manner to long-distance and otherwise inaccessible targets. Furthermore, EVs can pack multiple cargoes at once, which allow the simultaneous distribution of potent cocktails with different modes of action. Also, EVs' lumen acts as native microenvironment that facilitates and preserve original properties of cargoes (Bonnington & Kuehn, 2014). Drawbacks of this strategy lies on the consequent activation of host immune responses, evolved to eliminate bacterial intruders (McMillan & Kuehn, 2021). Yet, one should remember that bacteria too evolved several forms to resist and surpass host attacks. Remarkably, bacterial EVs fulfil a key role in these counteroffensives. As previously discussed, fast EV-mediated bacterial cell membrane restructuring is an effective form of resistance. On one side, the host offensive is designed against protein and lipid species of EVs that no longer dominate the bacterial envelope landscape. On the other side, enriched in those amphiphilic molecules and antigens, EVs are themselves decoys packed with substances that can actively degrade host's antimicrobial compounds. Worthwhile noticing that, in these particular cases, host antibody response is not fully against the bacterial cell, so allowing momentarily pathogen evasion (Band & Weiss, 2015; Nadeem *et al.*, 2020). The benefits for releasing EVs are not limited

to the context of pathogenesis. Aggressive environments are often replete with antimicrobials and phages. Comparable tactics are employed there, as EVs bind and inactivate these threats, protecting the vesicle-producing bacteria (Biller, 2020). In complex microbial communities, like biofilms, some microbial members can cheat the system being favoured by these nano-decoys, without any energetic cost. Actually, some of them can even use EVs as source of carbon and energy (Biller *et al.*, 2014). Therefore, on top of being involved in bacterial protection, EVs are in some cases directly implicated in pathogenesis, and in complex environmental niches can act as public-goods.

Another level of the benefits of EVs concerns bacterial communication. Bacterial quorum-sensing is a process described to metabolically synchronize local microbial populations to manage extracellular resources (Waters & Bassler, 2005). Thought to occur solely using diffusible signalling molecules through bulk gene regulation, today, it is known that quorum-sensing can be triggered by EVs (Toyofuku, 2019). Actually, one single vesicle, loaded with signalling molecules above the threshold concentration, is enough to activate this type of communication (Toyofuku *et al.*, 2017). This shifted the chemical gradient paradigm, in which free-diffusion results in infinite dilution of the signal, to a binary system approach (Toyofuku, 2019). In the latter, loaded vesicles are distributed more randomly, leading to heterogeneous gene expression amid the population. Long-distance signalling is thus viable, as signal concentration is temporally and spatially maintained within EVs. Moreover, the implication of vesicles in these processes uncovered the possibility of hydrophobic molecules to work as quorum-sensing molecules. This was unconceivable under the classical approach, as these molecules have trouble diffusing freely in aqueous environments, namely the ones with higher molecular weight or with unfavourable chemical properties. Signals like *N*-acyl homoserine lactones (AHLs) are widely used among Gram-negative bacteria (Toyofuku *et al.*, 2020). AHLs consist in a lactone ring and a modifiable acyl side chain with length ranging from 4 to 20 carbons (Arashida *et al.*, 2018). Interestingly, these hydrophobic signalling molecules are carried by EVs. This was first demonstrated in the human pathogen *P. aeruginosa*, which evolved an elegant loop mechanism of communication (Mashburn & Whiteley, 2005). *P. aeruginosa* vesicles associate with a highly hydrophobic molecule, PQS. PQS elicits vesicle formation by intercalating into the outer leaflet of the outer membrane. Released vesicles, containing PQS in their envelope, can then simultaneously induce the expression of genes involved in the synthesis of siderophores and further promote vesiculation in other cells (Toyofuku *et al.*, 2020). As PQS has also high affinity for ferric ions (Fe^{3+}), EVs act as sequestors of this micronutrient (Bredenbruch *et al.*, 2006; Diggle *et al.*, 2007), conferring a nutrient acquisition role to EVs. Remarkably, exogenous addition of PQS promotes vesicle formation in other

bacterial species (Tashiro *et al.*, 2010). Additional cases of quorum-sensing mediated by EVs are reported. For instance, *Paracoccus denitrificans*, a denitrifying bacterium inhabiting sludge, produces the strongly membrane associated C16-HSL (Schaefer *et al.*, 2002). *P. denitrificans* C16-HSL-vesicles have been shown to preferentially attach to cells of the same type, keeping away the signal from other bacteria. Yet, *P. denitrificans* recognizes AHLs signals from other bacteria, such as C12-HSL to C18-HSL, which also bind to their vesicles (Morinaga *et al.*, 2018). Hence, this bacterium can hack the microbial communication system, by tweaking the quorum-sensing response in its favour. From the “bacterial point of view”, this can be regarded both as an advantage and disadvantage. The same applies when addressing bacterial biofilms, which are composed of several bacterial species locally competing for resources. Thanks to their features, vesicles are used to communicate through the biofilm matrix. In this context, vesicles can promote biofilm dispersal by regulating bacterial adhesion, by blocking or not attachment sites. *Xylella fastidiosa*, a serious crop-threatening pathogen, colonizes plant xylem vessels. Modulating EVs release and thus attachment sites availability, *X. fastidiosa* spreads within the plant host, or stops when ideal for aphid transmission (Ionescu *et al.*, 2014). Altogether, these examples highlight the bacterial communication role fulfilled by EVs and their impact on using available resources for survival or host invasion.

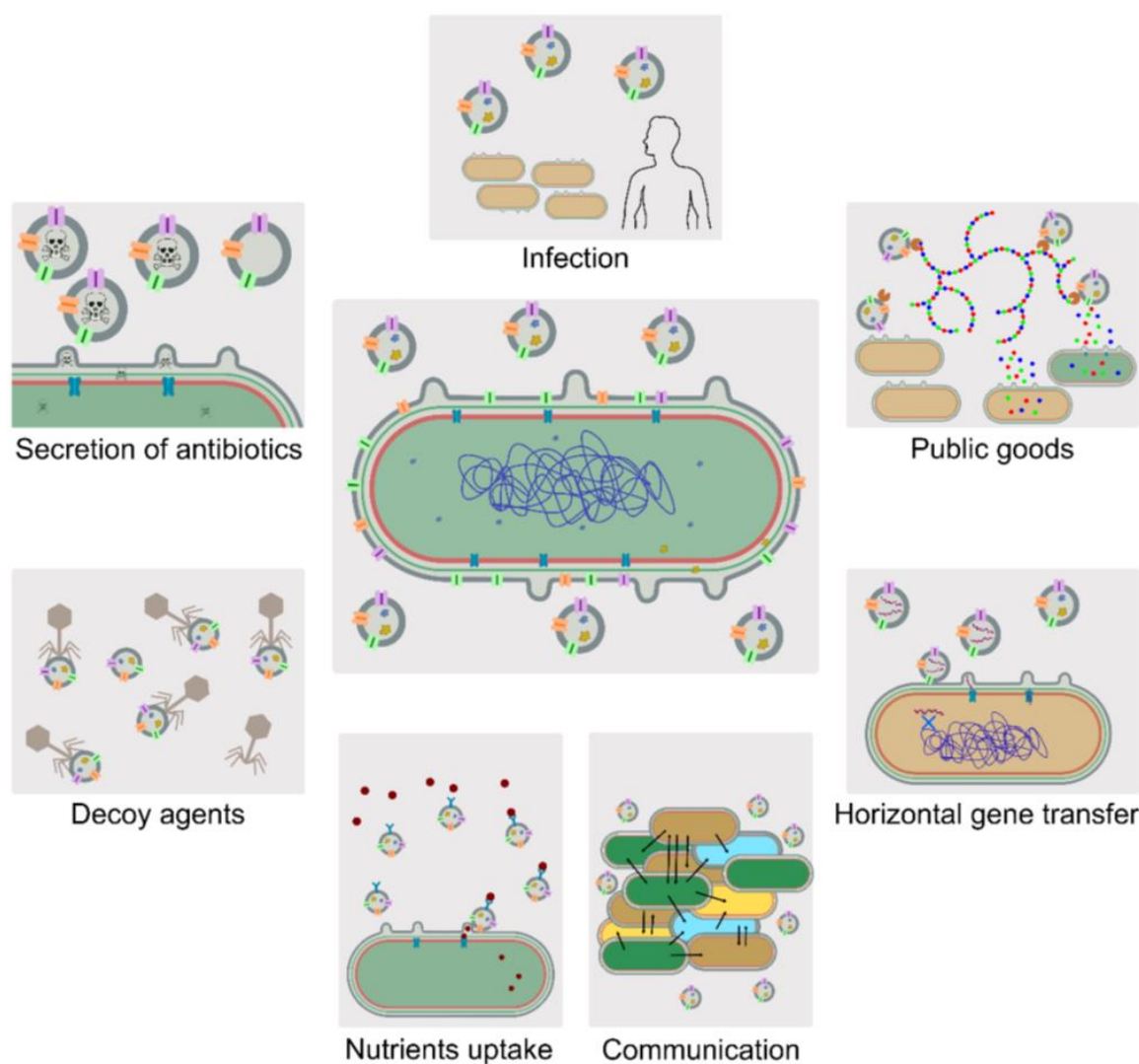


Figure 4. Examples of biological roles proposed for extracellular vesicles from Gram-negative bacteria. EVs have been described to be involved: in infection, by eluding the host immune response; as public goods, as they may carry catalytic enzymes that help deconstructing complex extracellular biomolecules, making them available for the EVs-releasing bacteria and for other organisms; in horizontal gene transfer, as they transport nucleic acids that can be transferred between organisms; in communication, e.g., in the context of biofilms; in the uptake of nutrients; as decoy, for example to evade phage infection; and in the secretion of antibiotics. Retrieved from (Lima *et al.*, 2020)

In summary, benefits for bacteria producing EVs seem to largely compensate their intrinsic energetic cost. Why? Because vesiculation is a key and distinct secretory process. As any other biological relevant mechanism, EVs production was progressively streamlined by natural selection. Understanding their regulation and formation processes, and how these reflect vesicle composition, is fundamental.

1.3.4 Biotechnological applicability

We live in an era when the interactions between Human and surrounding world are being progressively unveiled, particularly related with the microbial world. Now, conscious that the first steps of human speciation were engaged with our microbial partners, there is an increasing awareness of their contribution for a wide range of processes. From health-related to ecological and industrial ones, which also directly affect us, these microbial allies are everywhere. Hence, the use of these microbes to advance human society is an attractive topic of research (Caruana *et al.*, 2020). Bacterial EVs, due to their intrinsic and distinctive characteristics, display potential to be bioengineered. Accordingly, EVs have been associated with several technological purposes, such as to develop novel vaccines/vaccine platforms, to employ them as therapeutics capable of specialized drug delivery, or even exploring their innate potential for commercial applications (Caruana *et al.*, 2020). In this work, the potential of bacterial EVs as nano-carrier systems are presented, with a particular focus on vaccine and bioremediation research.

The vaccine concept is based on the capacity to acquire protective immunity against a particular pathogen. Inactivated/attenuated pathogens or parts that mimic it provoke the activation of the host natural immune system, never actually triggering the related disease (Caruana & Walper, 2020). It is worthwhile noticing that most of the effective known vaccines that have been commercialized apply a general Pasteur's principle determining the strain isolation, inactivation and injection, or the use of attenuated strains. Inactivated biomolecules such as proteins, carbohydrates or peptides can also be used (Zepp, 2010). Despite being one of the best implemented and proven health technologies of our existence, these methods can always be enhanced. For instance, vaccines based on an antigen that has a feeble reaction need to be combined with adjuvants or conjugated with specific carriers to ignite a satisfactory immune reaction (Coffman *et al.*, 2010). Classical adjuvants, such as aluminium, often leads to uncontrolled immune responses like inflammation. Therefore, a consensus is reached today that a vaccine must combine the minimum possible side effects with the maximum efficiency. Now, popular DNA/RNA vaccines are based on particular isolated genetic sequences that encode a protein from a target pathogen. Uptake of specific DNA/RNA by antigen-presenting cells, will lead to the transcription and/or translation into a protein, normally one presented at the cell envelope of that pathogen, which stimulates an immune response. However, the correct combination with adjuvants is still necessary.

An “all in one” approach of the 21st century to circumvent this for vaccine development is the use of nano/micro-acellular systems (e.g. virus-like particles, polymeric/inorganic

nanoparticles, liposomes, bacterial EVs) (Grandi *et al.*, 2016). Currently, bacterial EVs present exciting benefits for vaccine research comparing to other nano-systems, which still require the involvement of adjuvants. EVs are by nature enriched with immunomodulatory components (e.g. LPS, OM proteins) that act as pathogen-associated molecular patterns (PAMPs), which are introduced to the host innate immune system and detected by specific pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Kaparakis-Liaskos & Ferrero, 2015). Hence, EVs are able to naturally trigger an immune response. Beyond this often disregarded self-adjuvantivity, it is not necessary to treat them with inactivating agents because EVs are unable to replicate. Also, they are normally regarded as the best candidates to target the lymph nodes, where particular immune cells inhabit, and once there, in promoting their phagocytic uptake, all due to their natural size range and composition (Gerritzen *et al.*, 2017). Moreover, the ability to engineer the loading of specific antigens into EVs (lumen and/or surface), to manipulate their envelope decoration and so, the delivery to target cells, and to tweak when necessary the expected level of immune response by LPS remodelling, are other attractive advantages to explain the substantial amount of dedication to this topic (Caruana *et al.*, 2020).

To obtain EV-based vaccines, two alternative approaches have been employed. On one hand, the pathogenic bacterium of interest can be cultivated to produce EVs (either EVs artificially formed in solution or EVs spontaneously released) that can be directly administered to the subject (in some cases, further chemical treatments are required). The best example of this technology is the case of the EV-containing meningococcal vaccine against *Neisseria meningitidis*, for which already exists a commercially available product for human usage, Bexsero® (Balhuizen *et al.*, 2021; Gnopo *et al.*, 2017; Vernikos & Medini, 2014). On the other hand, recombinant EVs can be obtained directly from a more user-friendly bioengineered non-pathogenic bacterium model, harbouring heterologous antigen(s) derived from the target pathogenic bacterium, leading EVs to work as modular multiplex antigen delivery platforms (Gnopo *et al.*, 2017).

Despite the hype regarding the use of bacterial EVs for vaccination, nearly all highlighted advantages for their use are valid for an array of distinctive technological applications, including bioremediation (Caruana *et al.*, 2020). In this case, there is this notion that native microorganisms should be privileged and employed to degrade the contaminant at the polluted site. As spills occur in random locations, it is complicated to follow this approach. Instead, it is possible to understand their enzymatic action and replicate it at the laboratory scale, despite the cost of manufacture using recombinant technologies. Then, deploying these tailored enzymes will help decontaminate the polluted site. Unfortunately, when free enzymes face non-ideal extracellular conditions (e.g. salinity

and pH variations), they show stability and activity performance limitations. Bacterial EVs are able to shield their soluble cell-like microenvironment against extracellular threats. Therefore, loading these enzymes within vesicles would aid in local decontamination by extending their period of action. It is even possible to isolate EVs directly from decontamination suitable bacteria, surpassing the cost of bioengineering another bacterium, if these enzymes are naturally loaded. Moreover, bacterial EVs have extended shelf-periods, can be lyophilized for storage and/or distribution (Caruana *et al.*, 2020). As a proof of concept study, Alves *et al.* (Alves *et al.*, 2018) demonstrated the functionalization of *E. coli* EVs to degrade the organophosphate chemical warfare agent simulant paraoxon, not only in environmental water samples, but also in potential sites of contamination like glass, painted metal, and textiles. This was possible through an elegant engineering approach to packing phosphotriesterase (PTE) into vesicles. Directing a recombinant PTE enzyme, harbouring a C-terminal fusion SpyCatcher (PTE-SC), to the periplasmic space, in a cell coexpressing an engineered form of the abundant transmembrane porin OmpA, holding a Spy-catcher complementary periplasmic peptide tag (OmpA-SpyTag), PTE-SC is pulled into the vesicle during its formation, ultimately packaging in its lumen. Then, isolated EVs packed with PTE-SC are ready to hydrolyse the organophosphate compound at the contaminated site (Alves *et al.*, 2018; Alves *et al.*, 2015). In addition, authors showed that the heterologous expression of PTE, and its targeting to EVs, helped mitigate the often toxic effect that production of heterologous proteins result in the respective expressing bacteria. Notably, coexpression of OmpA-SpyTag with PTE-SC greatly enhanced the overall PTE production levels (Alves *et al.*, 2015). Taken together, bioremediation using bacterial EVs appear as a valid and environmentally friendly approach, even more assuming the still existing distrust from society regarding the use of genetically modified organisms (GMOs) (Caruana *et al.*, 2020).

Before these technological applications are realized with bacterial EVs, some issues still need to be addressed. For instance, neither all bacterial species possess the capacity to be genetically edited, nor the bioengineering tools to do so are available. For the ones where a genetic editing toolbox is accessible, there is still a need to understand and control vesiculation, as well as to evaluate EVs triggered immune response. In cases of host immune overstimulation, LPS-remodelling was already demonstrated to be a functional strategy (Gnopo *et al.*, 2017). Also, the recurrent question of industrial scalability will be at stake. Hyper-vesiculating mutants need to be created and streamlined to improve recovering yields, being selected heterologous proteins more efficiently expressed and packaged into EVs. Only after meeting all these requirements, will bacterial EVs become

sufficiently attractive to respond to diverse technological challenges in several areas of human society.

After this overview about bacterial EVs, this thesis will now focus on cyanobacteria, an evolutionary relevant group of microorganisms. In a challenging world, where circular economy and right sustainable ecological choices should dictate the path of Human future, cyanobacteria show attractive green features. Their environmentally friendly characteristics, allied with a capacity to be genetically edited and regulated through a still developing toolbox, could unroll novel avenues in the bacterial EVs field, namely for technological applications.

2. Cyanobacteria: a crown group of prokaryotes

Cyanobacteria are a crown group of Gram-negative prokaryotes. They pioneered, at least 2800 Ma ago, oxygenic photosynthesis. As true alchemists, they converted, using sunlight energy and reducing power extracted from water molecules, a common substrate, carbon dioxide, into precious reduced carbon compounds, rich in chemical energy (McFadden, 2014). They are the only known prokaryotes capable of this biochemical feat (Sánchez-Baracaldo & Cardona, 2020). In addition, by releasing molecular oxygen as a by-product of this process, they altered drastically the early atmosphere and contributed to the emergence of new life forms. This oxygenic photosynthetic ability was later acquired, via endosymbiosis, by eukaryotes. Cyanobacteria are generally accepted as present-day green algae and higher plants chloroplasts' ancestors, underlining their relevance through the course of eukaryotic evolution (McFadden, 2014). They are also one of the most morphologically diverse groups of prokaryotes, ranging from unicellular to filamentous or multicellular forms. In some cases, cyanobacteria are even capable of cellular differentiation (Herrero *et al.*, 2016). Moreover, their minimal nutritional requirements, namely sunlight, water, air and a few minerals, sustain their cosmopolitan distribution. They virtually can occupy almost all illuminated aquatic and terrestrial habitats. Cyanobacteria have been detected in the most inhospitable environments, including hot springs and Antarctic ponds (Singh, 2018). Accordingly, these prokaryotes present a great metabolic plasticity. On top of carbon dioxide fixation, several strains have the remarkable capacity to perform dinitrogen fixation (Eigemann *et al.*, 2019). Cyanobacterial impact on carbon and nitrogen global cycles is significant. These primary producers are one of the most abundant organisms on Earth (Flombaum *et al.*, 2013). Unsurprisingly, all these relevant biological, evolutionary and ecological aspects attracted the attention of the bioindustry.

World's thirst for sustainable biotechnologies is, comprehensibly, at its peak. Their implementation will certainly cause economic shifts and disrupt petrified lifestyles. Yet, policies to replace fossil fuels and investing in greener technologies are compulsory for Earth inhabitants' wellbeing. Accordingly, this immense challenge could have the aid of cyanobacteria (Sutherland *et al.*, 2021). Performing oxygenic photosynthesis, presenting minimal culture conditions and being genetically tractable, are seductive biotechnological characteristics. One should not forget that most biotechnological processes have elevated production costs, as they rely on microorganisms that require expensive carbohydrate compounds to grow (Ducat *et al.*, 2011). Also, massive cultivation of cyanobacteria does not require arable land, limiting the competition with food production. Several species can grow in seawater or are genetically amenable to do so, thereby avoiding the use of freshwater supplies. Cyanobacteria have been shown to synthesize a wide range of high-value commercial products, including antioxidants, biodegradable polymers, biofertilizers, nutrient supplements, colour substances and biofuels (Zahra *et al.*, 2020). Therefore, initiatives are ongoing toward the development of cyanobacteria as robust, self-sustaining, microbial cell factories.

But, why are we not widely using these sustainable biotechnologies based on cyanobacteria yet? What is the catch? It is not quite ready to fully triumph at an industrial scale. A certain number of technical limitations need to be tackled, which include cyanobacterial cultivation, genetic engineering and harvesting. Limited cyanobacterial species are efficiently and independently cultured in open pond conditions, as they adapt to resist environmental shifts and do not direct their energy for desired compound production. Actually, most species are unable to grow competently in highly selective environments, resulting in low biomass productivity (Zahra *et al.*, 2020). Closed systems with suitable dimensions and parameters control, as outdoor industrial photobioreactors are the solution. However, they are expensive to design and assemble, as they need to consider aspects like light exposure, intensity and distribution. Sunlight varies around the planet, so photobioreactors need to be adjustable (Chisti, 2007; Zahra *et al.*, 2020). In addition, they need to consider and overcome the self-shading effect as the culture optical density increases (Richmond, 2004). Regarding strains bioengineering, the cyanobacterial genetic toolbox needs to expand, thereby allowing regulation and production of commercial quantities of the desired compound (Sun *et al.*, 2018). Biomass and/or product of interest harvesting are laborious, costly and problematic to apply at a large scale. Ingenious approaches as streamline product secretion to the culture medium to ease recovery could be employed (Gonçalves *et al.*, 2019). Only investing in all these scattered cyanobacterial aspects will the leap from bench to large-scale industrial production be possible.

2.1 Cyanobacterial EVs as non-classical secretion mechanism

One of the ways by which cyanobacteria interact with their surroundings is through the secretion of a wide range of compounds. Classical membrane protein-dependent gates or portals, located at the cell envelope, has always been recognized as intrinsic to survival (Costa *et al.*, 2015). However, non-classical EV-mediated secretion have been largely overlooked. This last topic was addressed under the form of a review article, which corresponds to the second chapter of this thesis (*Chapter II - Extracellular vesicles: An overlooked secretion system in cyanobacteria*). This chapter summarises the state-of-the-art on cyanobacterial EVs as it was in the beginning of this doctoral thesis work, since it was written before the main results were obtained and reported. More, it identifies a number of aspects that represented the starting point for establishing the main goals of this work.

3. Thesis aims/Objectives

Cyanobacteria are a diverse group of Gram-negative prokaryotes. Pioneers of oxygenic photosynthesis, they not only are an evolutionary relevant group of bacteria, but continue to significantly influence Earth's ecology. Unveiling cyanobacterial secrets and developing technologies associated with these microorganisms will certainly contribute to greener sustainable solutions. Knowledge on cyanobacterial EVs lags behind compared to that of other bacterial species. Aspects regarding the biogenesis, composition and biological roles of cyanobacterial EVs are still incipient, as well as their potential to be bioengineered.

Synechocystis sp. PCC 6803 (Figure 5) is a unicellular model cyanobacterium, considered by some as the “Green *E. coli*” (Branco dos Santos *et al.*, 2014). Holding plenty of genetic editing tools at hand, and with genome-scale metabolic network models available describing and predicting metabolic fluxes, *Synechocystis* was shown to secrete vesicles in 2015 (Pardo *et al.*, 2015). Hence, *Synechocystis* represents one of the best candidates for the study of cyanobacterial EVs. Moreover, a hyper-vesiculating *Synechocystis* mutant was already available in the research group where this work was developed (Oliveira *et al.*, 2016), which further motivated to study fundamental questions of the cyanobacterial EVs biology. Therefore, the main objectives of this work were:

- i) To isolate and characterize cyanobacterial EVs.

Work performed within the scope of this thesis aimed at establishing procedures and consensus guidelines in order to collect and analyse cyanobacterial vesicles.

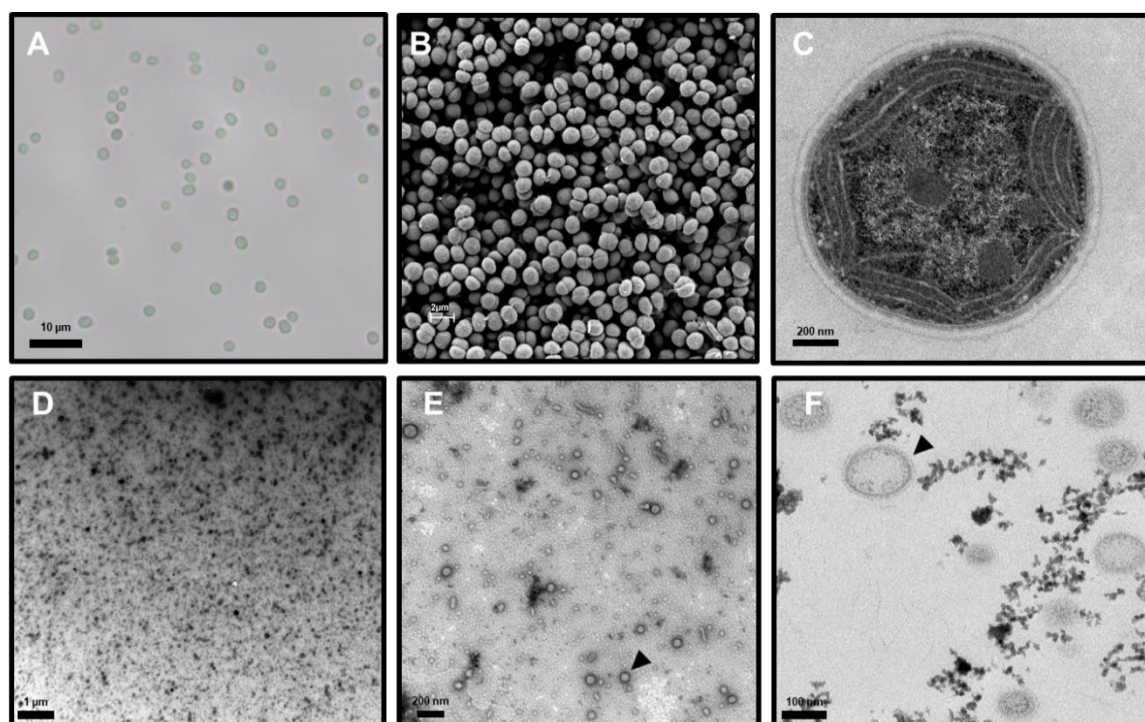


Figure 5. *Synechocystis* sp. PCC 6803, the unicellular cyanobacterium used as model organism throughout this thesis, releases EVs. From A to C (upper panels), photographs of *Synechocystis* using different microscopy techniques, namely light microscopy (A), scanning electron microscopy (SEM) (B) and transmission electron microscopy (TEM) (C). From D to F (lower panels), TEM photographs of *Synechocystis* EVs isolated from the extracellular medium. D and E are micrographs of negatively stained EVs, using two magnifications, while F shows ultrathin sections of EVs, revealing their ultrastructural details. Arrows in E and F indicate examples of EVs. Scale bars are referred in each panel.

ii) To uncover molecular mechanisms underlying cyanobacterial EVs production.

Membrane blebbing has been observed in cyanobacteria during the process of EV formation (Biller *et al.*, 2014). However, the mechanisms related to cyanobacterial EVs biogenesis remain unclear. This thesis aimed at identifying key players and processes involved in cyanobacterial vesicle formation and further release.

lii) To identify biological roles of cyanobacterial EVs.

One of the main questions in the field of EVs' biology is to understand the biological roles fulfilled by vesicles. Therefore, studies were carried-out to unveil possible

physiological and/or cellular advantages of cyanobacterial cells from the process of vesicles release.

- iv) To explore cyanobacterial EVs with customized protein content for biotechnological applications.

Cyanobacteria harbour an enormous potential as sustainable microbial cell factories. Efforts related to the modulation of cyanobacterial EVs production and customization of their protein content must be carried out, in order to evaluate the potential of cyanobacterial EVs to work as nano-carrier systems of proteins of interest. In this work, this biotechnological potential was tested in fish, envisioning the application of cyanobacterial EVs in aquaculture.

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CHAPTER II - Extracellular vesicles: An overlooked secretion system in cyanobacteria

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^{*} Performed literature searches and wrote the sections regarding composition, biogenesis and functional roles of EVs in bacteria and in cyanobacteria.



Review

Extracellular Vesicles: An Overlooked Secretion System in Cyanobacteria

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Abstract: In bacteria, the active transport of material from the interior to the exterior of the cell, or secretion, represents a very important mechanism of adaptation to the surrounding environment. The secretion of various types of biomolecules is mediated by a series of multiprotein complexes that cross the bacterial membrane(s), each complex dedicated to the secretion of specific substrates. In addition, biological material may also be released from the bacterial cell in the form of vesicles. Extracellular vesicles (EVs) are bilayered, nanoscale structures, derived from the bacterial cell envelope, which contain membrane components as well as soluble products. In cyanobacteria, the knowledge regarding EVs is lagging far behind compared to what is known about, for example, other Gram-negative bacteria. Here, we present a summary of the most important findings regarding EVs in Gram-negative bacteria, discussing aspects of their composition, formation processes and biological roles, and highlighting a number of technological applications tested. This lays the groundwork to raise awareness that the release of EVs by cyanobacteria likely represents an important, and yet highly disregarded, survival strategy. Furthermore, we hope to motivate future studies that can further elucidate the role of EVs in cyanobacterial cell biology and physiology.

Keywords: cyanobacteria; extracellular vesicles; composition; biogenesis; biological roles; applications

1. Introduction

The phylum Cyanobacteria consists of a group of prokaryotes that typically carry out oxygenic photosynthesis with water as an electron donor and use carbon dioxide as a carbon source, or those secondarily evolved from such organisms [1]. Cyanobacteria are generally regarded as Gram-negative bacteria that can be found in a wide range of habitats [2], from aquatic to land-based ecosystems. In addition, these microorganisms can also be found in the harshest environments, such as hot springs, polar lakes, deserts and even polluted wastelands [3]. To colonize and thrive in such places, cyanobacteria evolved niche-specific adaptation strategies. They exhibit a great tolerance to environmental stresses, such as desiccation, radiation, nutrient deficiency and exposure to heavy metals [3]. Cyanobacteria adjust to these life threats through a series of adaptive physiological responses, resulting from the balance between competition for resources and intra- and interspecies cooperation [4]. This vital game of transmitting and receiving signals enable them to activate survival pathways and to maintain homeostasis. In this context, the extracellular milieu plays an important role,

as it is a source of nutrients and structural components, but also of cell-damaging agents. Additionally, it can work as a sink for compounds, a channel for exchanging information, and a platform for interaction and for extending cellular functionalities [5].

In cyanobacteria, several uptake and secretion systems have been described, which mediate the transport of various substrates. However, the knowledge regarding some of the most fundamental aspects underlying transport across the cyanobacterial cell envelope remain unknown (details of the cyanobacterial cell envelope are shown in Figure 1). For example, only recently has the outer-membrane permeability of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 been investigated [6]. Remarkably, the permeability of this cyanobacterium outer membrane was found to be 20-fold lower than that of *Escherichia coli* [6], highlighting once more that what is known for the most well-documented Gram-negative bacteria should not be directly extrapolated to cyanobacteria.

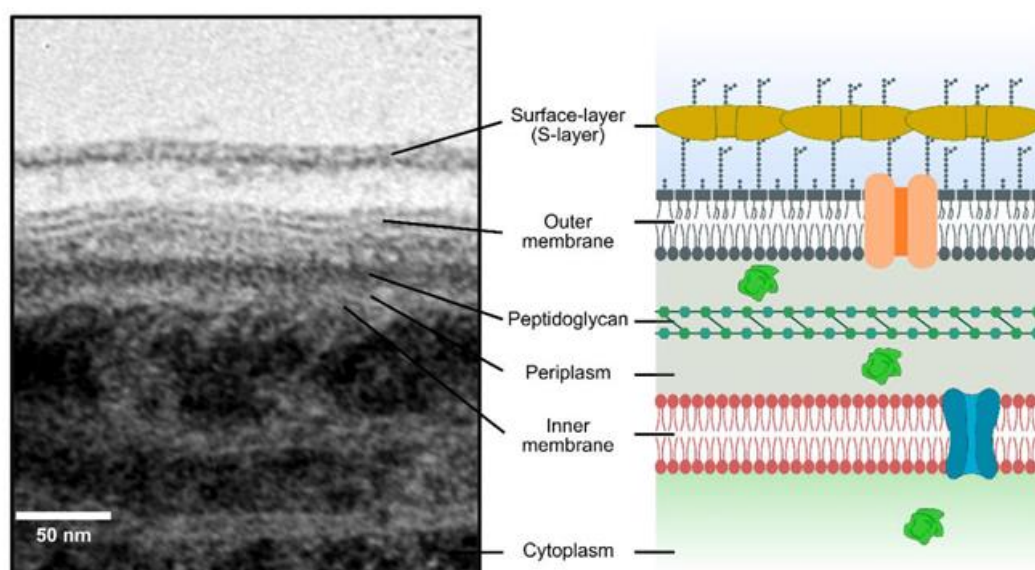


Figure 1. Cell envelope of the model, unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Electron micrograph showing details of the *Synechocystis* sp. PCC 6803 cell envelope (left panel), which is schematically represented on the panel on the right-hand side (not to scale).

A number of biomolecules have been shown to be produced and secreted by cyanobacterial cells, including proteins (e.g., [7–10]), polysaccharides (e.g., [11–13]), glycolipids (e.g., [14–16]), and even fatty acids (e.g., [17–19]). Moreover, secretion is also vital to rid the cyanobacterial cell from harmful, exogenous compounds that reach the cell’s interior, such as antibiotics (e.g., [9,20,21]) or excess of metals (e.g., [22,23]). Thus, product export represents an important survival strategy, playing crucial roles in a number of aspects of cyanobacterial physiology, like motility [24], adhesion [25], protection against desiccation [26], detoxification of extracellular reactive oxygen species [8,27], noxious compound efflux [9,20,21,28], etc. Throughout the years, several reports have focused on the identification and characterization of molecular machines mediating the translocation of products of metabolism, and others across the cyanobacterial inner and outer membranes (for reviews, see [29,30]). These systems, in which substrates are transported to the extracellular space via secretory portals located at the cell’s inner and outer membranes, are classified as “classical secretion systems”. Nevertheless, in terms of secretion strategies, one system has been highly overlooked in cyanobacteria—the release of extracellular vesicles (EVs), a mechanism of “non-classical secretion” (Figure 2).

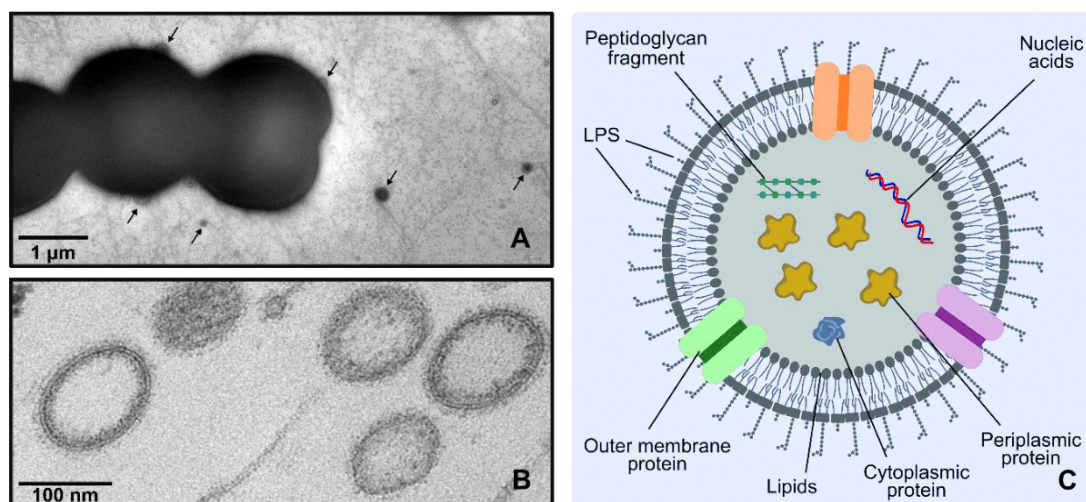


Figure 2. Cyanobacterial extracellular vesicles (EVs). **(A)** Transmission electron micrograph of negatively stained *Synechocystis* sp. PCC 6803 cells showing EVs (highlighted by arrows) on the cells' surfaces and in the extracellular medium. **(B)** Transmission electron micrograph showing ultrastructural details of EVs isolated from *Synechocystis* sp. PCC 6803 culture medium. **(C)** Diagram showing the general composition of EVs from Gram-negative bacteria, including those of the marine cyanobacterium *Prochlorococcus* MED4 [31]. These vesicles are composed mainly of outer-membrane components (lipopolysaccharides (LPS); outer-membrane proteins; and lipids), periplasmic content (proteins; peptidoglycan fragments; metabolites), and, in some instances, even cytoplasmic proteins and nucleic acids (DNA and/or RNA).

EVs have been detected in a number of cyanobacterial strains, including the unicellular *Synechococcus* sp. PCC 7002 [32], *Prochlorococcus* spp., *Synechococcus* sp. WH8102 [31], *Synechocystis* sp. PCC 6803 [9,33], *Synechococcus elongatus* PCC 7942 [34] and *Cyanothece* sp. CCY 0110 [35], in the filamentous, non-heterocystous *Jaaginema litorale* LEGE 07176 [36], and in the filamentous, heterocystous *Anabaena* sp. PCC 7120 [8], *Cylindrospermopsis raciborskii* (CYRF-01) [37] and the *Azolla microphylla* symbiont [38]. However, the study of EVs in cyanobacteria has not received as much attention as in other bacteria. In this work, we focus on EVs released by Gram-negative bacteria, highlighting several aspects related to their composition, biogenesis and, most importantly, proposed biological roles. In addition, we gather the limited and fragmented information available on cyanobacterial EVs, and discuss this in light of what has been reported for other bacteria. As the biotechnological potential of cyanobacteria is becoming increasingly explored and established, we also draw special attention to several applications that have been tested and implemented with bacterial EVs. Altogether, our hopes are that researchers studying cyanobacteria become more acquainted with EVs, and that they decide to embark on the journey of elucidating further how relevant this unconventional secretion mechanism is for cyanobacterial cell survival.

2. EVs in Gram-Negative Bacteria

EVs were first reported more than 50 years ago [39]. Initially, they were regarded as mere artefacts of the electron microscopy technique or byproducts of bacterial growth [40]. However, subsequent studies definitively showed their production by different microorganisms [41]. Today, practically every microbial species tested, ranging from fungi to bacteria and archaea, has been shown to produce EVs, which suggests that EVs are a feature of every living cell [42]. EVs are discrete and non-replicable proteoliposomal nanoparticles [43], ranging in size between 20 and 500 nm in diameter [44]. Released by the bacterial cell envelope, EVs from Gram-negative bacteria naturally contain lipopolysaccharides, phospholipids and outer-membrane proteins; nevertheless, as EVs are

not just small vesicles derived entirely from the outer membrane, periplasmic proteins, peptidoglycan fragments, and even cytoplasmic proteins and nucleic acids have been identified therein [40]. Due to the EVs' structure and composition, their content is strongly shielded against the activity of lytic enzymes present in the environment [45], preventing cargo degradation after leaving the cell. This way, the content of EVs can reach far longer distances than biomolecules released by the classical secretion systems. Moreover, EVs contain more than one cargo, all of which are simultaneously delivered to otherwise inaccessible targets. EVs' lumen, which resembles that of the periplasm in terms of its composition, contributes to the conservation of the cargos' features [45]. In particular, hydrophobic compounds, or those that demand targeted deliverance, can be transported in such nanostructures. Importantly, just as in the classical secretion systems, the formation and release of EVs can be tightly regulated by the cell, and so confer an adaptive advantage under stressful conditions.

3. EVs' Composition

One of the most well-characterized features of EVs is, perhaps, their biochemical composition. Several technical solutions are available to identify and quantify proteins, lipids and genetic material, which, combined with an ever increasing array of isolation methods (ultracentrifugation (including density gradient), ultrafiltration, hydrostatic dialysis, gel filtration (size-exclusion chromatography), precipitation, or even microfluidic devices and immunoprecipitation) [46], have helped clarifying many aspects of EVs' composition and biology. As briefly presented above, and despite cell envelope particularities found among Gram-negative bacteria, EVs are generally enriched in outer-membrane components [40]. Other cellular constituents have also been detected in EVs, namely periplasmic proteins and peptidoglycan fragments [47], and also metabolites, cytoplasmic proteins and nucleic acids [40,48] (Figure 2).

Increasing evidence supports the belief that bacteria modulate EVs' composition. For example, in *Salmonella* sp., EVs isolated from cultures grown under different conditions showed different compositions [49]. While proteins involved in translation and cellular metabolism were preferentially detected in EVs recovered from cultures grown with nutrient replete media, membrane proteins involved in nutrient acquisition were identified in EVs isolated from cultures maintained under limited nutritional conditions [49]. This study highlights the fact that EVs' content may vary according to the physiological state of the cell, and suggests that EVs' composition mirrors that of the parental cell at the moment of release. However, several others reported that the bacterial cell is capable of sorting what goes into EVs. The enrichment of a specific cargo that is scarce in the cell is illustrative of this fact. Conversely, the absence in EVs of highly abundant molecules in the cell and/or cell surface also indicate an EV-regulated packaging mechanism. In many bacterial species, whole-cell or outer-membrane and periplasmic proteome analyses showed differences when compared to isolated EVs [47,50]. An excellent showcase can be found in the bacteria of the genus *Bacteroides* [51–53]. These bacteria play a crucial role in decomposing complex polysaccharides in the human gut, being a typical resident of the intestinal microbiome. The ability is conferred by the expression of genes located in polysaccharide utilization loci (or PULs). A PUL represents a single genomic locus, which encodes proteins required to bind a specific polysaccharide at the cell surface, to perform an initial cleavage to oligosaccharides, to import these oligosaccharides into the periplasmic space, to complete the degradation into monosaccharides, and to regulate the PUL gene expression [54]. Comparative analyses between EVs and outer-membrane proteomes performed in *Bacteroides fragilis* and *B. thetaiotaomicron* revealed proteins exclusively detected either in EVs or in outer-membrane fractions [51,53]. Furthermore, EV proteomes were rich in PUL-encoded proteins, namely acidic lipoproteins with hydrolytic or carbohydrate-binding activities [51,53]. Thus, it appears that a selective packaging mechanism exists to load PUL-encoded hydrolytic enzymes into EVs [52]. Moreover, the regulated encapsulation of cargos into EVs is commonly observed in the context of pathogenesis, in which EVs work as a vehicle for virulence factors. Within the several cases reported, one highlight is the human oral *Porphyromonas gingivalis* that secretes EVs preferentially loaded with gingipains,

a family of proteases that degrade host cytokines, leading to a decrease in the inflammatory response and facilitating bacterial invasion [55]. A three- to fivefold enrichment of gingipains was observed in EVs derived from *P. gingivalis* 33277 and W83, respectively, compared with levels in their parent bacterial strains [56], while major outer-membrane proteins seem to be excluded [56,57].

Because of the EVs' composition modulation capacity demonstrated by bacteria, the population of EVs isolated from a given bacterium at a specific time point can be highly heterogeneous. To illustrate this fact, one should note that within a determined population of recovered EVs, not all the vesicles are equal regarding their physical properties, namely in terms of their size and buoyant density. Thus, when studies report the detection of certain molecules in isolated EVs, it does not mean that all vesicles contain the same types of molecules. In a study that focused on a biological comparison of EVs from *E. coli*, the authors could separate EV subgroups from crude EV preparations [58]. The isolated EV subgroups presented molecularly distinct characteristics in terms of protein and endotoxin content, and amount of RNA [58], illustrating the natural biological heterogeneity of bacterial EVs.

Composition of Cyanobacterial EVs

Very limited information is available regarding the composition of cyanobacterial EVs. What is known is that EVs isolated from the marine cyanobacterium *Prochlorococcus* sp. have been shown to contain lipopolysaccharides, a number of typical cyanobacterial lipids (namely monoglycosyldiacylglycerol and sulfoquinovosyldiacylglycerol), and a diverse set of proteins, including periplasmic and membrane proteins (nutrient transporters, proteases, porins, hydrolases), but also cytoplasmic proteins (ribosome-associated proteins, and even RuBisCO) [31]. *Prochlorococcus* MED4 EVs were also reported to contain DNA and RNA: interestingly, DNA fragments detected in EVs measured at least 3000 bp, potentially encoding several genes. In addition, within the whole population of DNA fragments identified, an overabundance of reads was found, corresponding to a specific region of the chromosome [31]. As for RNA, sequences from as many as 95% of all open reading frames in the genome could be detected [31].

Altogether, the budding and detachment of EVs from the bacterial cell during active growth seem to constitute a regulated process rather than an accidental cell envelope disorganization event or a simple byproduct of cell lysis [40]. EVs' composition varies in agreement with whole-cell proteomic and lipidomic changes and adaptations, or as a result of specific sorting mechanisms, both of which are intimately dependent on how EVs are formed and released, i.e., on their biogenesis. While the presence of periplasmic and outer-membrane components in EVs can be easily perceived, the detection of cytoplasmic proteins and nucleic acids require an in-depth analysis of alternative routes for vesicle production. In the following section, we focus on the EVs' formation mechanisms.

4. EVs' Biogenesis

When addressing EVs' biogenesis, a point that requires clarification is their nomenclature. In the beginning, when EVs were first described, as the vesiculation process appeared to occur exclusively from the outer membrane, EVs were designated outer-membrane vesicles (OMVs). Even today, a great majority of the published articles refer to the vesicles isolated from the extracellular milieu as OMVs. Nevertheless, recent studies have shown additional routes for vesicle formation. Accordingly, the new types of vesicles were named outer-inner-membrane vesicles (OIMVs), explosive outer-membrane vesicles (EOMVs) and tube-shaped membranous structures (TSMs) [41]. Hence, the term extracellular vesicles (EVs) is used here to refer to the whole population of vesicles released by cells, irrespective of their origin. However, it should be noted that Gram-negative EV studies predominantly discuss OMVs, and so most of the models describing vesicle formation and release address the classic concept of OMVs [59]. Here, we try to present a general overview of the various biogenesis hypotheses (Figure 3).

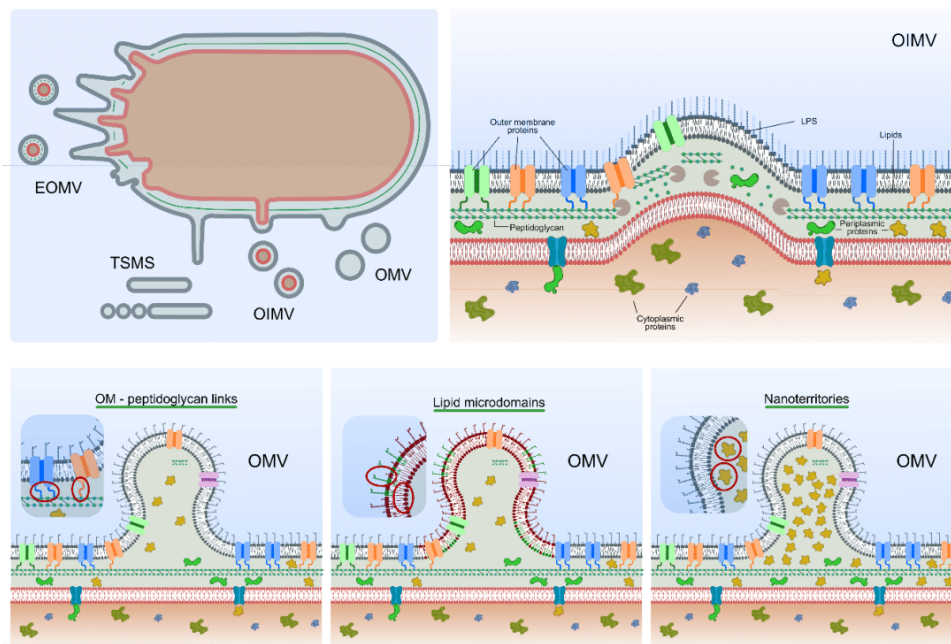


Figure 3. Gram-negative extracellular vesicles biogenesis models. The term “extracellular vesicle” is broadly used to describe various type of vesicles and structures released by bacterial cells. Four sub-groups of EVs have been described [41]: explosive outer-membrane vesicles (EOMV); tube-shaped membranous structures (TSMSs); outer-inner-membrane vesicles (OIMV), which have been proposed to form as a result of the action of peptidoglycan hydrolases in specific regions of the cell envelope, promoting the protrusion of the inner membrane into the periplasm and forcing the outer membrane to bulge [41,59,60]; and the classical outer-membrane vesicles (OMV) (lower panels). Different hypotheses have been formulated to explain the formation of OMVs [40,48,59], including modulation of the outer membrane–peptidoglycan links (left panel), lipid/LPS differential composition in specific regions of the outer membrane, termed lipid microdomains (middle panel), and accumulation of misfolded periplasmic proteins or envelope components in nanoterritories (right panel). Inset in each panel highlights different key components putatively involved in OMV formation, as per the respective model.

One aspect that is likely common for any EV formation model is the absence of linkages between the outer membrane and the underlying peptidoglycan in the blebbing region [61]. Envelope stability in most Gram-negative bacteria results from a number of interactions between the peptidoglycan and different components located in the outer and inner membranes [40]. The peptidoglycan-interacting outer-membrane components include the lipoprotein Braun (Lpp), the outer-membrane protein A (OmpA, which is a porin), and the Tol–Pal complex [40]. Thus, areas in the envelope devoid or depleted of attachments may represent hotspots for blebbing and EV formation. Accordingly, the overall number of Lpp–peptidoglycan crosslinks has been shown in some instances to inversely correlate with EV production. In *E. coli*, NlpI is an outer-membrane protein with a role in cell division, but it has also been shown to regulate the activity of a peptidoglycan endopeptidase (Spr, or MepS) that attacks peptide bonds in the peptidoglycan, modulating its crosslinks [62–64]. The *nlpI* mutant is a hypervesiculating strain, presenting approximately 40% less Lpp–peptidoglycan crosslinking than the wild-type strain [63]. Conversely, the loss of the diaminopimelic acid (a component of the peptidoglycan; DAP)–DAP peptide crosslinks resulted in an increase in Lpp–peptidoglycan crosslinking levels, which, in turn, led to hypovesiculation [65]. Furthermore, mutants lacking OmpA in several genera of Gram-negative bacteria resulted in EV overproduction, likely as a consequence of reduced outer membrane–peptidoglycan crosslinking [66–68]. Whether or not a specific mechanism exists to modulate these linkages remains unknown [61]. Consistent with the hypothesis of the

outer membrane's dissociation from the underlying peptidoglycan, without compromising envelope integrity, portions of the peptidoglycan may be locally weakened by hydrolases in a targeted manner, allowing the invasion of the inner membrane into the periplasmic space. This protrusion, initiated by the inner membrane, ends up blebbing as an OIMV, whose envelope is similar to that of a cell. This model puts forward a possible explanation for the presence of nucleic acids and cytoplasmic proteins in EVs, as the OIMVs' lumen originates from the cytoplasm [41,60]. Nevertheless, it is not completely certain that these OIMVs are the only EVs to contain nucleic acids, and the mechanism that enables DNA fragments to be generated and incorporated therein is far from understood.

Despite the crucial role of outer-membrane components–peptidoglycan interactions in modulating EV formation, vesicle production has also been shown to occur independently of the total level of interactions and crosslinks. In fact, mutants with high “periplasmic pressure”, resulting from an increase in misfolded periplasmic proteins, or accumulating high concentrations of envelope proteins, peptidoglycan fragments or LPS, were found to hypervesiculate [65]. This suggests an alternative route of EV biogenesis, in which these envelope components accumulate in nanoterritories, forcing the outer membrane to bulge outwards and to form a vesicle, relieving the cell from unwanted envelope components [40,59]. However, this model relies on a reduction in the crosslinking level in very specific regions, while the level of outer-membrane components–peptidoglycan crosslinks throughout the cell envelope remains constant [40,59,65].

As bacterial EVs are structurally composed of various lipid species and LPS (with rather different biophysical properties in terms of size, charge, and rigidity), determining membrane curvature and fluidity, some suggest their direct involvement in EV biogenesis. Thus, regions of the outer membrane may be enriched in particular types of lipids, LPS and/or specific LPS-associated molecules, representing lipid microdomains [40,59]. A combination of these lipid species and associated molecules may significantly increase the chance of producing outer-membrane blebs, resulting in EV production. To illustrate the role of lipid/LPS-associated molecules in the process of EVs biogenesis, one can highlight the major breakthroughs achieved in the study of the *Pseudomonas* quinolone signal (PQS). The PQS has been studied primarily in the context of its role as a quorum-sensing signaling molecule [69]. However, the exogenous addition of the hydrophobic PQS was found to affect the curvature of the outer membrane in *Pseudomonas* spp. as well as in other bacteria [70–72], promoting EV formation, as it incorporates into and/or fuses with LPS aggregates [70–72] and interacts with phospholipids [71].

Specific cellular responses may also lead to EV formation, which is particularly important in the context of environmental settings. This is the case for EOMVs, which are EVs formed as a consequence of cell lysis, triggered by phage-derived endolysins that degrade the peptidoglycan, after which the cell rounds up and explodes [41,73,74]. This mechanism of EV release has only been observed and reported in *Pseudomonas aeruginosa* [74], but is likely to occur in many other Gram-negative bacteria. EOMVs are formed due to the normal tendency of shattered membrane fragments to reorganize into vesicles, randomly capturing buoyant cellular material [41]. Hence, EOMVs' luminal content may vary significantly in comparison to other EVs.

Finally, TSMSs represent a specialized type of EVs, comprising nanotubes, nanowires and nanopods. These structures are tube-like protrusions of the outer membrane, forming bridges between cells at the level of the periplasmic space, particularly produced in biofilms [41] but also detected in liquid media [75]. It has been suggested that TSMSs may facilitate the transfer between the cells of periplasmic proteins and metabolites, membrane proteins, and lipids [41,59]. Not much is known about how these structures are formed, and the simplest version detected was a chain of EVs in *Myxococcus xanthus* [76]. However, EVs have also been observed to pinch off from nanotubes in *Vibrio vulnificus* [77].

The models presented above elegantly address the contribution of various factors in EVs' biogenesis, and no vesicle formation process proposed thus far is widely accepted as the consensus. As the hypotheses discussed are not mutually exclusive, it is possible that a combination of factors and mechanisms operate to produce vesicles. Still, in the field of EV biogenesis, and in light of the presented models, a challenge that remains largely unsolved is to explain how soluble cargo is selected for EV packaging. It has been proposed that the sorting of soluble bacterial proteins into EVs may result from

direct or indirect interactions with the periplasmic face of specific integral or auxiliary outer-membrane components, or even lipids, which are likely to be included in future vesicles. Conversely, components may be excluded from EVs by similar interactions with outer-membrane components that are not prone to budding [40]. Nevertheless, more experimental data are needed to validate this model.

EVs' Formation in Cyanobacteria

Several electron micrographs have shown EVs on the cell wall of different cyanobacterial strains (Figure 2; e.g., [9,31,36,78]). Nevertheless, the knowledge regarding EVs' biogenesis in these microorganisms is still very limited. Owing to the unique features presented by the cyanobacterial cell envelope, it can be anticipated that cyanobacterial EVs may have a distinct composition and rather specific formation mechanisms. On the outer-membrane level, structural differences in the lipid A moiety of LPS compared to that usually found in enterobacteria (in most cyanobacteria, the glucosamine disaccharide backbone is not phosphorylated, and the hydroxyl fatty acid chains are different [30]), the presence of species-specific carotenoids [79], the absence of the Braun lipoprotein Lpp (which establishes covalent interactions with the peptidoglycan; see above), are just a few of the factors that may determine specific mechanistic adaptations for EVs' biogenesis in cyanobacteria. Others may include peptidoglycan thickness (significantly higher when compared to other Gram-negative bacteria), the extent of peptidoglycan crosslinking (close to 60%, as opposed to the usual 20–30% found in most Gram-negative bacteria), the presence of specific polysaccharides complexed with the peptidoglycan [80], and unique lipid composition (while most bacteria contain phospholipids as major glycerolipids, cyanobacteria generally contain three glycolipids, monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol, and a phospholipid, phosphatidylglycerol [81]). In addition, some cyanobacterial strains possess a surface layer (S-layer) (see Figure 1), the outermost structure of the cell envelope, composed of a glycoprotein with the capacity of self-assembly [82]. The S-layer in *Synechocystis* sp. PCC 6803 has been proposed to contribute to the integrity of the cell wall [83], conferring mechanical and osmotic cell stabilization, almost as an exoskeleton [84]. Using a number of *Synechocystis* sp. PCC 6803 mutant strains, impaired in several secretory functions, Gonçalves et al. observed that strains lacking the S-layer consistently presented a higher vesiculation capacity [28]. The authors hypothesized that the S-layer in *Synechocystis* sp. PCC 6803 represents a physical barrier for the biogenesis and release of EVs [28].

In summary, Gram-negative bacteria produce different types of EVs (OMVs, OIMVs, EOMVs and TSMs). Remarkably, the production of EVs is apparently a vital mechanism as neither a bacterial species nor a mutant without the capacity to release EVs has been reported thus far [42]. As EVs' composition is intimately determined by the biogenesis process, an in-depth understanding of these mechanisms will continuously contribute to appreciate how bacteria selectively package EVs, and uncover their biological roles.

5. EVs' Biological Roles

Today, bacterial EVs are well established and are accepted to be involved in several biological functions. Intra- and interspecies communication, defense, the uptake of resources, the release of metabolic byproducts, detoxification, responsiveness to abrupt envelope stresses, horizontal gene transfer, acting as 'decoy' agents, as well as functioning as public goods, are among the proposed roles of bacterial EVs [40,48] (Figure 4). For instance, *Helicobacter pylori* releases EVs selectively decorated with catalase KatA on their surface, which contributes to neutralizing reactive oxygen species from the host, working as a mechanism to aid in evading host immune responses during infection [85]. Interestingly, *H. pylori* EVs showed a better performance in hydrogen peroxide detoxification than whole-cell lysates [85]. Moreover, Lekmechai et al. demonstrated that these KatA-decorated EVs were able to protect *H. pylori* katA-null mutants from the hydrogen peroxide noxious effects, while EVs from these mutants or heat-inactivated KatA-decorated EVs could not [85]. With this example, it is possible to highlight that: (i) EVs can be enriched with a crucial biological component, which is active in the extracellular environment; (ii) EVs contribute to host infection;

and (iii) EVs released by specific bacteria may aid other bacteria to promote survival. Moreover, there are numerous cases in which antibiotic resistance enzymes have been detected in isolated EVs [86–88], resulting not only in a defense strategy, but also in large-spectrum community tactics. In this context, nucleic acid exchange between bacterial cells via EVs [89–91] represents a serious health-threatening issue, as antibiotic resistance determinants encoded in genetic material may be transferred by horizontal gene transfer. Another example to highlight EVs importance is their presence in biofilms [70,92,93], which are competitive niches that can comprise several bacterial species. It is thought that, due to the natural structure and secreting advantages of EVs, they could have a role in communication through the biofilm matrix to ease bacterial cooperation [70]. Biofilms may also function as well as a way to adhere to certain surfaces to facilitate the process of invasion, in which EVs may also play a critical role. That is the case described for EVs from the oral pathogen *P. gingivalis*, which enables the recruitment and adhesion of other bacterial players to the biofilm, increasing the chance of dental plaque formation [50,94]. The role of EVs in biofilms can be further illustrated by *Xylella fastidiosa*, a serious crop-threatening pathogen. During plant colonization, *X. fastidiosa* migrates and proliferates within xylem vessels. It has been shown that *X. fastidiosa* hypervesiculating mutants present a higher virulence capacity than the wild-type bacterium [95]. It was proposed that the production of EVs by *X. fastidiosa* represents a system that helps to control the state of biofilm formation: as the surface composition of EVs released by *X. fastidiosa* is very similar to that of the cells, EVs bind to the xylem vessels and block the attachment of *X. fastidiosa* to surfaces [95]. This allows the bacterial cell to modulate its adhesion capacity, in order to change from an “exploratory” lifestyle, for spreading within the plant host, to a more adhesive type, prone to insect transmission [95].

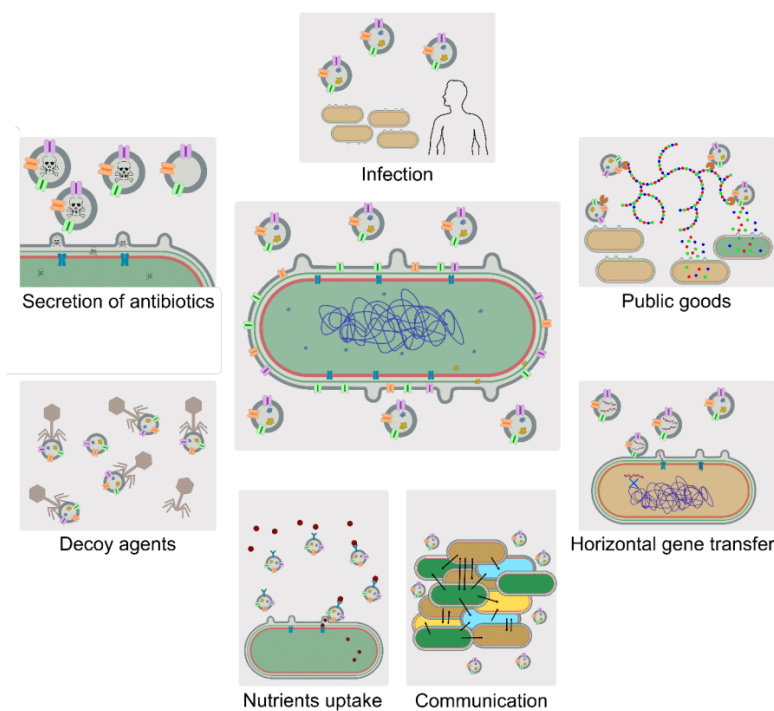


Figure 4. Diagram summarizing the biological roles proposed for extracellular vesicles from Gram-negative bacteria. EVs may have the following functions: in infection, by eluding the host immune response; as public goods, as they may carry catalytic enzymes that help deconstructing complex extracellular biomolecules, making them available for the EVs-releasing bacteria and for other organisms; in horizontal gene transfer, as they transport nucleic acids that can be transferred between organisms; in communication, e.g., in the context of biofilms; in the uptake of nutrients (e.g., [96,97]); as decoy, for example to evade phage infection; and in the secretion of antibiotics (e.g., [98,99]).

Biological Functions of EVs in Cyanobacteria

Cyanobacterial EVs have also been associated with key physiological roles. For instance, an increase in vesiculation was observed in *Cylindrospermopsis raciborskii* CYRF-01 in response to UV radiation [37]. A similar hypervesiculation response was detected when this cyanobacterium was co-cultured with *Microcystis aeruginosa* (MIRF-01). The authors suggested that hypervesiculation was an adaption response to the physical and biological environmental stressors [37]. Moreover, the work by Biller and co-workers on *Prochlorococcus* sp. EVs suggested that these spherical nanostructures can work as a “smoke-screen”, preventing cyanophage infection [31]. Due to the similar composition between outer membranes from *Prochlorococcus* sp. and their EVs, cyanophage receptors are equally found on living cells and vesicles. This could explain the numerous accounts of phages bound to vesicles reported by Biller et al. [31], some of which seemed to have injected their genetic material into the EV. Thus, EVs may help the cell to escape phage infection, and so represent a crucial element of cyanobacterial population dynamics in the ocean. As *Prochlorococcus* sp. are the main primary producers in open oceans [100], this work represents also a major breakthrough in the understanding of the role of cyanobacterial EVs in other ecological aspects, namely as vectors for carbon fluxes in the ocean, as the metabolites and biomolecules packaged in *Prochlorococcus*-derived EVs were shown to be able to exclusively sustain the growth of heterotrophic bacteria [31]. Moreover, nucleic acids were found in EVs released by this highly abundant microorganism. Thus, vesicles from *Prochlorococcus* sp. truly represent a reservoir of genetic information, proposed to be active in horizontal gene transfer in marine ecosystems [31,101]. Another report supports the presence of DNA in cyanobacterial EVs: Zheng et al., showed that the cyanobiont present in the water fern *Azolla microphylla* sporocarp abundantly releases EVs [38]. Remarkably, up to 90% of the detected vesicles that were released by the cyanobiont filaments upon their initiation of prokinete/akinetes differentiation contained DNA [38]. This observation suggests possible lateral gene transfer between the symbiotic partners. In addition, the released EVs may also contribute to cyanobacterial biofilm formation and outer envelope development during akinetes differentiation and during sporocarp development, as proposed [38].

In line with reports from other Gram-negative bacteria, vesiculation in cyanobacteria also seems to be adjusted in response to specific genetic modifications, further suggesting that EVs may contribute to the homeostasis of the cyanobacterial cell. The deletion of the two glycogen synthase genes (*glgA-I* and *glgA-II*) in *Synechococcus* sp. PCC 7002 resulted not only in an accumulation of soluble sugars, but also in the spontaneous secretion of high levels of soluble sugars into the medium, without the need for additional transporters [32]. Moreover, numerous EVs budding from the outer membrane of the mutant could be observed, and the authors hypothesized that the release of EVs could mediate soluble sugar excretion [32]. In a different work, the deletion of the gene encoding the outer-membrane protein TolC in *Synechocystis* sp. PCC 6803 resulted in a strain with a high vesiculation capacity [9]. TolC is crucial for the export of several types of biomolecules, ranging from proteins [7,9,25,28] to fatty acids [17], and exogenous compounds that reach the cytoplasm [9,20,21,28]. It was proposed that the vesiculation was a response to the impaired classical secretion [9]. In a follow up study, deletion mutants in some inner-membrane and periplasmic components, parts of the TolC-dependent efflux pumps, showed as well-altered vesiculation [28]. Mutants with a higher vesiculation capacity than the wild-type strain were found to lack the S-layer (see above, EVs’ biogenesis). Nevertheless, none of these mutant strains reached the high vesiculation level presented by the *tolC*-deletion mutant [28]. It remains to be fully determined why the *tolC*-deletion mutant hypervesiculates, but there is evidence that the strain is under some sort of envelope stress, as specific periplasmic chaperones and proteases are highly upregulated (*spy* and *degQ*) and overexpressed (*Spy* and *DegP*) compared to the wild-type [28]. Genetic modifications in cyanobacteria that affect vesiculation do not always result in hypervesiculation. In a recent work, a hypovesiculating *Synechocystis* sp. PCC 6803 mutant was reported, in which the gene encoding the alternative sigma factor SigF was deleted [102]. Nonetheless, SigF was found to be a broad player in the control of several metabolic and cellular processes, including different secretion

mechanisms. The lower vesiculation capacity was therefore proposed to be part of a large adaptation process, rather than a specific target of SigF regulation [102].

As more bacterial species become characterized in terms of their EVs production capacities, more biological roles are attributed to EVs. The fact that many of these bacteria can be genetically modified and engineered has contributed towards the recognition that bacterial EVs may be used to fulfil specific technological goals.

6. Biotechnological Applications of Bacterial EVs

Fundamental research carried out in the past 20–30 years has significantly contributed to our better understanding of the mechanisms of EVs biogenesis in bacteria, their composition and properties, as well as their biological roles. The capacity of EVs to package multiple cargos, the fact that cargo properties are maintained in EVs, and the extraordinary advantage of EVs in protecting and trafficking cargo to otherwise inaccessible targets [45] are just a few of the EV attributes that have promoted their application in biotechnology. Furthermore, through the constant developments in bioengineering and genetic editing techniques, it is now possible to tailor EVs' exterior features, as well as modulate their cargo [103,104]. As a result, EV-based nanotechnology has been evolving quickly into a powerful and innovative toolkit for various fields (Figure 5), even though several challenges still need to be addressed, mainly regarding their scalability, commercial viability, and safe and efficient clinical use [103].

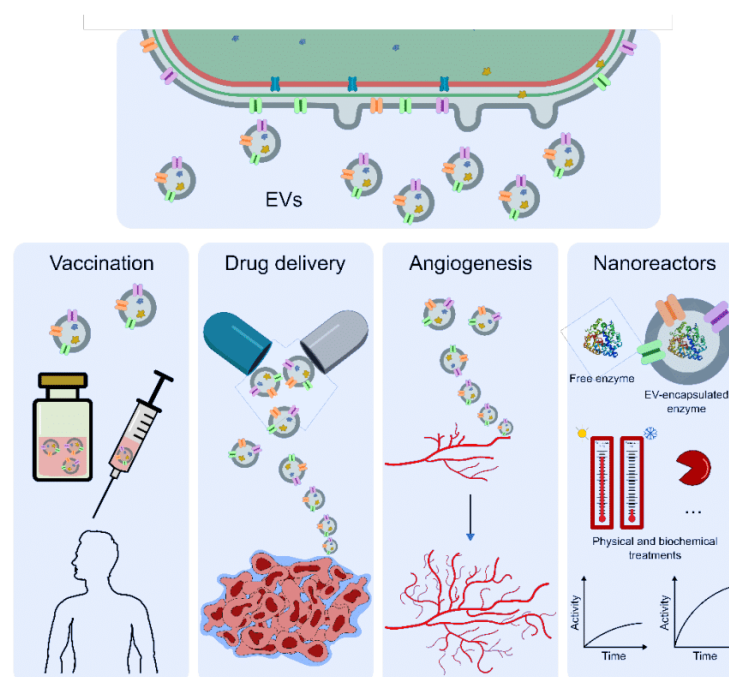


Figure 5. Illustration summarizing some applications that have been tested with bacterial EVs. One field that has benefited enormously from the potential of bacterial EVs is biomedicine. The best example is perhaps the use of EVs in vaccination, for which the commercial vaccine Bexsero® is a case of widely accepted success. Bacterial EVs have also been tested with promising results to deliver cargo in a cell-specific manner, in particular in the context of cancer research (e.g., [105,106]). EVs from the cyanobacterium *Synechococcus elongatus* PCC 7942 have been shown to positively modulate and even promote angiogenesis in the context of wound healing. Bacteria have also been genetically engineered as to release EVs packaged with enzymes, contributing to the protection of enzymatic functions (e.g., [107]), in what the EVs may be regarded as nanoreactors.

Inherent to the constant necessity of developing novel and more efficient vaccines against various pathogenic agents, EVs have been successfully implemented as potential vaccines/antigen-delivery platforms. Two major strategies have been followed for the development of EV-based vaccination. On one hand, naturally derived EVs from the target pathogenic bacterium can be isolated and directly administered to the subject. In this case, EV protection is mediated by the abundance of pathogen-associated molecular patterns, known to play a key role in stimulating innate immunity and promoting adaptive immune responses. The best example is the EV-containing meningococcal vaccine against *Neisseria meningitidis*, currently commercialized as Bexsero® [96,108–110]. On the other hand, through a genetic engineering approach, recombinant EVs harboring heterologous antigen(s) can be directly obtained from a non-pathogenic bacterial model. In this way, it is possible to develop safe and standardized EVs, capable of eliciting an adequate immune response with great versatility and flexibility [111–113]. The use of engineered *E. coli*-derived EVs loaded with heterologous proteins to work as antigen-delivery vehicles has been tried with very promising results [111]. All of these efforts towards the development of EV-based vaccine strategies offer the opportunity to accomplish highly effective, easy to produce and multi-valent vaccines.

EVs have also been used as specialized carriers for the delivery of therapeutic compounds [103,104]. For instance, bioengineered EVs produced by *E. coli*, presenting a human epidermal growth factor receptor 2-specific affibody molecule on their surface, could target cancer cells in a mouse model in a cell-specific manner [105]. As EVs were loaded with a small interfering RNA (siRNA), the specificity of the EVs' delivery resulted in targeted gene silencing, and thus in a reduction in the protein levels of a key enzyme involved in mitosis, ultimately inhibiting cancer cell proliferation [105]. Moreover, these nanostructures have also revealed their great potential in the delivery of chemotherapeutic agents, as demonstrated with EVs from an attenuated *Klebsiella pneumonia* strain that efficiently delivered the drug doxorubicin to lung cancer cells [106]. Moreover, in the context of cancer, EVs have also been reported as a promising tool for immunotherapy strategies, not only through EV-induced long-term antitumor immune responses [114], but also through their decoration with tumor antigens towards antigen-specific antitumor therapy [115]. Despite the fact that most efforts have been directed towards anti-cancer/anti-tumor therapies, other delivery applications have also been associated with EVs, including their development as antibiotic delivery vehicles. Recently, through the identification of a novel drug resistance mechanism mediated by EVs in *Acinetobacter baumannii*, antibiotic-loaded EVs were shown not only to efficiently deliver and facilitate the entry of antibiotics into pathogenic bacteria, but also to support the sustained bactericidal effect in the intestinal tract of a mouse model [99]. In an era of global concern regarding drug-resistant bacteria, this reported novel class of EVs mediating the delivery of antibiotics may turn out to be extremely useful in reducing antibiotic dosage, and in increasing the bactericidal effect.

Other applications are also being considered and developed, including the use of EVs as natural and versatile nanoreactors. The systematic organization of enzymes for the efficient operation of cascade reactions, resulting in cellulose degradation, has been demonstrated through the use of *E. coli*-derived EVs [116]. The functional assembly of multiple enzymes on the EV surface was accomplished by the construction of a system comprising three orthogonal cohesion domains and a cellulose-binding module, all assembled and anchored through OmpA [116]. By adding their species-specific dockerin parts, all cellulases could be activated, resulting in the amplification of cellulose hydrolysis by more than 20-fold over that of non-complexed enzymes [116]. In addition to performing complex biocatalysis, EVs have also been recently described to protect the native properties and enzymatic activity of phosphotriesterase (PTE), an ideal enzyme for bioremediation [107]. In light of this discovery, it is plausible to extend this EV-mediated enzymatic packaging/protection strategy to other biotechnological fields, including the health sciences context, for example, where the prevention of enzyme degradation on human serum would lead to a more efficient enzymatic delivery and bioactivity.

Cyanobacterial EVs in Biotechnology

Although most of the reports available on cyanobacterial EVs are fundamentally directed towards basic research on EV biology, these microorganisms represent an untapped tool with attractive features and promising applications. Indeed, EVs from *Synechococcus elongatus* PCC 7942 have already been linked to the promotion of angiogenesis and wound healing in vitro and in vivo through the promotion of interleukin 6 expression, highlighting their potential in the field of biomedicine [34]. As novel insights are gained in respect to cyanobacterial EVs, this can be the first of a broad range of prospective applications. In fact, cyanobacterial EVs might have a significant advantage over other bacteria in relation to a relevant issue concerning EVs' structural components, namely regarding LPS. In high dosages, LPS from some Gram-negative bacteria, including *E. coli*, *Salmonella* and *Pseudomonas*, can lead to pathological reactions, including systemic inflammatory responses [117]. Consequently, to avoid toxicity in biomedical applications, administration to the subjects of EVs derived from such organisms must follow rigorous and complex administration schemes, and/or demand vesicle bioprocessing approaches by implementing attenuated mutants or additional LPS detoxification steps [118,119]. Remarkably, evidence from different sources strongly suggests a significantly reduced toxicity in cyanobacterial LPS [120–122]. This low immune reactivity against cyanobacterial LPS can be explained by the missing phosphorylation at positions 1-4' of the glucosamine disaccharide in the cyanobacterial lipid A backbone [30]. The possibility that cyanobacterial LPS do not possess true endotoxic effects that can harm human health [30] underlines the potential of cyanobacteria-derived EVs as valid and safe alternatives to overcome this LPS-induced toxicity hurdle. With the current efforts regarding the development of more robust and reliable molecular tools to metabolically engineer cyanobacteria, EVs may become the target for packaging products of interest that can range from soluble and membrane-bound enzymes, to lipids, long-chain carbon molecules, and metabolites. With this in mind, an array of interesting applications may soon be associated with cyanobacterial EVs.

7. Conclusions

The EV research field is not novel, but there has always been some skepticism among researchers as to the existence and physiological function of EVs [42]. Some of the frequent arguments include the possibility of vesicles being artefacts of lipid self-aggregation, or debris from lysed cells, or even just ordinary waste products. Moreover, other argue that EV release represents a waste of energy, and thus serves no purpose, or that bacteria lack the complex machinery present in eukaryotic organisms determining the release of vesicular bodies, or even, as there are no mutants lacking vesiculation capacity, that EV release cannot be a regulated process [42]. Recent technical advances to isolate and analyze EVs have contributed significantly to dismissing these doubts. As the field develops and EVs become increasingly accepted, there is a demand for standardizing EV research through increased systematic reporting, which can already be followed on the Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research (EV-TRACK) database [123]. In addition, the International Society for Extracellular Vesicles has also published guidelines for EV research, which are frequently updated.

In cyanobacteria, research into EVs is only just emerging, but the limited reports available already suggest that cyanobacterial EVs fulfil important ecological functions, such as evading phage infection [31]. In other instances, vesiculation was detected in response to the deletion of key metabolic enzymes [32] or transporters [9,28], but the full biological meaning of such observations remains to be elucidated. Nevertheless, it is clear that vesiculation in cyanobacteria can be modulated in response to environmental cues [37], thus pointing to the strong possibility of contributing to cell homeostasis and, ultimately, adaptation and survival. The detection of EVs in the context of symbiotic associations [38] opens the door to poorly addressed inter-kingdom communication, with large implications. In particular, the detection of genetic material therein may represent a route of gene transfer, maybe accounting for the presence of cyanobacterial genes in plant nuclear genomes [124].

In the past few years, the advances in the understanding of cyanobacterial biology and metabolism have been intimately related to rapid developments in synthetic biology and metabolic engineering. In addition, these contributions have also placed cyanobacteria in the spotlight in biotechnology, garnering increasing interest from several sectors. Combining cyanobacterial genetic tractability with metabolic plasticity, cyanobacteria can easily become attractive models for the production of EVs with tailored cargo. Some reports indicate that cyanobacterial lipopolysaccharides are less toxic to mammals compared to those isolated from other bacteria [121,122], which can be relevant for EV-mediated delivery.

In summary, the study of EVs in cyanobacteria definitely deserves more attention. Several open questions related to physiological responses, cellular modifications, differentiation, communication strategies and adaptability might be re-analyzed in light of the existence of EVs. Future advances in this field will surely contribute to a better understanding of how cyanobacteria have made their way through the last 2.1 Ga of the Earth's history, while shaping life as we know it.

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CHAPTER III - Isolation and characterization of cyanobacterial extracellular vesicles



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*Participation in: the development of a standardized protocol to isolate and analyze EVs from *Synechocystis* sp. PCC 6803.



Isolation and Characterization of Cyanobacterial Extracellular Vesicles

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Abstract

Cyanobacteria are a diverse group of photosynthetic, Gram-negative bacteria that play critical roles in global ecosystems and serve as essential biotechnology models. Recent work has demonstrated that both marine and freshwater cyanobacteria produce extracellular vesicles - small membrane-bound structures released from the outer surface of the microbes. While vesicles likely contribute to diverse biological processes, their specific functional roles in cyanobacterial biology remain largely unknown. To encourage and advance research in this area, a detailed protocol is presented for isolating, concentrating, and purifying cyanobacterial extracellular vesicles. The current work discusses methodologies that have successfully isolated vesicles from large cultures of *Prochlorococcus*, *Synechococcus*, and *Synechocystis*. Methods for quantifying and characterizing vesicle samples from these strains are presented. Approaches for isolating vesicles from aquatic field samples are also described. Finally, typical challenges encountered with cyanobacterial vesicle purification, methodological considerations for different downstream applications, and the trade-offs between approaches are also discussed.

Introduction

Extracellular vesicles (EVs) are spherical structures, ranging between ~20-400 nm in diameter, released by virtually all organisms into their surrounding environment^{1,2,3}. Vesicles

are delimited by a lipid bilayer and cannot replicate themselves. In Gram-negative bacteria, these structures are thought to primarily arise by 'blebbing' of small portions from



the outer membrane. Still, other processes, including flagellar motion, cell lysis, and secretion of both inner and outer membrane material, can produce vesicles as well^{4,5}. EVs can contain various biomolecules, including lipids, soluble and membrane proteins, nucleic acids, and metabolites, and can transport this material between cells^{4,5,6}. Given these features, EVs are being studied to understand their possible roles in a wide range of biological processes, including cellular communication, biofilm formation, horizontal gene transfer, host-phage dynamics, and nutrient exchange^{4,6}.

Cyanobacteria are a large and diverse group of Gram-negative bacteria, including unicellular and filamentous organisms. They are of interest from many perspectives, including understanding their physiology and diversity^{7,8}, the critical ecosystem functions they serve^{9,10}, and their utility for biotechnology^{11,12}. Cyanobacteria are found in a wide variety of habitats, either as free-living organisms in marine, freshwater, and terrestrial environments, or in symbiotic associations with mosses, ferns, plants, or in lichens and sponges¹³. They serve as crucial primary producers in aquatic ecosystems, producing oxygen and organic carbon through oxygenic photosynthesis^{9,10}, and some are capable of fixing atmospheric nitrogen as well⁷. Marine and freshwater cyanobacteria, including *Prochlorococcus*, *Synechococcus*, and *Synechocystis*, produce EVs under laboratory conditions^{14,15,16}, and cyanobacterial vesicles can also be found in natural environments^{14,17}. The biological and ecological functions of cyanobacterial vesicles are unknown, but further research in this area is likely to provide new insights into questions on cyanobacterial physiology, differentiation, communication strategies, evolution, and trophic interactions. In addition, the

ability of cyanobacterial EVs to carry diverse categories of biomolecules may have commercial applications^{18,19}.

The present protocol describes methods for isolating and characterizing vesicles from cyanobacterial cultures and field samples to enable and encourage a broader examination of cyanobacterial extracellular vesicle biology. While the workflow described here is analogous to protocols for isolating and characterizing EVs from other bacteria, cyanobacterial cultures and field samples typically contain lower cell and vesicle concentrations than are commonly observed with host-associated or pathogenic model systems^{20,21,22}. Thus, studies of cyanobacterial EVs require special considerations and optimizations for cultivation and vesicle isolation, which vary further between strains and media backgrounds. As no single protocol will work equally well for all strains, growth conditions, and downstream applications, we provide multiple options and discuss the trade-offs involved so that researchers may determine the approaches most appropriate for addressing their experimental questions.

Protocol

The cyanobacterial strains are obtained from several culture collections (see **Discussion** for details).

1. Cyanobacterial cultivation

1. Cultivate marine cyanobacteria *Prochlorococcus* and *Synechococcus* following the steps below.
 1. Cultivate *Prochlorococcus* and *Synechococcus* strains in polycarbonate flasks using an appropriate medium such as Pro99 or SN (see **Table of Materials**). For details, please see previously published References^{23,24}.



2. Acclimate the cultures by growing them across two to three transfers (1:20 dilutions of culture into fresh media for each passage) under the desired temperature and irradiance conditions required for the experiment.

NOTE: Typical growth conditions^{23,25} include temperatures between 15-30 °C at irradiance between 8-150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, but individual strain tolerances and optima vary widely²⁶ and need to be set based on guidance from the relevant culture collection instructions or literature. Monitor growth rate using culture fluorescence or flow cytometry counts²³, and ensure that cultures grow at a consistent steady-state exponential rate before beginning the experiment.

3. Carry out a set of successive gradual transfers from smaller to larger volumes once cells reach late exponential phase (e.g., 20 mL < 250 mL < 2 L < 10 L < 20 L).

NOTE: Large volume cultures cannot be directly inoculated from small amounts of starting culture. Note that larger cultures may require the addition of buffers (such as 1 mM HEPES, pH 7.5 or 3.75 mM TAPS, pH 8), along with supplementary sodium bicarbonate or bubbling with sterile air²⁴.

2. Cultivate freshwater cyanobacterium *Synechocystis* sp. PCC 6803.

1. Prepare a culture of *Synechocystis* sp. PCC 6803 to be used as inoculum by cultivating it with aeration (bubbling air at 1 L/min), at 30 °C, under a 16 h light (photons count of 50 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) / 8 h dark regime, up to an optical density at 730 nm (OD730) of 1.0 (exponential phase) in BG11 medium²⁷.

2. Inoculate 30 mL of this culture *Synechocystis* sp. PCC 6803 in 570 mL of BG11 medium to an OD730 of 0.05 in 1 L glass gas washing bottles.
3. Allow cells to grow with aeration, at 30 °C, under a 16 h light (photon count of 50 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) / 8 h dark regime, up to a final OD730 of 1.0-1.5.

2. Separation of cyanobacterial biomass from the small particle (vesicle) fraction

NOTE: First, separating cells from the supernatant through centrifugal pelleting of cells is recommended. However, when culture volumes are too large for this to be feasible, it is possible to skip centrifugation and proceed directly to filtering whole cultures using capsule filtration (step 2.4).

1. Perform centrifugation to separate cells (best for volumes up to ~4 L, depending on available centrifuge capacity).
 1. Autoclave or thoroughly wash and clean the centrifuge bottles using Type I ultrapure grade water (see **Table of Materials**), ensuring that no residual soap or other material remains.
 2. Load culture samples into an appropriate number of centrifuge bottles and balance them.
 3. Spin cultures for >10,000 x g at 4 °C for 10 min. If the supernatant remains visibly turbid, increase centrifugation conditions to 20 min at maximum speed and retry.
 4. Carefully decant or pipet supernatant into a clean vessel and proceed to one of the following filtration options mentioned in steps 2.2-2.4.
2. Option 1: perform syringe filtration using 0.2 μm filters (for sample volumes up to ~50 mL).

1. Fill a sterile 50 mL syringe with a largely cell-free culture medium, and filter it through a 0.2 (or 0.45) μm polyethersulfone (PES) filter (see **Table of Materials**). Collect the filtrate in a clean, sterilized receptacle.
2. Repeat this step until the filter is clogged (e.g., it becomes difficult to push with the syringe). Replace with a new filter, and proceed until the sample is thoroughly filtered.
3. Option 2: perform 0.2 μm vacuum filtration (up to ~4 L).
 1. Thoroughly wash and clean the vacuum apparatus using Type I - ultrapure grade water, connecting it to a vacuum trap and pump (see **Table of Materials**).
 2. Insert a 0.2 μm filter of an appropriate diameter and clamp the vacuum apparatus.
 3. Add a small amount of culture sample, ensuring that the vacuum pressure remains <10 psi.
 4. Continue filtering the culture in small increments until complete. If the filtration rate slows significantly, stop the vacuum and replace the filter.
 5. Collect the <0.2 μm fraction containing vesicles into a clean container.
4. Option 3: perform 0.2 μm capsule filtration (for sample volumes >~4 L)
 1. Clean appropriately sized flexible tubing and collection vessel by washing with water and mild detergent. Rinse the material with distilled and deionized water.

NOTE: Prior to utilization, the material should be rinsed with Type I - ultrapure grade water.
 2. Place the culture to be filtered in a secure, elevated location with the final collection vessel below.

Connect a 0.2 μm capsule filter (see **Table of Materials**) outflow port to one piece of tubing and place it in a collection vessel.

3. Place another tubing into the sample, begin a gravity siphon by pulling some sample into the tubing with a syringe, and connect the tubing to the capsule filter. Use the vent to release excess air and fill the chamber with supernatant.
4. Allow material to move through the capsule until the sample is thoroughly filtered. If the flow rate becomes too slow (<~1/10th the starting flow rate, or coming out dropwise as opposed to a continuously flowing stream), wait or apply gentle force with a peristaltic pump until backpressure increases. Alternatively, partially restore the flow rates by temporarily disconnecting the filter, backflushing accumulated biomass from the inflow side with ultraclean water until the material is no longer visibly turbid, and then restart the filtration process.

3. Concentrating the vesicle sample

1. Option 1: perform centrifugal ultrafiltration for concentrating small (<500 mL) volume of samples.
 1. Rinse the 15-20 mL ultrafiltration centrifugal concentrators of choice with Type I - ultrapure grade water.
 2. Load the concentrators into the centrifuge and spin at 4,400 x g at 4 °C. Discard the run through and repeat this step at least twice more.
 3. Load the supernatant sample into water-rinsed concentrators and spin under the same conditions. Depending on experimental goals, the sample filtrate can be discarded or collected for further



concentration (with concentrators with a nominal molecular weight limit of 3 kDa) and analysis.

4. Repeat this step until the sample is concentrated to a final volume of ~15-30 mL.

2. Option 2: perform tangential flow filtration (TFF) for concentrating a large (>500 mL) volume of sample.

1. Set up the TFF apparatus according to the manufacturer's guidelines (see **Table of Materials**). Attach a peristaltic pump to the intake line and place adjustable clamps on the retentate line. Sanitize the TFF as recommended by the manufacturer, and then flush the device with 1 L of Type I - ultrapure grade water.

NOTE: The intake and retentate lines should be placed into a clean sample reservoir (glass bottle). Filtrate line(s) should be directed into a waste vessel or sink.

2. Add <0.2 µm filtrate into the sample reservoir. Slowly increase pump speed and backpressure levels on the retentate line to increase output from the filtrate lines.
3. Continue to run the TFF, refilling the reservoir with culture supernatant as the material is removed. Avoid allowing the intake line to come out of the sample during processing to avoid introducing air bubbles. Ensure that feed pressure does not exceed ~10 psi and that the retentate flows out of the TFF at a consistent pace back into the reservoir.
4. Stop concentrating the sample once the volume in the reservoir reaches the lowest possible amount needed to maintain flow into the intake line without introducing air bubbles.

5. Close the outflow line with a clamp. Remove the backpressure on the retentate line and recirculate the concentrated supernatant through the filter for ~10 min, reducing the recirculation rate to 20-40 mL/min to maximize recovery.

6. Move the retentate line into a clean vessel, remove the intake line from the sample, and collect the concentrated material. Recover any remaining material in the sample reservoir using a pipette.

7. Filter the concentrated supernatant through a 0.2 µm syringe filter (step 2.2) to ensure no cells remain.

NOTE: Step 3.2.7 is optional.

8. If needed, store the final concentrate at 4 °C for ~3 weeks before moving to vesicle purification (step 4).

4. Vesicle isolation and purification

1. Pellet directly by ultracentrifugation following the steps below.

1. Place the concentrated <0.2 µm culture sample in a clean ultracentrifuge tube. Add clean media or buffer as necessary to ensure that the tube is completely filled.

NOTE: If the final culture sample is too large to fit in one tube, either pellet the material in multiple tubes to be combined before the wash steps or pellet serially in the same tube.

2. If needed, create a balance and spin at ~100,000 x g for 3 h at 4 °C.
3. Carefully remove the supernatant with a pipette. While cyanobacterial vesicle pellets can show some coloration, they are frequently invisible.

4. Wash the pelleted material by adding fresh culture media or wash buffer such as 1x phosphate-buffered saline (PBS) (see **Discussion**) to the ultracentrifuge tube, mix by gentle pipetting, and spin again as in step 4.1.2. Repeat the wash process a second time.
 5. Resuspend the final pellet in fresh culture by repeatedly but gently pipetting up and down around the bottom of the tube with a 1 mL pipette. Transfer to a clean vessel.
2. Perform density gradient ultracentrifugation.
 1. Prepare iodixanol stocks (see **Table of Materials**) by making a 4x concentrated version of the buffer background desired for the sample (see **Discussion**).
 2. Mix one part of the 4x buffer with three parts of 60% iodixanol stock to make a 45% iodixanol solution.
 3. Dilute 45% iodixanol with volumes of 1x buffer to create stocks of gradient media at 40%, 35%, 30%, 25%, 20%, 15%, and 10% final iodixanol concentrations.
NOTE: The total amount needed will vary with the capacity of the ultracentrifuge rotor/tubes.
 4. Set up an ultracentrifuge density gradient by carefully overlaying equal amounts of 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, and 0% iodixanol into an ultracentrifuge tube such that the entire volume of the tube is utilized.
NOTE: The extracellular vesicle sample to be purified should be placed at the top of the gradient as part of the 0% iodixanol layer. If the final volume of the vesicle sample exceeds the size of the 0% layer, mix any excess vesicle sample with enough 45% iodixanol and/or buffer to generate the required volume of the 10% or higher concentration optiprep stocks, and use these in the corresponding location in the gradient.
 5. Spin the gradient at $\sim 100,000 \times g$ at 4 °C for 6 h.
 6. Collect fractions (typically 0.5 mL each for a ~ 4.5 mL gradient) by careful pipetting or using a fraction collector (see **Table of Materials**).
 7. Determine the density of each fraction (in g/mL) using an analytical balance and calibrated pipette to measure the weight of a known volume of sample. Remove the sample from the tube, determine the weight, and return the sample directly.
 8. Dilute individual fractions in a new ultracentrifuge tube with clean buffer and wash the material as mentioned in steps 4.1.2-4.1.4. Alternatively, use dialysis or ultrafiltration columns to recover particles.
NOTE: Cyanobacterial extracellular vesicles typically migrate to buoyant densities of ~ 1.14 - 1.19 g/mL in iodixanol.
 3. Store the vesicles at 4 °C if they are to be used within 1-3 weeks. Freeze the vesicles at -20 °C or -80 °C if they are not used for longer than that period.

5. Characterization of the isolated vesicles

1. Perform negative stain transmission electron microscopy (see **Table of Materials**) (TEM; particle size, structure, purity).
 1. To improve the image quality, glow discharge the surface of a former-coated TEM grid using a glow discharge system according to the manufacturer's guidelines (see **Table of Materials**).

2. Carefully apply ~5 μL of the vesicle sample and let sit for 5 min.
NOTE: Samples with vesicle concentrations $>10^9 \text{ mL}^{-1}$ will typically give the best results; more dilute samples will have few vesicles per image.
 3. Remove the sample by touching the edge of a grid to a piece of clean filter paper.
 4. Pipet a 20-50 μL drop of 2% uranyl acetate (see **Table of Materials**) onto a flat surface covered by plastic film, and place the grid floating on top of it for 2 min.
 5. Remove uranyl acetate using filter paper and float briefly on a drop of ultrapure water to wash. Repeat the water wash a second time.
NOTE: The final dry grid is ready for visualization after step 5.1.5.
2. Perform ultra-thin section staining for TEM (particle size, internal structure).
 1. Resuspend the pellet from step 4.1.5 with 1 mL of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7) (see **Table of Materials**) and fix the sample for 2 h at 4 °C.
 2. Spin at ~100,000 x g for 1 h at 4 °C.
 3. Wash the pellet carefully with 0.1 M sodium cacodylate buffer (pH 7), and then spin at ~100,000 x g for 1 h at 4 °C.
 4. Fix the pellet with 1 mL of 1% osmium tetroxide (see **Table of Materials**) (prepared in 0.1 M sodium cacodylate buffer) for 1 h, and then wash as in step 5.2.3.
 5. Dehydrate the sample with 1 mL of an ascendant series of ethanol (50%, 70%, 90%, and two times in absolute ethanol) in steps of 10 min each at 4 °C.
 6. Add 250 μL of epoxy resin (see **Table of Materials**) mixed with absolute ethanol (1:1) to the pellet and incubate overnight at 4 °C.
 7. Transfer the pellet to 500 μL of pure resin for 24 h. Then, incubate the resin at 65 °C for 48 h.
NOTE: The resin is now ready for sectioning by ultramicrotome, following the manufacturer's instructions (see **Table of Materials**).
 8. Stain the grids containing sections with 2% uranyl acetate (in 50% ethanol) for 5 min. Wash the slides gently under running deionized water for 10-15 s each. Place a drop of lead citrate (see **Table of Materials**) and stain for another 5 min.
 9. Wash as before, remove the water by blotting the grid on a filter paper, and air-dry the grids. Visualize by TEM according to the manufacturer's guidelines (see **Table of Materials**).
3. Perform nanoparticle tracking analysis (NTA) (particle size, concentration).
 1. Using lens paper, carefully wipe any dust or visible material off the optical flat. Check whether all O-rings and other seals are clean and intact. Turn on the instrument and start up the software.
 2. Using a clean syringe, fill the chamber with ultra-pure water. Ensure that no air bubbles are present.
 3. Click on **Start Camera** and visualize the optimal region of the chamber about the 'thumbprint'. Adjust the microscope stage horizontally or vertically as needed so that signal intensity is even across the



imaged region. Move the imaging region horizontally relative to the thumbprint (to the right, toward the vertical line), finding a region nearby with low background signal.

4. Slide the **Screen Gain** and **Camera Level** sliders to maximum, and then decrease to the lowest level to see the dimmest particles.

NOTE: Typical settings for cyanobacterial vesicles utilize a Screen Gain of 7 and Camera Level between 10-12.

5. Adjust the focus as necessary to ensure that particles are roughly equally visible across the field of view. Continue to push ultra-clean water through the chamber until you have visually confirmed that the chamber is clean.
6. Remove residual water from the chamber using a different syringe.
7. Examine a sample of the media/buffer your sample is in to determine the background particle concentration by filling the chamber with media/buffer using a clean 1 mL syringe.
8. Select Standard measurement from the **SOP** dropdown box. Change settings to collect at least three technical replicates of 60 s videos each. Press **Create and Run Script** and follow the prompts. Push ~100 μL of sample into the chamber between replicates.
9. When the acquisition is complete, remove the residual buffer with a syringe, flush 3-5 mL of ultra-pure water through the chamber, and remove the remaining water.
10. Add the vesicle sample to the chamber using a 1 mL syringe and confirm the acquisition settings. If

particle count is not within the instrument's linear range (typically between 20-80 particles/frame), the sample will need to be diluted or concentrated. Push at least 500 μL of sample into the chamber before collecting data.

11. Collect video data as in step 5.3.8, making sure to clean the chamber with ultra-pure water between different samples.
12. Collect all subsequent samples from the same organism/experiment using identical camera settings to ensure comparability of data; changes in the Camera Level setting can have a notable effect on the final values obtained.
13. Determine particle parameters using the analysis section of the software. Select **Analysis > Open Experiment**, and load the sample file. Select **Process Selected Files** and wait for the analysis to complete. Ensure to use the same **Detection Threshold** for all subsequent samples from the same experiment.
NOTE: As cyanobacterial vesicles are frequently dim, the Detection Threshold will typically need to be adjusted to the most sensitive (lowest) value.
4. Perform dynamic light scattering (DLS) (particle size, zeta potential).
 1. Filter through a 0.2 μm pore size filter for any sample that goes into the DLS to screen out large particles that interfere with measurements.
 2. Add 1 mL of the media/buffer into a clean cuvette to examine possible aggregations or other nanoparticles coming from the media. Put the cuvette with the frosted side on the left into the micro



sampler, and close the lid. Let the sample equilibrate for at least 5 min.

3. Enter the refractive index of the material and the material absorption if they differ significantly from the defaults for water.

NOTE: The material properties would have very little influence if the vesicles are smaller than 100 nm. When measuring the surface potential (zeta potential) of EVs, vesicles should be resuspended in the original growth media.

4. Click on **Instrument Control Panel** to check the readings and start data acquisition by clicking on the green icon. If the values look good and your media/buffer does not contain aggregations or other particles, you are now ready to measure your sample.
5. Add 1 mL of vesicle sample into a clean cuvette and collect data as mentioned in step 5.4.4. Collect at least three technical replicates for 10 min each.
5. Perform lipopolysaccharide (LPS) analysis to confirm that the Gram-negative EVs are present in the sample.
 1. Heat denature vesicle sample at 95 °C for 10 min in standard 1x Laemmli sample buffer (see **Table of Materials**).
 2. Incubate with 0.2 U of type XIV protease from *Streptomyces griseus* (see **Table of Materials**) at 37 °C for 30 min to remove contaminating glycoproteins.
 3. Separate treated samples by electrophoresis on denaturing 16% (w/v) SDS-polyacrylamide gels following the previously published protocol¹⁶.

4. To detect LPS, stain the gel either with commercially available staining kits or with a modified silver staining technique²⁸. Quantify the relative LPS abundance by densitometry analysis of stained gels as per previously published References^{29,30}.

6. Vesicle production rate measurements

1. Using nanoparticle tracking analysis as in step 5.3, or equivalent technology, measure the particle concentration in the growth media for the experiment. If a high particle count is found, filter through a 0.1 µm pore filter and recheck.

NOTE: To minimize the error, the media background particle concentration should be less than 10% of the particle concentration found in cultures at the lowest density.
2. Acclimate the culture by growing cells for at least two successive serial transfers under the media and environmental conditions desired for the experiments as in step 1.1.2. Cultures need to be transferred while still in the exponential growth phase. Ensure that the measured culture growth rates are consistent across transfers; if not, continue transferring cultures until growth rates are reproducible.
3. Collect time-course samples upon inoculation and regularly timed intervals across the growth curve into the early stationary phase. Stagger time points to have a minimum of 3-4 samples collected across the full range of exponential growth. To sample at each time point, perform the following steps.
 1. Filter 1 mL or more of culture directly through a clean, sterile 0.2 µm syringe filter and save the filtrate to measure vesicles concentration as in step

- 5.3. Freeze the time-course samples if desired. Use relative fluorescence to follow culture growth dynamics.
2. Save a sample of the whole culture to determine population size using a method appropriate for the organism (flow cytometry; colony plating; or otherwise).
4. Obtain measurements of cells and vesicle-like particles (per mL) at each timepoint.
5. Identify the timepoints which occurred during the exponential growth phase. To calculate r , the number of vesicles produced per cell per generation, between two points during exponential growth (typically the beginning and end of the time-course), use the equation provided in **Supplementary File 1**.

NOTE: This method is only valid during steady-state growth conditions; underlying assumptions and derivation of the calculation are detailed in Reference¹⁴.

Representative Results

Figure 1 presents an overview of the cyanobacterial vesicle isolation process, highlighting key aspects of the protocol described here. Of particular note are the steps detailing separation of cyanobacterial biomass from the small particle (vesicle) fraction, concentrating the vesicle sample and vesicle isolation and purification (**Figure 1**, steps 2 to 4), which are critical to obtaining reproducible preparations

of vesicles. **Figure 2** presents representative results from the isolation and characterization of extracellular vesicles from the cyanobacterium *Synechocystis* sp. PCC 6803. The outer membrane of this cyanobacterium has been shown to contain carotenoids³¹, which confer a characteristic orange coloration to vesicles' samples collected by direct pelleting through ultracentrifugation (**Figure 2A**). Once the vesicle pellet is resuspended, vesicles can be examined by transmission electron microscopy (TEM), either by negatively staining samples with uranyl acetate (**Figure 2B**) or by observation of ultrathin sections (**Figure 2C**). Vesicle size distribution and concentration can be assessed using dynamic light scattering (DLS) (**Figure 2D**) and nanoparticle tracking analyses (NTA) (**Figure 2E**). With the described protocol, typically $\sim 3.5 \pm 1.0 \times 10^8$ nanoparticles per mL were obtained in an exponentially growing culture of *Synechocystis* sp. PCC 6803 at an OD730 of 1.0. Biochemical analyses of isolated EVs can be carried out to complement the physical characterization of the isolated vesicles. As an example, purified *Synechocystis* sp. PCC 6803 vesicles were separated on an SDS-polyacrylamide gel and stained for lipopolysaccharides (LPS; **Figure 2F**). As LPS are specific to the outer membrane³², LPS detection can validate the presence of membrane-bound vesicles and serve as a marker for analyzing relative vesicle content between preparations from the same strain³³. Inspection of the relative abundance of low vs. high molecular weight LPS can indicate changes in vesicle composition between samples^{34,35}.

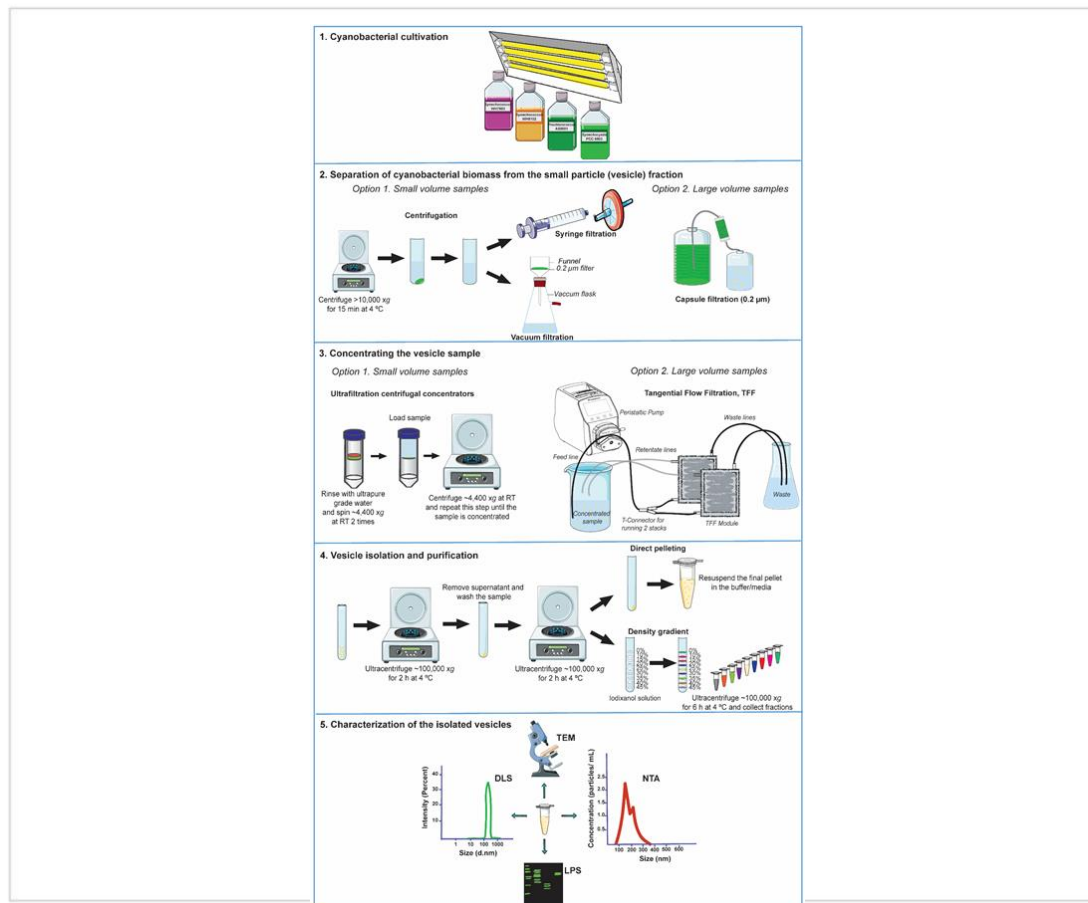


Figure 1: Experimental procedures used for cyanobacterial EV isolation and characterization. Schematic representations of growing cultures (1) and separation of cyanobacterial biomass from the growth medium (2). Cell-free samples containing vesicles are concentrated (3), and then the EVs are isolated and purified (4). Depending on the application, vesicle preparations can be characterized using one or a combination of transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and lipopolysaccharide (LPS) profiling (5). RT, room temperature. [Please click here to view a larger version of this figure.](#)

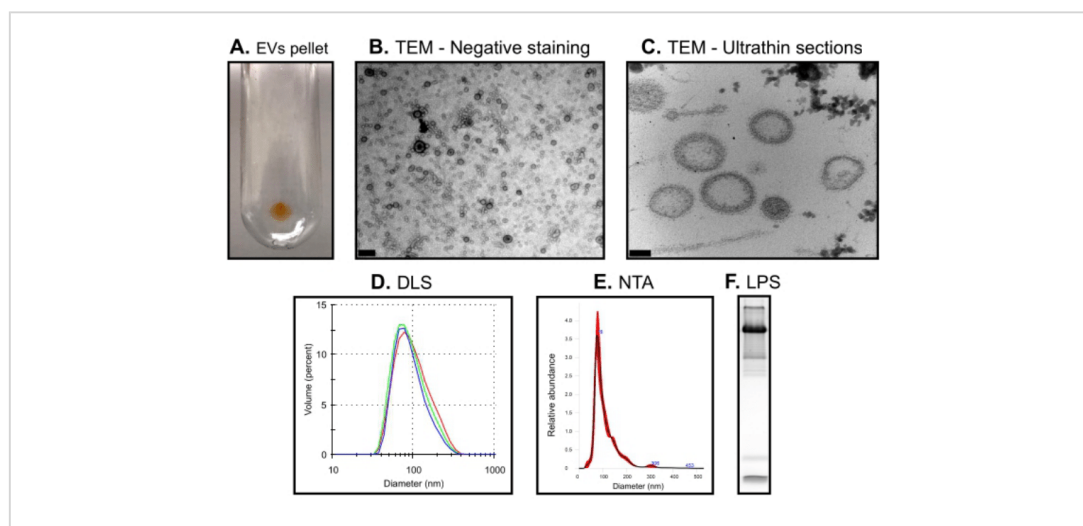


Figure 2: Representative results of the extracellular vesicle isolation and characterization for the cyanobacterium *Synechocystis* sp. PCC 6803. (A) Photograph of the extracellular vesicle pellet (EVs pellet) obtained after ultracentrifugation of the concentrated cell-free extracellular medium from a 600 mL *Synechocystis* sp. PCC 6803 culture grown to an OD730 of 1.0-1.5. Note the typical orange coloration of the vesicle pellet derived from the carotenoids found in the outer membrane. (B,C) Transmission electron microscopy (TEM) photographs of negatively-stained (B) and ultrathin sections (C) of *Synechocystis* sp. PCC 6803 vesicle samples. Scale bars: 200 nm and 50 nm, respectively. Samples were visualized on a transmission electron microscope operated at 80 kV. (D) Typical dynamic light scattering (DLS) plot depicting the distribution of vesicle volume as a function of vesicle diameter (in nm). Colored lines indicate data from three technical replicate measurements of the same sample. (E) Representative nanoparticle tracking analysis (NTA) data of *Synechocystis* extracellular vesicle size distribution (in nm). The black line indicates the mean of three technical replicates, and the red lines represent the standard error of the mean. (F) Lipopolysaccharides (LPS) profile was detected after separating the vesicle preparation by electrophoresis on a 16% (w/v) SDS-polyacrylamide gel and staining with a commercial LPS staining kit. Low- and high-molecular-weight LPS, corresponding to rough- and smooth-LPS forms, respectively, are detectable. [Please click here to view a larger version of this figure.](#)

Supplementary File 1: Equation to calculate r . The number of vesicles produced per cell per generation between two points during exponential growth (typically the beginning and

end of the time course) is determined using this equation. [Please click here to download this File.](#)

Discussion

General considerations



The protocol (**Figure 1**) is presented as a set of options to highlight that there is no 'one-size-fits-all' method for working with cyanobacterial extracellular vesicles. Interested researchers can utilize the sections of this protocol that are compatible and appropriate for their particular model organism, experimental questions/goals, and equipment availability. All vesicle isolation approaches involve trade-offs and will unavoidably result in some degree of bias. While one should seek to minimize this whenever possible, the most crucial consideration is to ensure that the detailed methodology used is reported according to appropriate MISEV (Minimal Information for Studies of Extracellular Vesicles) guidelines³⁶.

Culture growth

Cyanobacterial cultures can be readily obtained from one of the many culture collections available worldwide. A few examples are the Roscoff Culture Collection (Station Biologique de Roscoff, France), the Pasteur Culture Collection (Institute Pasteur, Paris, France), and the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, Maine, USA). The cyanobacterial strain of choice must be cultivated in the appropriate medium and environmental conditions, varying significantly among different strains. A list of commonly used media for cyanobacterial cultivation can be found at culture collection websites or other publications^{37,38,39}.

Working with cyanobacterial extracellular vesicles presents some unique challenges compared with the methodologies reported for many typical model laboratory heterotrophs. Cyanobacterial cultures have vesicle concentrations of orders of magnitude lower than those found in other microbes such as *Escherichia coli*^{14,16,40}. These differences, presumably arising from lower cell densities and/or vesicle production

rates, mean that relatively large cultures (~1-20 L or more) may be required to yield sufficient material for bulk analyses. Thus, researchers are encouraged to test vesicle yields from smaller-scale cultures to determine how much material will be necessary to achieve their desired end goals. The importance of establishing whether the media used for the experiment has a detectable particulate background also needs to be stressed before beginning any experiment, as that material can potentially confound vesicle population quantification, reduce the sensitivity of vesicle concentration/size measurements, or contaminate the final vesicle preparations.

Another challenge for the field is that not all cyanobacteria grow well, or at all, in pure culture. Until rendered axenic, interpretations of physical vesicle characteristics, production rates, or contents cannot necessarily lead to clear conclusions about vesicles produced by any one strain in the community. Researchers are also advised to consider what other types of particles could be present and potentially confused with vesicles by specific nanoparticle analysis tools, which cannot necessarily discriminate between different particle types. For instance, it may be essential to verify that the strain being used lacks a prophage, either *via* genome sequencing, induction assays, or other means or does not release other types of particulate matter. In our experience, most cyanobacterial vesicles are <0.2 µm in diameter, but when looking at a new strain or growth condition, one should confirm whether using a 0.45 µm pore-size filter alters the size distribution of purified particles.

Many aspects of culture conditions can influence vesicle production and its contents^{41,42}. Thus, the physical and chemical conditions used for culture growth (including light irradiance, temperature, and media composition) need to



be documented and controlled to the degree possible to ensure the reproducibility of results. Any chemical analysis of vesicle contents must account for background composition, particularly when carrying out 'omics' style high-throughput analyses. This can be especially critical when using undefined media, such as those based on a natural seawater background or supplemented with yeast extract or tryptone. Using a defined growth medium may be preferable depending on the experimental goals.

Researchers need to carefully monitor culture growth dynamics at regular intervals to ensure that they know where in the growth phase a given batch culture is and not just collect samples after an arbitrary amount of time. Vesicle composition can vary across growth phases, particularly between exponential and stationary phases^{41,42}. For instance, at least some fraction of vesicles sampled in the stationary phase may arise from a different cellular mechanism, such as cell lysis, that will not occur during exponential growth. While this may still be of great biological interest, it is essential to know the sample. If a cyanobacterial culture reaches the desired growth phase at a time when it is not possible to proceed directly to sample concentration, separating the cells from the <0.2 µm fraction immediately (with centrifugation and/or direct 0.2 µm filtration), and then storing the cell-free filtrate at 4 °C is recommended. The material can be stored in this way for days with little to no noticeable impact on the concentration or size distribution of vesicles.

Vesicle purifications

The frequent need to isolate vesicles from significant volume cultures is vital in the cyanobacterial vesicle isolation workflow. When working with larger volumes of material, the vesicles will need to be concentrated before downstream

separation workflows. Concentrators (tangential flow filter membranes or centrifugal columns) with a nominal molecular weight limit of 100 kDa are generally advised, as they allow separation from soluble material with low molecular weight while keeping concentration times reasonable, but 30 kDa filters are also frequently used with success. While several non-ultracentrifugation-based methods of purifying vesicles (e.g., size exclusion chromatography, microfluidic-based systems, affinity-capture techniques, and precipitation-based approaches) are becoming popular in the extracellular vesicle field, in our experience, these approaches can result in decreased yields and are typically incompatible with the culture volumes needed.

Researchers need to consider the composition of the iodixanol background and the wash/resuspension buffers used during vesicle purification to ensure that they are compatible with the desired downstream applications. In many cases, the final vesicle sample can be resuspended in growth media or a defined buffer comparable in composition to the growth medium (e.g., natural vs. artificial seawater). However, this may not be possible with marine cyanobacterial vesicles, which will require further experimental manipulation for analysis, as high salt concentrations similar to seawater levels can inhibit many enzymatic reactions. In such cases, standard laboratory buffers such as 0.2 µm filtered, 1x PBS typically work well for maintaining the stability of marine cyanobacterial vesicles and can be more compatible with downstream experimental processes.

Density gradient purification can be considered optional depending on the experimental goals and culture composition but is strongly recommended for producing a more rigorously pure and reproducible sample. EV populations are heterogeneous and can be found across a range of buoyant



densities, which further vary by strain, growth conditions, and other factors^{4,5,6}. The densities listed above represent those typically found for vesicles from cyanobacterial cultures and field samples in iodixanol, but results in other strains may vary. Other density gradient materials such as sucrose and CsCl can be utilized for vesicles, but they will migrate to different buoyant densities in these backgrounds. Different gradient media backgrounds can bias the recovery of lipid-enclosed viruses⁴³ and might potentially influence the recovery of vesicles from different strains.

Vesicles can be lost at multiple points through the vesicle isolation, and gradient purification process described here, reducing yields and increasing the amount of starting material needed to achieve a given final vesicle yield for downstream applications. Particular care should be taken when working with vesicle pellets following ultracentrifugation. While some cyanobacterial vesicles can have carotenoids or other compounds which may lend vesicle samples some amount of pigmentation (**Figure 2**), depending on the strain or amount of material, it is not necessarily expected to be able to visualize the vesicles pellet directly. Be aware of where the pellet is expected to be given the type of centrifuge rotor used. When possible, purified vesicle samples are suggested to be examined by electron microscopy to verify the composition of the final material recovered.

The impact of storage conditions on vesicles and their contents remains an open question. Though it is found that the storage does not have a notable effect on cyanobacterial vesicle size or concentration¹⁴, the functionality of isolated vesicle preparations may change over time⁴⁴. While freeze/thaw cycles should be avoided whenever possible, the impact of freezing samples on overall vesicle numbers and sizes appears to be minimal. One should be aware of the potential

for freeze-thaw cycles to influence the composition of vesicle contents, such as the length of vesicle-associated nucleic acids or the stability of proteins.

Vesicles from field samples

Current methods for isolating extracellular vesicles from natural aquatic environments are conceptually and operationally similar to those described here for large-volume cultures. Still, they can require even greater volumes of material. Such field samples can involve collecting, filtration, and concentration of hundreds to thousands of liters of water to obtain sufficient material for analysis. Depending on the turbidity of the sample being used, incorporation of one or more pre-filtration steps before the 0.2 μm filter may be needed. The specific TFF device used should be appropriate for working with such large volumes in a reasonable amount of time (ideally in the order of hours) and without putting excessive pressure on the sample. In practice, this often involves using a much greater total surface area than might be applied to culture-based samples, as well as larger-diameter tubing to facilitate increased flow rates. This increased filter surface area will likely lead to a marginal increase in particle losses compared to smaller TFF arrangements and a larger final volume of concentrate; however, these concerns must be balanced with considerations of total processing time. For situations such as an extended oceanographic cruise where samples will not return to a lab for many days post-sampling, we recommend carrying out initial 0.2 μm filtration and TFF steps in the field. This smaller volume of concentrated material can then be stored at 4 °C or -80 °C on board (depending on availability and downstream analytical considerations) until it is returned to the lab for final processing.



Isolation and separation of extracellular vesicles from other small particles, both organic and inorganic, can be challenging, and methods for separating different particles are not yet perfect. For instance, iodixanol density gradients may not readily separate all classes of vesicles and viruses present in a given sample. As the types of confounding particles, and their physical properties, will vary between sampling sites, it is currently impossible to provide a protocol that will robustly partition all classes of small aquatic particles. Trial and error are essential, and experimentation with the iodixanol range used in the gradient and ultracentrifugation conditions will be required to maximize the separation; a collection of smaller volume, more finely resolved density fractions may also be required. Depending on the context, using CsCl gradients instead of iodixanol may help separate environmental particles⁴⁵. Still, the change in osmotic conditions could lead to biases in the final recovered products, as discussed above.

Vesicle characterization

Nanoparticle analysis instruments are not yet routinely available in microbiology laboratory settings but are becoming increasingly available. All methodologies have pros and cons, and we make no specific endorsement of one platform as being better than all others for cyanobacterial vesicle work; indeed, all have particular trade-offs concerning costs, resolution, ease of use, detection limits, compatibility with different growth media/buffer backgrounds, and data reproducibility. In addition to instruments based on nanoparticle tracking analysis described above, other approaches, including nanoflow cytometry, microfluidic resistive pulse sensing, and tunable resistive pulse sensing^{46,47} can be applied. Users should be careful to learn the details of their available instrumentation and verify that it works well with their system, as we

have encountered difficulties when using some platforms with seawater-based media. We encourage the field to move toward quantitative characterization of vesicle size, concentrations, and production rates. Measuring vesicle concentrations on a fundamental particle per mL basis, and not in terms of protein content or other metrics, will allow for the integration of vesicles into more quantitative frameworks and enable intercomparisons among strains and conditions. Further efforts to improve the ability to calibrate concentration measurements for <100nm particles are needed.

The fact that vesicle production rate measurements are done directly from 0.2 μ m filtered culture supernatant to minimize particle losses would be associated with other purification steps described above. However, this does mean that the approach does not necessarily discriminate between actual extracellular vesicles and other small particles found in the cultures. Only counting particles within specific size ranges (e.g., 50-250 nm diameter) may help exclude some outliers, but visual confirmation that pelleted <0.2 μ m culture contents appear to be membrane-bound vesicles (by TEM or other approaches) is needed to be able to specifically claim that one is measuring the production of vesicles as opposed to the production of vesicle-like particles.

An essential factor in vesicle characterization is to ensure that the vesicle sample is being analyzed in the appropriate linear sensitivity range of the nanoparticle analysis device. When a sample is too concentrated, it is straightforward to dilute that material with a clean buffer and re-analyze it. On the other hand, the relatively low cell and/or vesicle density of some cyanobacterial cultures can sometimes yield vesicle preparations that are below some instruments' detection limits. In cases where this occurs with bulk vesicle purifications, one can consider growing larger volume



cultures, re-pelleting and resuspending the final material in a smaller volume, or evaluating whether there is a step in the isolation process where excessive losses are occurring and could be mitigated. When measuring vesicle production rates, the fixes are not necessarily so straightforward. Samples can be concentrated if necessary, but the first one should see whether adjustments to the media could result in higher cell densities or whether sufficiently concentrated samples could be obtained from later time points during the exponential phase where concentrations will be higher.

Limitations

As with any protocol, vesicle isolation using these approaches has clear limitations. These approaches do not rely on their guarantee that an utterly pure preparation of vesicles will be isolated. Both cultures and field samples may contain other materials, which migrate similarly to vesicles in density gradients. Still, at a minimum, these types of additional purification methodologies are essential for ensuring rigor and reproducibility of vesicle analysis. While we describe vesicle isolation approaches in the context of cyanobacteria, cultures of many other microbes will also contain vesicles at comparatively low concentrations, and the procedures described here should be generally applicable. It is expected that these methods will serve not as a permanent protocol but instead as a starting point for spurring future advancements in working with extracellular vesicles from diverse microbes. Future efforts are required to merge these methods with other approaches such as size exclusion columns or asymmetric field flow fractionation to improve the discrimination and separation of different categories of small particles from cultures and environmental samples. We are also hopeful that these techniques can continue to evolve alongside improvements in nanoparticle characterization technologies to improve the ability to examine heterogeneity within vesicle

populations, their contents, and their exact functional roles in the environment.

Disclosures

The authors declare no conflicts of interest.

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CHAPTER IV - The role of outer membrane protein(s) harboring SLH/OprB-Domains in extracellular vesicles' production in *Synechocystis* sp. PCC 6803

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*Participate in: the protein analysis of *Synechocystis* EVs that led to the identification of outer membrane SLH/OprB porins; attempts to generate an *slr1841* deletion mutant; isolation and characterization of EVs released by the wild-type and Δ *slr1908* mutant strains.



Article

The Role of Outer Membrane Protein(s) Harboring SLH/OprB-Domains in Extracellular Vesicles' Production in *Synechocystis* sp. PCC 6803

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Abstract: Cyanobacteria are a group of photosynthetic prokaryotes that contribute to primary production on a global scale. These microorganisms release vesicles to the extracellular environment, spherical nanosized structures, derived essentially from the outer membrane. Even though earlier works in model Gram-negative bacteria have hypothesized that outer membrane stability is crucial in vesicle formation, the mechanisms determining vesicle biogenesis in cyanobacteria remain unknown. Here, we report on the identification of six candidate genes encoding outer membrane proteins harboring SLH/OprB-domains in the genome of the model cyanobacterium *Synechocystis* sp. PCC 6803. Using a genetics-based approach, one gene was found to encode an essential protein (Slr1841), while the remaining five are not essential for growth under standard conditions. Vesicle production was monitored, and it was found that a mutant in the gene encoding the second most abundant SLH/OprB protein in *Synechocystis* sp. PCC 6803 outer membrane (Slr1908) produces more vesicles than any of the other tested strains. Moreover, the Slr1908-protein was also found to be important for iron uptake. Altogether, our results suggest that proteins containing the SLH/OprB-domains may have dual biological role, related to micronutrient uptake and to outer membrane stability, which, together or alone, seem to be involved in cyanobacterial vesicle biogenesis.

Keywords: cyanobacteria; SLH-protein domain; OprB-protein domain; outer membrane proteins; extracellular vesicles; iron uptake

1. Introduction

Extracellular vesicles (EVs) are nanosized structures (20–400 nm in diameter) presumably released by the cells of all organisms [1–3]. Despite their reduced size, EVs are rich in content, as they are composed of different biomolecules, including lipids, proteins, nucleic acids, and metabolites [4,5]. Delimited by a lipid bilayer, and without the capacity of replicating, EVs are not just random fragments of the cell membrane/wall, as it has been repetitively shown that their biochemical composition can be the product of selective packaging e.g., [6,7]. In agreement, EVs have been implicated with a plethora of biological roles, ranging from secretion of target molecules to inter-species and even inter-kingdom communication [4,5,8]. Remarkably, bacterial EVs have been proposed to be at the very

origin of the eukaryotic endomembrane system [9]; most endosymbiotic theories postulate that eukaryotic cells must have originated from an archaeal host that engulfed an alphaproteobacterium (the mitochondrial ancestor) [10]. However, it remains highly debatable as to how the eukaryotic endomembrane system appeared, with such an elaborate vesicle trafficking organization. According to Gould et al. [9], the mitochondrial ancestor naturally released EVs, which, confined within the host's cytosol, accumulated and fused, either with each other or with the host's plasma membrane, giving rise to the primordial endomembrane system. In the particular case of green algae and plants, a better understanding of the origin, dynamics and response capacity of its cellular endomembrane system will require a deeper look into the evolutionary ancestors of chloroplasts, cyanobacteria.

The Cyanobacteria phylum is a large group of the domain Bacteria, including species living in, e.g., marine, freshwater or terrestrial environments, both as free-living organisms or in symbiotic associations. These photoautotrophic microorganisms play a key role in the biogeochemical cycle of carbon on a global scale. Estimates predict a global net primary production of 12 Gigatonnes each year for marine cyanobacteria only, which represents approximately 25% of ocean net primary production [11]. The first comprehensive study documenting the capacity of cyanobacteria to release EVs became available in 2014 [12]. In that study, cyanobacterial EVs were proposed to function as vehicles for the movement of carbon through marine food webs, as vectors for horizontal gene transfer, and as decoys for predators and phages [12]. After that work, others followed, mainly reporting the capacity of other cyanobacterial strains to vesiculate, leaving many aspects regarding cyanobacterial EVs biology unaddressed. One topic that has not been studied relates to how vesicles are formed and released in these microorganisms. In well-studied Gram-negative bacteria, such as *Escherichia coli* or *Pseudomonas aeruginosa*, several models have been proposed to explain the processes of EVs biogenesis, most of them positing that outer membrane (OM) stability is a fundamental aspect in EVs formation [4,5,8]. Related to such hypothesis, some players have been identified, such as different types of OM proteins, including the Lpp, or porins of the OmpA-family. However, these proteins are not universal, as not all Gram-negative bacteria harbor genes encoding those players. One such example is the Lpp protein, which is absent in cyanobacteria [8]. In fact, the cell wall of cyanobacteria is well known for its unique features, e.g., peptidoglycan thickness and extent of its crosslinking; structural differences in the lipid A moiety of lipopolysaccharides (LPS); and unique lipid composition (mainly composed of glycolipids, like chloroplasts, instead of the ordinary phospholipids present in bacterial membranes) [13], which anticipates that cyanobacterial EVs may have rather specific formation mechanisms [8].

The goal of this work was therefore to identify OM protein candidates that could play a role in EVs formation in cyanobacteria, using the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) as a model organism. Given the available knowledge on the OM proteome composition in *Synechocystis* [14–16], particular attention was paid to porins harboring the SLH (S-layer homology) and the OprB (carbohydrate-selective porin OprB) protein domains. While the SLH-domain in cyanobacterial OM porins was shown to bind the peptidoglycan layer [15], and so, could contribute to OM stability, the OprB-domain is predicted to assume a β -barrel structure, forming the porin in the OM [15,17]. Here, a combination of mutant generation, strains' growth analyses, identification of proteomic adaptations in response to target gene disruption, and isolation and characterization of vesicles, was carried out to give a comprehensive view of the investigated mechanisms. Results show that one of the highly abundant OM proteins (Slr1908) may have dual biological role, as the respective gene-disrupted mutant strain shows an unbalanced intracellular iron content, and produces more vesicles than the wild-type. This work highlights that the study of players involved in vesicle biogenesis in cyanobacteria must be accompanied by a broader evaluation of cyanobacterial-adaptation responses.

2. Results and Discussion

2.1. The Genome of *Synechocystis* Harbors Six Genes Putatively Encoding SLH/OprB-Domains Containing Proteins

This work began with the search in the genome of *Synechocystis* for genes that could putatively encode OM proteins containing simultaneously the SLH (PF00395; IPR001119) and the OprB (PF04966; IPR007049) protein domains. The in silico analysis revealed six genes meeting these criteria—*slr1908*, *slr1841*, *sll1271*, *sll0772*, *slr0042* and *sll1550*—whose protein products show E-values lower than 4.2×10^{-8} and 3.6×10^{-49} for the SLH and OprB domains, respectively. In Figure 1, a schematic representation of the *Synechocystis* genome and the relative position of each gene is shown (Figure 1A). The identified candidates are not simply duplicated genes, as the predicted encoded proteins have differences in size and domain length (Figure 1B). Next, we searched the literature to understand whether transcripts of the identified genes had already been detected in high-throughput RNA experiments. To that end, we looked particularly into the work of Kopf et al. [18], in which a comparative analysis of the primary transcriptome of *Synechocystis* under 10 different growth conditions is described. While transcripts of genes *slr1908*, *slr1841*, and *slr0042* have been detected in relatively high amounts (number of reads for the respective transcriptional units were, in average, above 22,000 reads), genes *sll1271* and *sll1550* showed low transcript abundance, and transcripts of gene *sll0772* could not be detected for any of the tested conditions [18] (Supplementary Material Table S1).

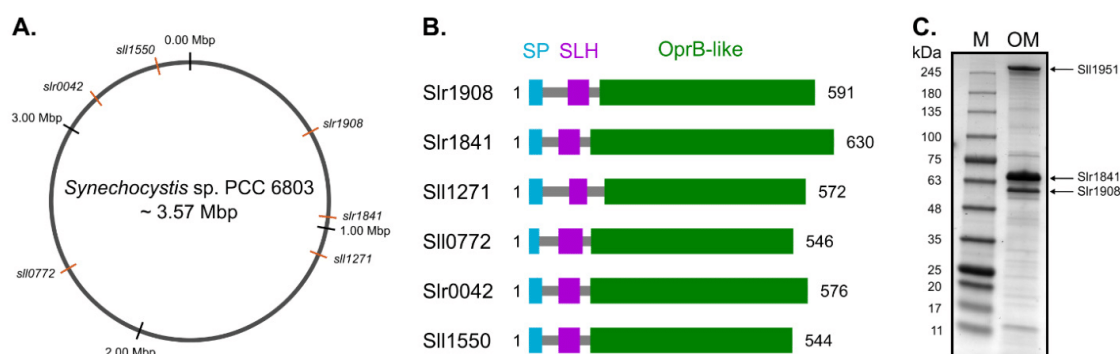


Figure 1. Distribution of the genes and outer membrane proteins containing S-layer homology (SLH) and carbohydrate-selective porin OprB (OprB)-domains in *Synechocystis* sp. PCC 6803. (A) Schematic representation of the *Synechocystis* genome, and relative position of the six genes encoding putative outer membrane (OM) proteins containing the SLH- and the OprB-domains. (B) Details of the size (in amino acids), and relative position of signal peptide (SP) and conserved SLH and OprB domains in the identified putative OM proteins. (C) PageBlue™ (Thermo Fisher Scientific, Waltham, MA, USA) stained gradient 4–15% (w/v) SDS-polyacrylamide gel showing the protein composition of isolated *Synechocystis* OM preparations. Highlighted peptide bands were identified by mass spectrometry. Sll1951: main protein constituent of the *Synechocystis* S-layer; Slr1841 and Slr1908: SLH- and OprB-containing outer membrane proteins.

We then proceeded by isolating OM fractions from *Synechocystis* wild-type cells, and separated the OM proteins by electrophoresis on SDS-polyacrylamide gels (Figure 1C). Identification by mass spectrometry of three of the most abundant proteins in the isolated OM fraction correspond to proteins Sll1951 (the main protein constituent of the *Synechocystis* S-layer), and the OM proteins Slr1908 and Slr1841. This result is in agreement with the relative abundance of transcripts of each gene (Supplementary Material Table S1), as well as with previous OM protein analyses [15,16]. Altogether, the differences detected between the identified candidates in terms of gene expression, amino acid length and composition, and protein abundance in isolated OM preparations, suggest that they may fulfill different biological functions.

2.2. *slr1841* Encodes an Essential Protein, While the Remaining SLH/OprB-Domain Containing Outer Membrane Proteins Are Not Essential for Growth

To assess the role of each candidate gene identified in this work and its protein product on the formation and release of EVs in *Synechocystis*, a genetics-based approach was undertaken. Constructs to generate single-gene disruption mutants by double homologous recombination of each of the identified gene candidates were prepared (Figure 2A), and *Synechocystis* cells were naturally transformed. Despite several attempts, disruption of gene *slr1841* could not be achieved. On one hand, transformation of *Synechocystis* with plasmid pDC1841K resulted in less transformants as compared to those with the other plasmids constructed in this work (data not shown). On the other hand, cells transformed with pDC1841K showed no sign of complete chromosome segregation even after several rounds of streaking onto BG11 plates with increasing antibiotic concentration (Figure 2B). Our results are in agreement with the work of Qiu et al. [19], in which the authors report to have failed in generating a full knockout mutant of gene *slr1841*. This suggests that Slr1841 likely plays an essential role in *Synechocystis* under the tested conditions, possibly involved in the uptake of micronutrients and/or in stabilizing the OM. Given the difficulty in obtaining a *Synechocystis* mutant strain lacking a functional Slr1841 protein, we therefore decided not to proceed with further studies of this gene/protein.

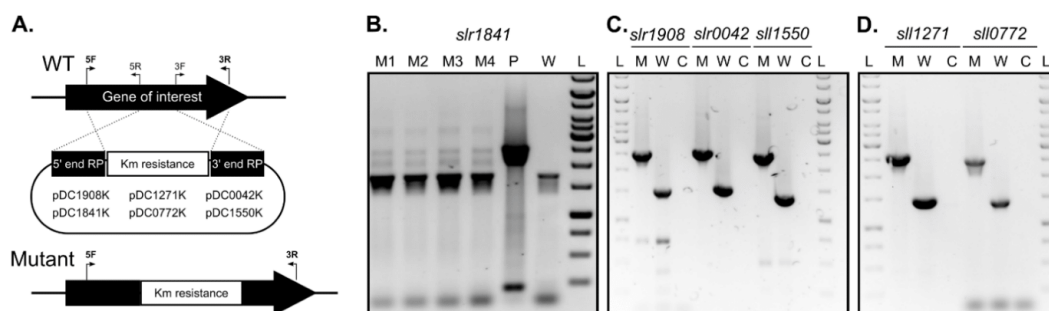


Figure 2. Generation of disruption mutants in genes encoding SLH-OprB-domain outer membrane proteins in *Synechocystis* sp. PCC 6803. (A) Scheme representing the double homologous recombination events (dashed lines) necessary to disrupt each gene of interest in the genome of *Synechocystis* wild-type (WT). The resulting mutant strain (Mutant), in which part of the gene's coding sequence was replaced by the insertion of a kanamycin resistance cassette is also shown. Relative position of the oligonucleotides (5F, 5R; and 3F, 3R), used for amplification of the 5' and 3' end recombination platforms (RP), respectively, is presented. Identity of the plasmids constructed in this work and used for mutant generation is displayed. (B) to (D) Photographs of DNA-stained agarose gels showing PCR analyses of chromosome segregation in *Synechocystis* transformed with plasmids pDC1841K (for disrupting gene *slr1841*—(B)), pDC1908K, pDC0042K, pDC1550K (for genes *slr1908*, *slr0042*, and *slr1550*—(C)), and pDC1271K, pDC0772K (for genes *slr1271* and *slr0772*—(D)). Oligonucleotides 5F and 3R (see section (A)) were used for amplification, using the following templates: M1–4, 4 independent mutant colonies transformed with pDC1841K; P, plasmid DNA of pDC1841K; W, genomic DNA of *Synechocystis* wild-type; M, genomic DNA from a clone of the respective disruption mutant; C, water (negative control). L, GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific).

In contrast, the remaining five genes could be successfully disrupted, as determined by complete chromosome segregation (Figure 2C,D). Growth phenotype characterization under photoautotrophic conditions in liquid medium indicated that none of the disrupted genes encode essential proteins for *Synechocystis*, at least under standard growth conditions, as all mutant strains showed similar growth rates as compared to that of the wild-type strain (Supplementary Material Figure S1). Qiu et al. were also capable of generating full knockout mutants of genes *slr1271*, *slr0772*, *slr0042* and *slr1550*, but not of *slr1908* [19]. This discrepancy could be related to differences in *Synechocystis* sp. PCC 6803 sub-strains used in the two different studies, and/or to the approach adopted for mutant generation (complete gene deletion in [19] vs. gene disruption by partial gene deletion in the present work).

2.3. Disruption of *slr1908* Results in Adjustments in the Outer Membrane and Extracellular Protein Composition Related to Iron Uptake

To determine whether disruption of SLH/OprB-protein encoding genes could result in specific proteomic adaptations in *Synechocystis*, exoprotein and OM protein fractions were isolated and analyzed by SDS-polyacrylamide gel electrophoresis (Figure 3).

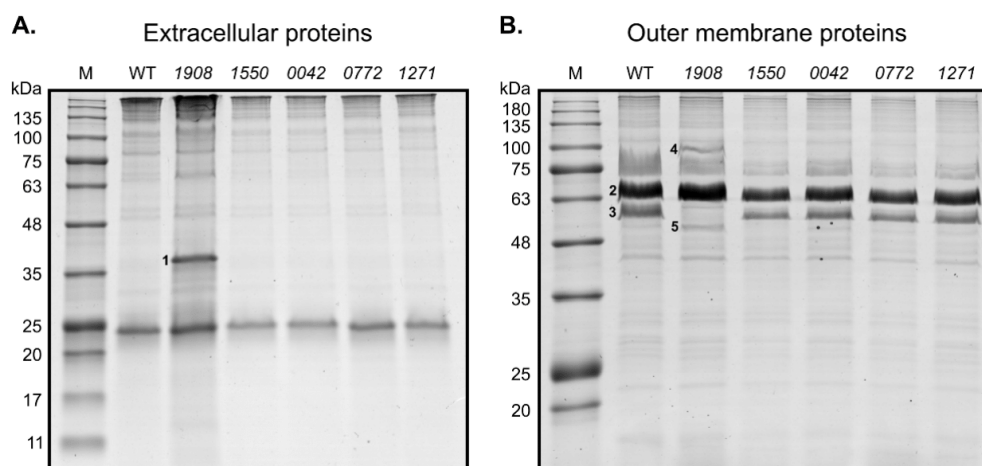


Figure 3. Analyses of the *Synechocystis* sp. PCC 6803 disruption mutants' cell-free extracellular medium and outer membrane protein composition. **(A)** Protein profile of the concentrated cell-free extracellular medium from the *Synechocystis* wild-type (WT) and mutant strains (1908, 1550, 0042, 0772, 1271). Proteins were stained with PageBlue™ Staining Solution after separation in a gradient 4–15% (*w/v*) SDS-polyacrylamide gel. Sample loading is normalized to each culture cell density (OD_{730}), volume of cell-free medium concentrated and concentration factor. **(B)** PageBlue™ stained 12% (*w/v*) SDS-polyacrylamide gel showing the protein composition of isolated wild-type and mutant strains' OM preparations. Proteins identified by mass spectrometry: 1, Slr0513 (FutA2); 2, Slr1841; 3, Slr1908; 4, Sll1406 (FhuA); 5, Sll1550; M, NZYColour Protein Marker II (NZYTech).

Regarding the exoproteome analysis, no significant differences could be observed in peptide profile between the wild-type and the different mutant strains, except for strain 1908 (*slr1908*-disruption mutant) (Figure 3A). In this mutant, over accumulation of a single peptide with approximately 38 kDa could be observed. Mass spectrometry analysis of the respective band indicated that it corresponds to protein Slr0513.

As to the OM protein composition (Figure 3B), more differences could be detected. In mutant 1908, the peptide band corresponding to Slr1908 is clearly absent, in agreement with the genetic modification introduced. In addition, two peptide bands showed increased accumulation as compared to the other tested strains; while the band of approximately 95 kDa was identified as protein Sll1406, the band of approximately 55 kDa was identified as the SLH/OprB-protein Sll1550. In line with the identification of Sll1550 in the OM preparation of the *slr1908*-mutant, it is possible to observe that the corresponding band in mutant 1550 is absent. Besides these differences, no further OM protein alterations could be detected in the remaining mutant strains. The reason for the lack of missing bands in OM preparations that could correspond to proteins Slr0042, Sll0772 and Sll1271 in the respective mutant strains could be related to their low abundance, and/or to the fact that the respective peptide bands separate together with other highly abundant OM proteins.

We then focused our attention on the differences observed in the exoproteome and OM protein composition of mutant 1908. Proteins Sll1406 and Slr0513 are known iron import-related proteins. Sll1406 is homologous to the FhuA protein from *E. coli* [20]. FhuA is one of the TonB-dependent transporters (TBDTs) localized in the OM of Gram-negative bacteria [20,21]. TBDTs mediate substrate uptake in an energy-dependent mode [21], mainly

iron siderophores, but also vitamins and carbohydrates [21]. It has been demonstrated that the Sll1406 protein from *Synechocystis* is a TonB-dependent transporter, together with three additional proteins [22]. These TBDTs were suggested to have redundant functions, as single knockout mutants of each of them resulted in strains lacking any distinguishable phenotype [22]. However, a quadruple knockout mutant of all TBDTs showed a sharp iron depleted phenotype, as the strain was almost completely unable to import iron siderophores, and showed strong impairment in inorganic iron uptake [22]. Moreover, Slr0513 is annotated as the iron uptake protein A2 (FutA2), a component of the Fut iron uptake system. This system is composed by two periplasmic binding proteins (FutA1 and FutA2), and two inner membrane proteins (FutB and FutC) [20]. The periplasmic proteins are iron binding proteins that mediate iron transport to the cytoplasm through the inner membrane protein complex [20,23]. In *Synechocystis*, only the FutA2 protein was found in the periplasmic space, where it is the most abundant protein therein, while the FutA1 protein was detected neither in the soluble protein fraction of the periplasm nor in the cytoplasmic membrane [24,25].

Differential accumulation of proteins in the OM (FhuA and Sll1550) and in the extracellular medium (FutA2) strongly indicates that iron uptake systems have been adapted to adjust for the absence of Slr1908. Higher abundance of FhuA in the OM can result from an attempt to increase iron uptake by other routes, namely by the TonB-dependent transport system. In addition, FutA2 accumulation in the extracellular medium may represent a strategy for iron scavenging from the medium, which would require further FutA2 internalization. Moreover, Sll1550 may also represent an additional mechanism for iron uptake; in fact, several reports show that *sll1550* gene expression is upregulated when *Synechocystis* cells are exposed to iron-limiting conditions [18,26]. Overall, the finding of increased abundance in iron uptake proteins in the *slr1908*-mutant is in agreement with the potential role proposed for the Slr1908 protein to work as a porin [16]. Kowata et al. showed that Slr1841, Slr1908 and Slr0042 are responsible for uptaking approximately 80% of inorganic ions [16]. Moreover, Slr1908 has also been suggested to work as an iron-selective porin [19]. Based on our and others' results, the *slr1908*-mutant strain was selected for further studies to uncover its altered iron uptake phenotype, and to better understand the function of the Slr1908 protein in *Synechocystis*.

2.4. Mutant Strain 1908 Shows Significantly Lower Intracellular Iron Content

To evaluate the involvement of Slr1908 in iron uptake, the total intracellular iron content of *Synechocystis* wild-type and *slr1908*-mutant cells cultivated under standard growth conditions was investigated. To that end, cell extracts were obtained and analyzed by atomic absorption spectrometry, which determined that *slr1908*-mutant cells have approximately 40% less iron content than wild-type cells (Figure 4A), further supporting that Slr1908 is involved in iron import in *Synechocystis*. As no difference in growth could be observed between the *Synechocystis* wild-type and 1908 cells under standard conditions (Supplementary Material Figure S1), we then decided to evaluate the role of the protein under conditions with different iron availability and light regimes (Figure 4B). The decision of combining these two environmental conditions was based on the fact that different enzymatic complexes involved in the primary metabolism of *Synechocystis* (e.g., light-dependent photosystems I and II, and cytochrome *b₆f*, but also terminal oxidases in respiration) require iron as a co-factor.

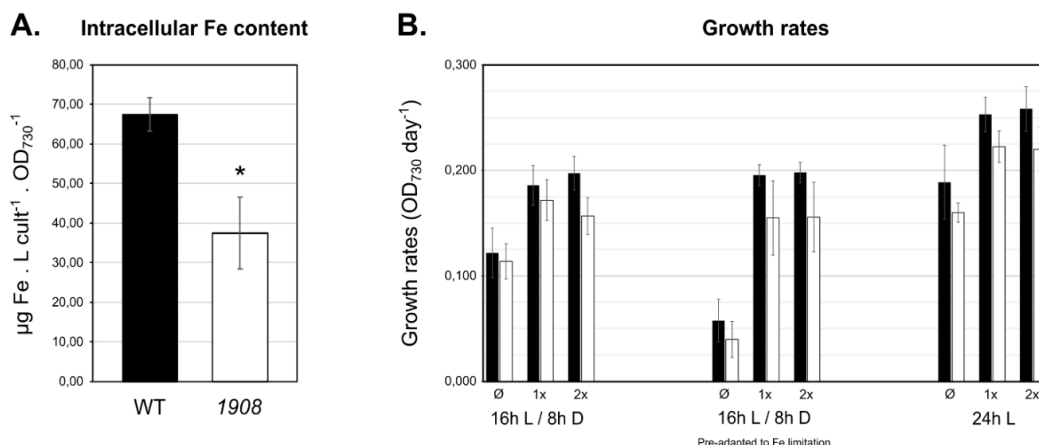


Figure 4. Analysis of the *Synechocystis* sp. PCC 6803 *slr1908*-mutant strain intracellular iron content and growth in medium containing different amounts of iron. **(A)** Intracellular iron concentration determined by atomic absorption spectrometry in *Synechocystis* wild-type (WT) and *slr1908*-mutant (1908) strains cultivated under standard liquid BG11 conditions, shown in $\mu\text{g of Fe} \cdot \text{L culture}^{-1} \cdot \text{OD}_{730}^{-1}$. Error bars represent the standard deviations of three independent biological replicates. *, $p < 0.05$. **(B)** *Synechocystis* wild-type and *slr1908*-mutant growth rates in BG11 medium with modified iron ammonium citrate concentration, under different cultivation setups. Cyanobacterial cells were cultivated in 96-well clear bottomed microplates at 30°C for four days. Standard BG11 medium was used as control condition (1 $\times$), or in BG11 either lacking iron ammonium citrate ($\emptyset$) or in BG11 medium containing two-fold excess iron ammonium citrate (2 $\times$). Different cultivation setups were tested: (1) cells were grown under a 16 h light ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)/ 8 h dark cycle regime (16 h L/8 h D); (2) cells were previously pre-adapted for seven days in BG11 medium without iron ammonium citrate, and then used as inoculum for the cultivation assay, under a 16 h light ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)/ 8 h dark cycle regime (16 h L/8 h D (pre-adapted to Fe limitation)); (3) cells grown under constant light (24 h L). Error bars represent the standard deviation of at least three independent biological replicates.

Overall, growth rates' analysis showed that cyanobacterial strains cultivated under continuous light grew faster than those in light/dark cycles, as cyanobacterial growth is closely dependent on light availability [27]. Moreover, it was also possible to observe that cells cultivated in medium lacking iron ammonium citrate had lower growth rates as to those cultivated in complete medium or supplemented with extra amounts of iron ammonium citrate, in agreement with previous reports [19]. Nevertheless, irrespective of the condition analyzed, strain 1908 displayed a consistently lower growth rate when compared to the wild-type, but the detected differences were never significant ($p > 0.05$), with at least three independent biological replicates. Altogether, these results indicate that under physiological conditions combining the light regime and iron availability, absence of *Slr1908* does not induce significant growth impairment, even when the intracellular iron content is reduced by 40% (Figure 4A). These results are somewhat different to those reported earlier by Qiu et al., who showed that downregulation of *slr1908* gene expression results in significantly lower growth [19]. However, the work was not carried out on a *slr1908* disruption mutant as described here, but rather on a strain in which *slr1908* gene expression was under the control of a copper inducible promoter (P_{petE}) [19]. In such conditions, *slr1908* was transcribed in a medium containing copper, while strong repression of gene expression was achieved by removing copper from the medium. Therefore, even though Qiu et al. observe significant impairment in iron uptake in a *slr1908*-knocked-down strain, followed by an impact in its growth, this could result from synergistic effects between altered iron uptake and induced copper low availability, which could generate an effect in, e.g., electron transport in photosynthesis [19]. It is also possible that absence of a functional *Slr1908* does indeed result in significant alterations in growth, but the difference is likely so small that more biological replicates would be required to validate

such phenotypes. Nevertheless, the results from both works complement each other in supporting the possibility of Slr1908 in working as an iron-selective import porin.

2.5. Disruption of *slr1908* Results in Hypervesiculation

The main goal of this work was to investigate the possible role of SLH-OprB OM proteins on the processes of EVs formation. As LPS are structural components of the cyanobacterial OM, their abundance in the extracellular medium was also evaluated, as a proxy for assessing possible differential vesiculation capacity between the different cyanobacterial strains, as previously determined [28,29]. Cell-free, concentrated extracellular medium samples isolated from *Synechocystis* wild-type and mutant strains were separated by electrophoresis on SDS-polyacrylamide gels, and further stained with an LPS specific protocol (Figure 5).

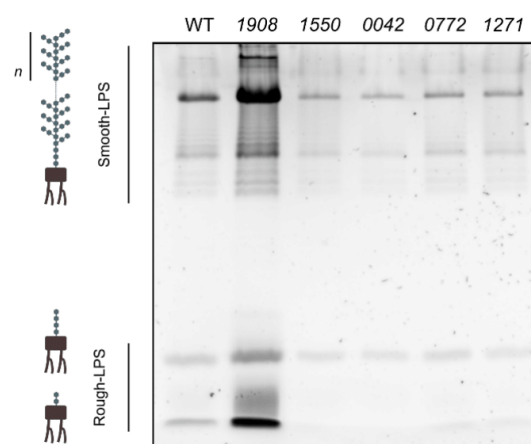


Figure 5. Analysis of the lipopolysaccharides (LPS) content in *Synechocystis* sp. PCC 6803 wild-type and mutant strains in cell-free extracellular medium samples. *Synechocystis* wild-type (WT) and mutant (1908, 1550, 0042, 0772, 1271) strains were cultivated under standard conditions, and the growth medium was collected and concentrated after cultivation. Cell-free, concentrated samples were separated on 16% (*w/v*) SDS-polyacrylamide gels and stained with Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit (Thermo Fisher Scientific). Sample loading is normalized to each culture cell density (OD_{730}), volume of cell-free medium concentrated and concentration factor. On the left-hand side, schematic representation of the smooth-LPS (lipid A, oligosaccharide core, and O-antigen), and rough-LPS (lipid A and oligosaccharide core) that are found in *Synechocystis* OM and vesicles.

Mutant 1908 was the only strain showing a significant increase in the total amount of extracellular LPS as compared to the wild-type strain, suggesting that it releases more EVs. To verify this result, we then proceeded with EVs isolation from wild-type and 1908. The resulting samples were then characterized by evaluating LPS bands' profile and abundance, nanoparticle tracking analysis (NTA), and transmission electron microscopy. In agreement with the analysis of complete extracellular medium concentrated samples (Figure 5), analysis of the LPS content in isolated EVs samples, followed by densitometry quantification, indicated that 1908 contains approximately five-fold more LPS than the wild-type strain (Figure 6A). Furthermore, NTA allowed for the quantification of the total amount of nano-sized particles in the samples (Figure 6B). This technique shows that *slr1908*-mutant strain releases up to four-fold more nanoparticles than the parental strain. Together, these results strongly suggest that 1908 is a hypervesiculating strain.

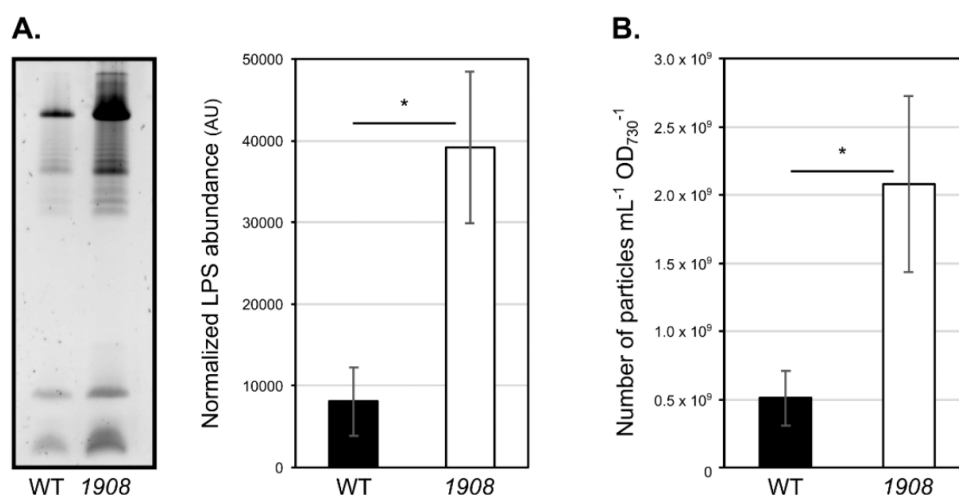


Figure 6. Vesiculation capacity of *Synechocystis* sp. PCC 6803 wild-type and *slr1908*-mutant strains. Cyanobacterial strains were cultivated under standard conditions and their extracellular vesicles (EVs) isolated by ultrafiltration and ultracentrifugation. **(A)** EVs samples from wild-type (WT) and *slr1908*-mutant were separated by electrophoresis on 16% (*w/v*) SDS-polyacrylamide gel and stained with Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain (Thermo Fisher Scientific). Sample loading is normalized to each culture cell density (OD₇₃₀), volume of cell-free medium concentrated and concentration factor. LPS band intensity was quantified by densitometry using the ImageJ software, resulting in the graph shown onto the right-hand side. **(B)** Particle concentration determination of the isolated EVs samples by nanoparticle tracking analysis. Error bars represent the standard deviation of three independent biological replicates. *, $p < 0.05$.

Transmission electron micrographs of negatively stained isolated EVs (Figure 7) show the accumulation of nano-sized spherical structures that resemble those of EVs reported earlier for *Synechocystis* [8,29,30] and other Gram-negative bacteria [4,5,12]. In line with LPS and NTA analyses, electron micrographs further support the higher vesiculation capacity of 1908. In addition, this technique also allowed the detection of shifts in size of the released EVs. Wild-type EVs have a broad diameter distribution, and more than 50% of the measured vesicles range between 45 and 75 nm in diameter. In contrast, EVs produced by 1908 have a more homogeneous size distribution, with approximately 50% of the measured EVs with a diameter between 30 and 45 nm (Figure 7). However, the biological aspects accounting for the differences in EVs' size between the wild-type and the mutant strain remain unknown.

The increase in vesicles' release by 1908 is in line with the proposed biogenesis mechanism for Gram-negative bacteria, based on the modulation of links between the OM and the remaining cell envelope components [5,8]. This suggests that cyanobacteria can release EVs following this particular EVs production route. Nevertheless, 1908 also shows extensive adaptations to iron uptake. Therefore, it should not be discarded that mutant cells may be using EVs as an alternative iron uptake system. In this regard, it has been suggested that bacterial EVs can be loaded with proteins or other molecules to scavenge iron from the extracellular medium, and later be internalized by the cells [4,5,31]. Once the protein content from the isolated EVs was not analyzed in this work, further testing is needed to clarify the roles played by the EVs in 1908.

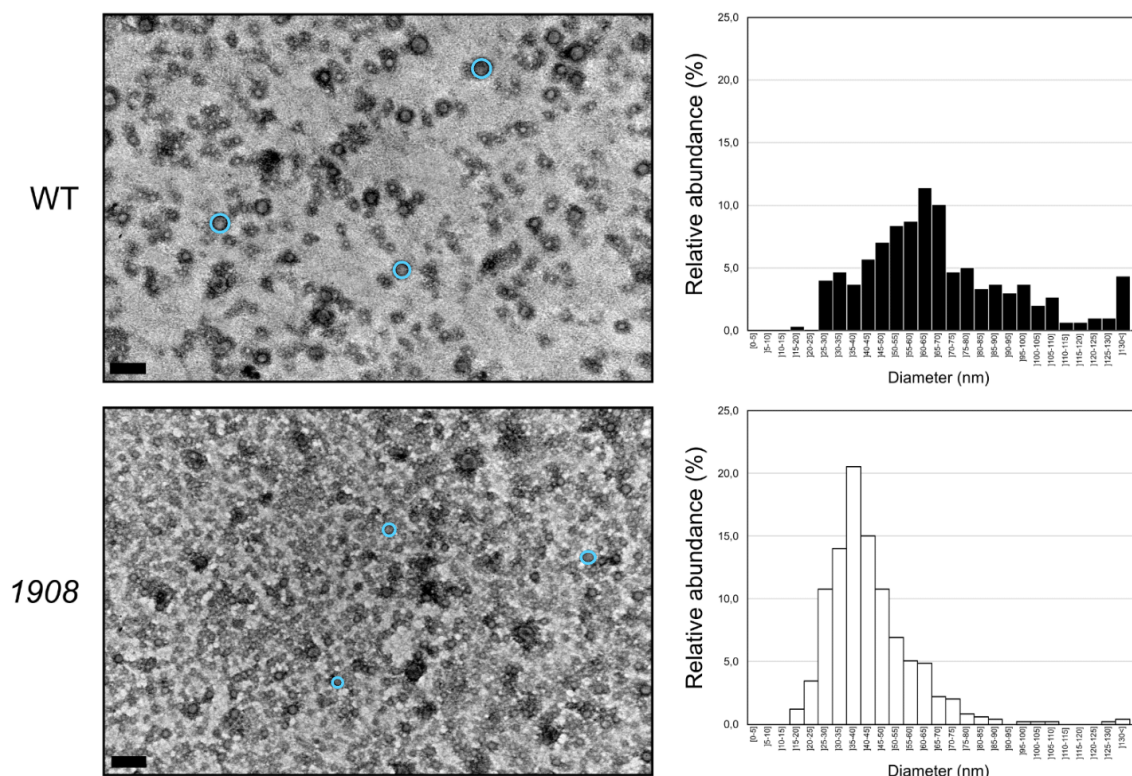


Figure 7. Structure and size distribution analysis of extracellular vesicles isolated from the *Synechocystis* sp. PCC 6803 wild-type and *slr1908*-mutant based on transmission electron microscopy. Cyanobacterial strains were cultivated under standard conditions, and their EVs isolated by ultrafiltration and ultracentrifugation. EVs from the *Synechocystis* wild-type (WT) and *slr1908*-mutant (1908) were negatively stained and visualized by transmission electron microscopy. Three selected vesicles in each micrograph are labeled as light-blue circles to indicate size and shape of representative EVs. Size bar: 200 nm. Vesicle diameter was determined by direct measurement from electron micrographs of three independent biological replicates, resulting in the histograms shown onto the right-hand side. EVs were separated in 5 nm size groups, distributed between 0 and 130 nm, and in a group comprising those with a size higher than 130 nm. Abundance of each size group is presented as relative figures, i.e., number of vesicles with a given diameter range relative to the total number of vesicles measured.

3. Conclusions and Future Perspectives

This work shows that inactivation of *slr1908*, encoding a major OM protein harboring SLH/OprB-domains, causes a reduction in intracellular iron content in *Synechocystis*, and results in a number of protein adaptations at the outer membrane and extracellular protein levels. In addition, absence of Slr1908 leads to an increased vesicle production. We suggest that outer membrane stability in cyanobacteria is dependent on the links established by proteins harboring SLH/OprB-domains and the underlying peptidoglycan layer. By removing Slr1908, we hypothesize that outer membrane stability is compromised, leading to hypervesiculation. Nevertheless, the molecular mechanisms determining SLH-protein domain binding to the peptidoglycan layer, and the players involved in modulating those interactions, represent two of the key aspects that deserve further examination. There are indications that pyruvylation of the peptidoglycan-associated polysaccharide is essential for the binding of the SLH-protein domain [32], but the picture is far from complete in cyanobacteria. Moreover, the dance between the different major OM proteins in response to different environmental and physiological conditions is an additional topic that warrants investigation. Altogether, this work highlights that the study of mechanisms determining

vesicle biogenesis in cyanobacteria must be closely combined with inorganic ions uptake, OM protein composition, and cell envelope stability, which will be essential for determining the cause–effect relationship among players with possible dual biological role.

4. Materials and Methods

4.1. Strains and Maintenance Conditions

The unicellular, non-nitrogen fixing cyanobacterium *Synechocystis* sp. PCC 6803 (non-motile and with S-layer) was routinely maintained in liquid BG11 medium [33], in Erlenmeyer flasks with agitation (70 rpm), under a light regimen of 16 h light (15 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)/8 h dark, at 28 °C. For cultivation in solid plates, BG11 medium was supplemented with 1% (*w/v*) Noble agar (Difco), and 4.79 g L^{−1} of sodium thiosulfate pentahydrate. *Synechocystis* mutant strains generated in this work were cultivated similarly, except that BG11 medium was supplemented with 100 $\mu\text{g mL}^{-1}$ kanamycin. For monitoring growth, optical density at 730 nm (OD₇₃₀) was determined by spectrophotometry.

4.2. In Silico Identification of Genes Encoding SLH/OprB-Domain Containing Proteins

The deduced protein sequences from the genome of *Synechocystis* sp. PCC 6803 were used as a query in the web resource SMART (Simple Modular Architecture Research Tool) [34,35]. The search of proteins with an architecture combining the specific protein domains SLH (PF00395; IPR001119) and OprB (PF04966; IPR007049) was carried out. Protein candidates with an E-value lower than 4.2×10^{-8} and 3.6×10^{-49} for the SLH and OprB domains, respectively, were selected for further analysis.

4.3. Isolation and Analysis of *Synechocystis* Outer Membrane Proteins

Synechocystis cultures were first prepared to an initial OD₇₃₀ of 0.5 in liquid BG11 medium, and cultivated in Erlenmeyer flasks with agitation (70 rpm) at 28 °C, under the same light and temperature conditions as described (see 4.1 *Strains and maintenance conditions*) up to an OD₇₃₀ of 1.0–1.5. These were then used to inoculate cultures (initial OD₇₃₀ of 0.05) maintained in liquid BG11 medium, in glass gas washing bottles with aeration (1 L min^{−1}), under a light regimen of 16 h light (40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)/8 h dark. When cultures reached an OD₇₃₀ of approximately 1.0–1.5, OMs were isolated as described initially by [36] and adapted by [37]. In brief, cells were pelleted at 4400 × *g* for 10 min at room temperature and the pellet corresponding to 50 mL of grown culture was washed in 1 mL of buffer O (10 mM Tris-HCl, pH 7.45). The mixture was then transferred to a 1.5 mL microfuge tube and centrifuged at 16,100 × *g* for 2 min at 4 °C. Cells were suspended in 400 μL of buffer O and lysed by sonication (Branson Sonifier 250 (output 3–4, 50% duty cycle)) in 5 cycles of 15 sec power, 15 sec on ice. Then, cell lysates were centrifuged for 15 sec at 16,100 × *g* (4 °C) to remove unbroken cells and heavy cellular debris, and the supernatant was further centrifuged for 30 min at 16,100 × *g* (4 °C). The resulting pellet was washed in 200 μL of buffer O, and centrifuged once again for 30 min under the same conditions. Next, the pellet was suspended in 200 μL buffer O, and mixed with 250 μL of buffer O containing 2% (*v/v*) N-Lauroylsarcosine. The mixture was incubated for 30 min with agitation in a Roto-Torque (Cole-Parmer) at room temperature. Outer membranes were then collected by centrifugation (45 min at 16,100 × *g* (4 °C)), and washed twice in 400 μL of buffer O. In the end, isolated OMs were suspended in 50 μL of buffer O and stored at −20 °C. Proteins were separated by electrophoresis either on gradient 4–15% (*w/v*) (Bio-Rad) or on homogeneous 12% (*w/v*) SDS-polyacrylamide gels. Peptide band detection was performed either with colloidal Coomassie brilliant blue (Sigma) staining or PageBlue™ Protein Staining Solution (Thermo Fisher Scientific), and pictures of the stained gels were acquired with a GS-800 calibrated imaging densitometer (Bio-Rad).

4.4. Protein Identification by NanoLC-MS/MS

Selected polyacrylamide gel bands were analyzed by mass spectrometry at the i3S Proteomics Unit. Gel bands were washed twice with 50% acetonitrile (ACN) in 50 mM

triethylammonium bicarbonate (TEAB) with shaking at 1500 rpm for 5 min, and further treated with ACN twice. Next, proteins were reduced with 25 mM dithiothreitol (DTT) for 20 min at 56 °C and alkylated with 55 mM iodoacetamide (IAA) for 20 min at room temperature in the dark, followed by the same wash procedure. Proteins were then digested with trypsin (240 ng) in 50 mM TEAB/0.01% (*w/v*) surfactant for 60 min at 50 °C. Peptide gel extraction was performed with 2.5% trifluoroacetic acid (TFA) followed by 50% ACN, 0.1% TFA. Then, samples were dried, suspended in 10 µL 0.1% TFA and cleaned by C18 reverse phase chromatography following the manufacturer's instructions (ZipTip, Merck, Darmstadt, Germany). Sample protein identification and quantification was performed by nano liquid chromatography mass spectrometry (nanoLC-MS/MS) with a 90 min chromatographic separation run. The specific MS parameters were: MS maximum injection time, 100 ms; dd settings: minimum AGC target 7.0×10^3 , intensity threshold 6.4×10^4 , and dynamic exclusion 20 s. Data acquisition was controlled by Tune 2.9 software (Thermo Scientific, Waltham, MA, USA). The UniProt database for the taxonomic selection *Synechocystis* sp. PCC 6803 was considered for protein identification. Protein identification was performed with the Proteome Discoverer software (v2.3.0.523, Thermo Scientific).

4.5. Mutant Generation

To generate mutants of the genes encoding OM porins in *Synechocystis*, a classical double homologous recombination approach was undertaken [38]. To that end, one plasmid was generated for each gene of interest. The recombination platforms (approximately 600 bp) for each gene disruption were amplified by PCR using specific oligonucleotides (see Supplementary Material Table S2), and with *Synechocystis* genomic DNA as template. As the oligonucleotides' sequences were designed to harbor recognition sites for various restriction enzymes, the DNA fragment corresponding to the 5' recombination platform was digested with XhoI and PstI, while the 3' recombination platform was digested with PstI and BamHI, following the manufacturer's instructions (Thermo Fisher Scientific). In addition, plasmid pBluescript SKII (+) was digested with XhoI and BamHI. Assembly of the three digested DNA fragments was performed by ligation with T4 DNA ligase (Thermo Fisher Scientific), and later transformed into *E. coli*. Identity of the successfully assembled plasmids was determined by Sanger sequencing (StabVida), resulting in plasmids pDC1841, pDC1908, pDC0042, pDC1550, pDC0772, and pDC1271. Later, the kanamycin resistance cassette from the plasmid pUC4K (GE Healthcare) was amplified using specific oligonucleotides (Supplementary Material Table S2). The resulting DNA fragment was digested with PstI, as well as each of the generated plasmids. The two DNA fragments were assembled by ligation and transformed into *E. coli*. Kanamycin resistant colonies were selected for plasmid isolation, and the resulting plasmids were named pDC1841K, pDC1908K, pDC0042K, pDC1550K, pDC0772K, and pDC1271K. The generated plasmids were used to naturally transform *Synechocystis* according to Heidorn et al. [38]. Transformants were transferred repeatedly over the course of 4–5 weeks to BG11 agar plates supplemented with increasing concentrations of kanamycin to ensure complete chromosome segregation.

4.6. Analysis of *Synechocystis* Wild-Type and Mutant Strains' Growth Capacities

To monitor growth of the various *Synechocystis* strains under standard conditions, cyanobacteria were first inoculated in 100 mL Erlenmeyer flasks to an initial OD₇₃₀ of 0.5, supplemented with kanamycin when necessary, and cultivated at 28 °C, under a light regimen of 16 h light (15 µmol photons m⁻² s⁻¹)/ 8 h dark, with agitation (70 rpm). Cultures with an OD₇₃₀ of approximately 1.0–1.8 were then diluted to an OD₇₃₀ of 0.4 with BG11 liquid medium, distributed onto 96-well clear bottomed microtiter plates in technical triplicates, and incubated at 30 °C with a 16 h light (50 µmol photons m⁻² s⁻¹)/ 8 h dark cycle with agitation (150 rpm). On a daily basis, cells were suspended by pipetting, and optical density at OD₇₃₀ was monitored on a microplate reader (Synergy Mx, BioTek, Winooski, VT, USA).

To evaluate the effect of combining iron and light availability on the growth capacity of *Synechocystis* wild-type and *slr1908*-mutant strain, similar analyses were performed on 96-well clear bottomed microtiter plates. Growth was monitored in cultures cultivated in BG11 medium, or in modified BG11 medium, in which iron ammonium citrate was either absent or supplied in a two-fold amount as compared to standard BG11. Moreover, three cultivation setups were tested, including: (1) cells cultivated in constant light; (2) cells cultivated in light/dark cycles (16 h light/8 h dark); or (3) cells pre-adapted for one week in modified BG11 medium lacking iron ammonium citrate (in that period, cultivation was carried out in Erlenmeyer flasks, in a 16 h light/8 h dark cycle regimen). Regardless of the tested condition, *Synechocystis* strains were washed three times in modified BG11 medium lacking iron ammonium citrate prior to being distributed onto the microtiter plates. Optical density was monitored daily on the microplate reader Synergy Mx.

At least three independent biological replicates were performed for each strain and condition. For analysis, growth rates were calculated between the first and fourth days of cultivation, and are expressed in $\text{OD}_{730} \cdot \text{day}^{-1}$. Statistical analyses were performed by one-way ANOVA, by comparing the growth rate of each strain in a given condition and that of the wild-type in the same growth condition.

4.7. Quantification of Intracellular Iron Levels by Atomic Absorption Spectrometry

The intracellular levels of iron in *Synechocystis* wild-type and *slr1908*-mutant strain were quantified by atomic absorption spectrometry. Cells were cultivated in standard conditions (BG11 medium, and in gas washing bottles, with aeration (1 L air min^{-1})) up to an OD_{730} of 0.8–1.2. Biomass was then collected by centrifugation at $4400 \times g$ for 10 min, and washed several times with extraction buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.6) for complete removal of BG11 traces. Later, cell pellets were mixed with 100 μL of 65% HNO_3 , and incubated at 75°C for 12 min, vigorously vortexed every 2 min. Then, Milli-Q[®] grade water was added, samples mildly vortexed, and centrifuged at $16,000 \times g$ for 5 min at room temperature. Iron levels were determined in the resulting supernatant with an atomic absorption spectrometer coupled to a flame atomization detector (Perkin Elmer). Quantifications (using three independent biological replicates for each strain) were performed against calibration curves using specific aqueous pattern solutions. Results are presented in μg of metal L^{-1} culture OD_{730}^{-1} , and statistical analysis was performed by one-way ANOVA.

4.8. Isolation, Concentration and Analysis of Cyanobacterial Extracellular Media

To analyze the exoprotein and extracellular LPS content in the growth medium, *Synechocystis* wild-type and mutant strains were cultivated as described above (see 4.3. *Isolation and analysis of Synechocystis outer membrane proteins*). After cell harvesting, the extracellular medium was filtered through 0.2 μm pore-size filters, and concentrated by ultrafiltration with Amicon Ultra-15 Centrifugal Filter units (Millipore, Burlington, MA, USA) with a nominal molecular weight limit of 3 kDa [29,39]. Prior to analysis, samples were normalized by the volume of cell-free extracellular medium concentrated, concentration factor, and respective culture's cell density (OD_{730}). For exoproteome analysis, samples were separated by electrophoresis either on gradient 4–15% (w/v) (Bio-Rad, Hercules, CA, USA) or on homogeneous 12% (w/v) SDS-polyacrylamide gels. Peptide band detection was performed with colloidal Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO, USA) staining, and pictures of the stained gels were acquired with a GS-800 calibrated imaging densitometer (Bio-Rad). For detection of extracellular LPS, the protocol described by Oliveira et al. [29] was followed. In brief, samples were heat denatured either in $1 \times$ solubilization buffer ($2 \times$: 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 0.05% (w/v) bromophenol blue in 125 mM Tris-HCl pH 6.8) at 95°C for 10 min. Heat denatured samples were incubated with 0.2 U of type XIV protease from *Streptomyces griseus* (Sigma-Aldrich) at 37°C for 30 min. Samples were then separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels, and LPS were stained with Pro-Q[®] Emerald 300 Lipopolysaccharide gel staining kit

(Life Technologies, Carlsbad, CA, USA). LPS were visualized on a Gel DocTM XR + UV transilluminator system (Bio-Rad), and LPS band intensity was quantified by densitometry using the ImageJ software.

4.9. Isolation and Characterization of Cyanobacterial Extracellular Vesicles

For isolation of cyanobacterial EVs, cells were cultivated and the extracellular medium recovered as described above. Next, the cell-free extracellular medium was concentrated by ultrafiltration with centrifugal filters (Pall, New York, NY, USA) with a nominal molecular weight limit of 100 kDa to approximately 10–20 mL. The concentrated solution was then ultracentrifuged (70 Ti fixed angle rotor, Beckman Coulter Optima L80 XP Ultracentrifuge, Brea, CA, USA) using polycarbonate bottles with cap assembly (#355618) for 3 h at 100,000× *g* at 4 °C. Finally, the resulting pellet was suspended in approximately 200 µL of sterile BG11 growth medium, and stored at −80 °C until further analysis.

To evaluate the amount and band profile of the LPS in the isolated *Synechocystis* EVs, samples were prepared, separated by electrophoresis, and gels stained as described above. In addition, EVs samples were visualized by negative-staining transmission electron microscopy mainly as described elsewhere [29]. A volume of 10 µL of sample were added on formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min. Using filter paper, the liquid in excess was removed, and 5 µL of 1% (*w/v*) uranyl acetate was used to cover the grids and left standing for 10 s, after which liquid in excess was again removed with filter paper. Observation was performed using a Jeol JEM-1400 transmission electron microscope at 80 kV. Finally, EVs were analyzed on a NanoSight NS300 NTA Dev Build 3.2.16 (Malvern, Malvern, UK), equipped with an sCMOS camera. Samples were diluted, and further analyzed as described [29]. In brief, three movies of 30 s were recorded for each sample (with a threshold of 10 to 50 particles per frame), with the following capturing settings: camera level 14–16, slider shutter 1300, slider gain 512, at 23 °C, and with a syringe pump speed of 40. Data acquisition and processing were performed using the NanoSight NS300 NTA 3.0 software, using a detection threshold of 5.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/plants10122757/s1>, Table S1: Transcript levels analysis of the *Synechocystis* sp. PCC 6803 transcriptional units (TU) harboring genes encoding SLH/OprB-domains outer membrane proteins under 10 different environmental conditions, as determined by [18]; Table S2: Oligonucleotides used in this study; Figure S1: *Synechocystis* sp. PCC 6803 disruption mutants' growth rates determined in cultures grown under standard conditions.

Author Contributions: Conceptualization, P.O.; Data curation, D.C., S.L. and J.M.-C.; Formal analysis, D.C., S.L., J.M.-C. and P.O.; Funding acquisition, P.T. and P.O.; Investigation, D.C., S.L., J.M.-C. and P.O.; Methodology, D.C., S.L. and J.M.-C.; Project administration, P.T. and P.O.; Resources, P.T. and P.O.; Supervision, P.T. and P.O.; Writing—original draft, D.C. and P.O.; Writing—review and editing, D.C., S.L., J.M.-C., P.T. and P.O. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material

Table S1 - Transcript levels analysis of the *Synechocystis* sp. PCC 6803 transcriptional units (TU) harboring genes encoding SLH/OprB-domains outer membrane proteins under 10 different environmental conditions, as determined by [18].

TU ID	TU start	TU end	Strand	5'UTR	genes	15 °C	42 °C	-CO ₂	Dark	-Fe	HL	-N	-P	Exp. p.	Stat. p.	Max reads	Average	Max/Avg	Sense tags
TU971	958084	960116	+	53	1	40362	45295	38272	9363	45113	95667	82402	66762	49195	29046	95667	50147,7	1,91	slr1841
TU599	610937	614020	+	120	3	21331	15443	7420	243	42214	53026	11858	43046	22829	4413	53026	22182,3	2,39	slr1908 , <i>slr1909</i> , <i>slr1910</i>
TU1141	1116114	1111170	-	93	4	1678	0	58	0	214	259	2823	0	13761	180	13761	1897,3	7,25	<i>slr1270</i> , slr1271 , <i>ssl2507</i> , <i>slr1272</i>
																			slr0772
TU3332	3168888	3176441	+	166	5	30343	1201	345174	2673	5711	1005917	5985	26025	207607	180	1005917	163081,6	6,17	<i>slr0040</i> , <i>slr0041</i> , slr0042 , <i>slr0043</i> , <i>slr0044</i>
TU3630	3456162	3454008	-	106	1	0	33	19	0	1382	199	75	39	210	45	1382	200,2	6,9	slr1550

In bold are highlighted the genes encoding SLH/OprB-domains outer membrane proteins studied in this work.

Table S2. Oligonucleotides used in this work.

Oligo	Application	Sequence (5' to 3')	Product size (bp)	Restriction Enzyme	T _a (°C)
<i>slr1841</i> 5'F	<i>slr1841</i> 5' recombination platform	Aaa <u>ctcgag</u> agtcctgttagtgagccct	651	XhoI	63
<i>slr1841</i> 5'R		Aaa <u>ctgcagc</u> gagacgagtgaccaacagg		PstI	
<i>slr1841</i> 3'F	<i>slr1841</i> 3' recombination platform	Aaactgcagtggttacttggttagcacg	663	PstI	63
<i>slr1841</i> 3'R		Aaaggatccgagagggttagcagtggtt		BamHI	
<i>slr1908</i> 5'F	<i>slr1908</i> 5' recombination platform	Aaa <u>ctcgag</u> agtcacaggtgaattggcc	580	XhoI	63
<i>slr1908</i> 5'R		Aaactgcaggcgccgaagtgtcaatgtt		PstI	
<i>slr1908</i> 3'F	<i>slr1908</i> 3' recombination platform	Aaactgcagctggattggcgttactggtt	668	PstI	63
<i>slr1908</i> 3'R		Aaaggatccagggtcatcaaacaccactc		BamHI	
<i>slr0042</i> 5'F	<i>slr0042</i> 5' recombination platform	Aaa <u>ctcgagg</u> tgcacatcggttcggagtt	647	XhoI	63
<i>slr0042</i> 5'R		Aaactgcagggttgagatttggggatt		PstI	
<i>slr0042</i> 3'F	<i>slr0042</i> 3' recombination platform	Aaactgcaggcctaacgatgtggtcgatt	629	BamHI	63
<i>slr0042</i> 3'R		Aaaggatcctagatgccttccggatta		PstI	
<i>slI1550</i> 5'F	<i>slI1550</i> 5' recombination platform	Aaactcgaggggacagatcacttccgtgt	566	XhoI	63
<i>slI1550</i> 5'R		Aaactgcagacactggctgaaagtaccg		PstI	
<i>slI1550</i> 3'F	<i>slI1550</i> 3' recombination platform	Aaactgcagaccgtagcccgatctattt	581	PstI	63
<i>slI1550</i> 3'R		Aaaggatcctcaatgtcagtgttggaat		BamHI	
<i>slI0772</i> 5'F	<i>slI0772</i> 5' recombination platform	Aaa <u>ctcgagg</u> gcaatggcgtaatacaagt	689	XhoI	63
<i>slI0772</i> 5'R		Aaactgcagacagtacccaagcgagagt		PstI	
<i>slI0772</i> 3'F	<i>slI0772</i> 3' recombination platform	Aaactgcagagctggcttaattggcaga	636	PstI	63
<i>slI0772</i> 3'R		Aaaggatccttaagcacgacccaaaacc		BamHI	
<i>slI1271</i> 5'F	<i>slI1271</i> 5' recombination platform	Aaa <u>ctcgaga</u> acgacaatgccaaaacctc	648	XhoI	63
<i>slI1271</i> 5'R		Aaactgcaggcgcaaagttaaatacag		PstI	
<i>slI1271</i> 3'F	<i>slI1271</i> 3' recombination platform	Aaactgcagatcgccccatttattacc	574	PstI	63
<i>slI1271</i> 3'R		Aaaggatcccggtgtagcggagtaata		BamHI	
pUC 4K F	Kanamycin resistance cassette	aaactgcagtgaggtctgcctcgtgaagaa	1226	PstI	64
pUC 4K R		aaactgcagaaagccacgtgtgtctcaaa		PstI	

The pair of oligonucleotide used for the amplification of the kanamycin resistance cassette was taken from [29].

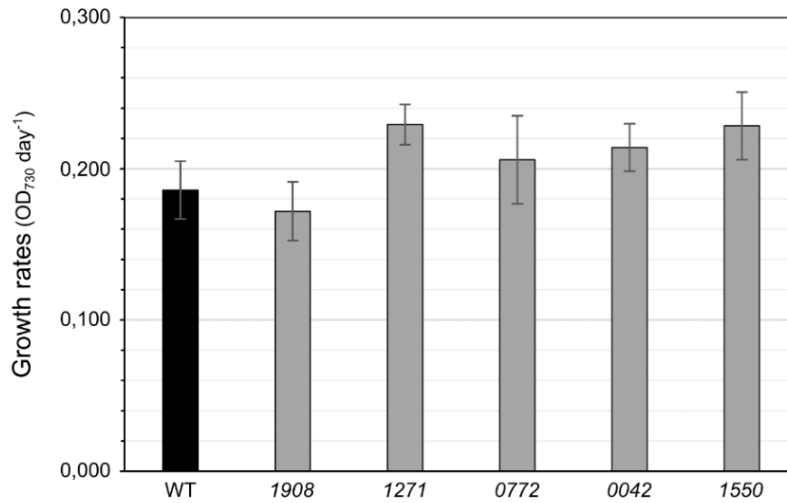


Figure S1. *Synechocystis* sp. PCC 6803 disruption mutants' growth rates determined in cultures grown under standard conditions. *Synechocystis* cultures of the wild-type (WT) and mutant strains *slr1908* (1908), *slr1271* (1271), *slr0772* (0772), *slr0042* (0042) and *slr1550* (1550) were grown in BG11 liquid medium, in 96-well clear bottomed microplates at 30 °C with a 16 h light (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) / 8 h dark cycle regime, with agitation, for 4 days. Every day, cell cultures were suspended and their optical density (OD₇₃₀) measured on a microplate reader. Growth rates were determined between the first and fourth day of cultivation. Error bars represent the standard deviation of three independent biological replicates.

CHAPTER V - Extracellular vesicles as an alternative copper-secretion mechanism in bacteria



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* Performed all the laboratory work leading to this publication, with the exception of: the proteomic analysis by iTRAQ; metal sensitivity screening; metal quantification; analysis of transcript levels by RT-qPCR; confocal microscopy analysis.



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Extracellular vesicles as an alternative copper-secretion mechanism in bacteria

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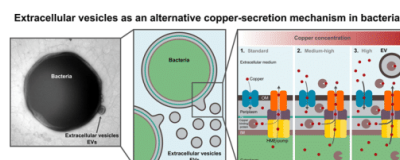
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HIGHLIGHTS

- Extracellular vesicles (EVs): overlooked metal resistance mechanism in bacteria.
- Vesiculation is a common response mechanism to copper-induced stressful conditions.
- Cyanobacterial EVs are packaged with metal binding proteins.
- Cyanobacterial EVs work as copper-secreting nanocapsules.

GRAPHICAL ABSTRACT



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ABSTRACT

Metal homeostasis is fundamental for optimal performance of cell metabolic pathways. Over the course of evolution, several systems emerged to warrant an intracellular metal equilibrium. When exposed to growth-challenging copper concentrations, Gram-negative bacteria quickly activate copper-detoxification mechanisms, dependent on transmembrane-protein complexes and metallochaperones that mediate metal efflux. Here, we show that vesiculation is also a common bacterial response mechanism to high copper concentrations, and that extracellular vesicles (EVs) play a role in transporting copper. We present evidence that bacteria from different ecological niches release copious amounts of EVs when exposed to copper. Along with the activation of the classical detoxification systems, we demonstrate that copper-stressed cells of the cyanobacterium *Synechocystis* sp. PCC6803 release EVs loaded with the copper-binding metallochaperone CopM. Under standard growth conditions, CopM-loaded EVs could also be isolated from a *Synechocystis* strain lacking a functional TolC-protein, which we characterize here as exhibiting a copper-sensitive phenotype. Analyses of *Synechocystis* tolC-mutant's

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EVs isolated from cells cultivated under standard conditions indicated the presence of copper therein, in significantly higher levels as compared to those from the wild-type. Altogether, these results suggest that release of EVs in bacteria represent a novel copper-secretion mechanism, shedding light into alternative mechanisms of bacterial metal resistance.

1. Introduction

Copper is an essential micronutrient. Many proteins and enzymes, such as cytochrome oxidases, laccases, superoxide dismutases, plastocyanin, or nitrite reductase (Argüello et al., 2013), which are involved in a variety of key biological functions in most living organisms require copper as a redox cofactor (Xu et al., 2021). Nevertheless, the ability to undergo redox reactions makes free copper a potential hazard (Argüello et al., 2013), catalyzing the formation of highly reactive hydroxyl radicals (Gaetke and Chow, 2003). This is particularly relevant when organisms are exposed to high copper concentrations. In such conditions, copper may also attack and destroy iron-sulfur clusters, thereby releasing iron, which can in turn cause further oxidative stress (Rensing and McDevitt, 2013), leading to severe cell damage.

Due to its toxicity in high concentrations, copper can be used to control unwanted microbial growth. This feature has been streamlined in the course of evolution: the innate immune system in mammals utilizes copper intoxication to reduce intracellular survival of bacterial pathogens (Djoko et al., 2015). Besides, several anthropogenic activities also employ copper-based solutions to control microbial biofilm formation, including in medical settings, industrial equipment and plumbing systems (Gomes et al., 2020). Even certain sectors of the agri-food industry, such as grape-wine production (EFSA et al., 2018) and aquaculture (Yanong, 2010) resort to copper-based compounds to tackle fungal and bacterial growth. Despite the toxicity and risk of contamination, copper-based compounds are easy and cheap to obtain, do not discolor the water, and are one of the few effective options for organic growers, which still motivates their use (Romanazzi et al., 2020; Yanong, 2010).

Throughout evolution, bacteria have developed copper-transport systems tightly controlling uptake and secretion, but also trafficking through different compartments, promoting a highly regulated balance of copper metabolism (Argüello et al., 2013; Rensing and McDevitt, 2013). Particularly, when exposed to high copper levels, bacteria have shown to activate several systems that effectively help in copper efflux, reducing its noxious effects intracellularly. In spite of different bacteria exhibiting different combinations of copper-efflux components (Argüello et al., 2013), the two essential mechanisms for copper export rely on: (i), transmembrane-protein complexes that cross the cell wall and pump copper from the cytoplasm, or periplasm in Gram-negative bacteria, directly into the extracellular space; and (ii), export of metallochaperones with copper binding capacity, which can work as chelating agents (Nies, 2003; Argüello et al., 2013; Rensing and McDevitt, 2013). Bacterial cells regulate these processes mainly by modifying transporter abundance through transcriptional control, which also facilitates co-regulation of chaperone and chelating metalloproteins (Argüello et al., 2013).

Similar to higher organisms, bacteria release extracellular vesicles (EVs), which have been shown to play fundamental roles in bacterial cell survival, including secretion, communication, and nutrient uptake (Lima et al., 2020). Many studies on vesicles from Gram-negative bacteria predominantly discuss the classic concept of outer membrane vesicles (OMVs). However, there has been a trend in the past few years to refer generally to vesicles from Gram-negative bacteria as EVs (e.g. Biller et al., 2021; McMillan and Kuehn, 2021; Biller et al., 2022). This is mainly due to the fact that recent studies have shown additional routes for vesicle formation, which are not limited to OMVs (Toyofuku et al., 2019). Thus, in this study, we collectively refer to the vesicles produced by bacteria as EVs.

The Cyanobacteria phylum is a large phylogenetic group of the domain bacteria, with the particular feature of being capable of performing oxygenic photosynthesis with water as an electron donor (García-Pichel et al., 2020), similarly to green algae and plants. These photoautotrophic microorganisms play a key role in the biogeochemical cycle of carbon on a global scale, as estimates predict a global net primary production of 12 gigatonnes each year for marine cyanobacteria only, representing approximately 25% of ocean net primary production (Flombaum et al., 2013). The capacity of cyanobacteria to release EVs was documented in detail for the first time in 2014, and cyanobacterial EVs were proposed to function as vehicles for the movement of carbon through marine food webs, as vectors for horizontal gene transfer, and as decoys for predators and phages (Biller et al., 2014). More recently, the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) was also shown to vesiculate (Pardo et al., 2015), and a *Synechocystis* mutant strain, lacking a functional TolC-protein, was described to release substantially more EVs than the parental strain (Oliveira et al., 2016). TolC is an outer membrane protein, essential for the export of proteins (Oliveira et al., 2016; Agarwal et al., 2018; Gonçalves et al., 2018), fatty acids (Bellefleur et al., 2019), and exogenous compounds that reach the cytoplasm (Oliveira et al., 2016; Gonçalves et al., 2018). It was proposed that hypervesiculation in the *Synechocystis* *tolC*-mutant was a response to the impaired classical secretion (Oliveira et al., 2016). Nevertheless, deletion of inner membrane and periplasmic adaptor components of TolC-dependent efflux systems showed that none of those mutants reached the high vesiculation capacity presented by the *tolC*-deletion mutant (Gonçalves et al., 2018). Thus, it remains to be fully determined why the *Synechocystis* *tolC*-deletion mutant hypervesiculates.

In this work, we carried out a comprehensive study of the *Synechocystis* *tolC*-mutant phenotype, in order to clarify why this strain releases more EVs than the wild-type. Combining a quantitative proteomic study with metal resistance tests, intracellular metal quantifications, and biochemical analyses, we show that the *Synechocystis* *tolC*-mutant accumulates copper intracellularly, uncovering the role of TolC in copper efflux in *Synechocystis*. More importantly, we show that EVs release is a common bacterial response upon exposure to high copper levels, and we detect the presence of a copper-binding chaperone in isolated cyanobacterial EVs. Moreover, detection and quantitation of copper in EVs isolated from the copper-sensitive *Synechocystis* *tolC*-mutant, cultivated under standard growth conditions, highlights that EVs in bacteria represent an overlooked copper-secretion mechanism. These results contribute to a deeper understanding of the intricate mechanisms warranting copper homeostasis in bacteria, further expanding the role of EVs in bacterial metal metabolism.

2. Experimental

2.1. Strains and growth conditions

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) wild-type strain and respective mutants used in this work, namely $\Delta tolC$, $\Delta copB$, $\Delta slr1180$ (or $\Delta hlyB$), $\Delta slr1053$ (or $\Delta acrA$), and EV-trc-GFP (see below) were routinely maintained in BG11 medium (Stanier et al., 1971) supplemented, when necessary, with appropriate antibiotics, in 100 mL Erlenmeyer flasks, with orbital shaking, under a regimen of 12 h light (25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)/ 12 h dark at 30 °C. Growth was assessed by measuring optical density at 730 nm (OD₇₃₀). Cyanobacterial strains were routinely streaked on solid BG11 medium

plates to assess the axenic state of the cultures. Unless stated otherwise, *Synechocystis* cells were inoculated at an initial OD₇₃₀ of 0.05 in BG11 and grown in glass gas washing bottles with aeration (approx. 1 L air min⁻¹), under a regimen of 16 h light (45 μmol photons m⁻² s⁻¹)/ 8 h dark at 28 °C, for approximately 3.5 days (unless stated otherwise), reaching a final OD₇₃₀ of approximately 0.8–1.0.

Escherichia coli [strain BL21 (DE3)] and *Pseudomonas aeruginosa* [PAO-1 (ATCC 47085)] were routinely maintained/streaked in LB medium agar plates. Both strains were grown over-night (ON) in 50 mL sterile conical centrifuge tubes, containing 5 mL of liquid LB medium, with orbital shaking (150 rpm for *E. coli*, or 200 rpm for *P. aeruginosa*) at either 37 °C (*E. coli*) or 30 °C (*P. aeruginosa*). ON-grown cultures were used to inoculate 200 mL of LB medium, LB supplemented with 0.5, 1 and 2 mM CuSO₄ for *E. coli*, or LB supplemented with 1 and 3 mM CuSO₄ for *P. aeruginosa*, in 1000 mL Erlenmeyer flasks with an initial OD₆₀₀ of 0.05, at the above-mentioned conditions for 15–16 h, reaching a final OD₆₀₀ of approximately 3.0–5.0.

2.2. Proteomics analysis by iTRAQ

The proteomes of *Synechocystis* wild type and *tolC*-mutant were analyzed by 8-plex isobaric tags for relative and absolute quantification (iTRAQ), using four biological replicates. Cultures were grown in BG11 in 500 mL Erlenmeyer flasks, with orbital shaking, under a regimen of continuous light (35 μmol photons m⁻² s⁻¹) at 30 °C. Upon reaching an OD₇₃₀ of approximately 1.0–1.5, cells were collected by centrifugation (4400 g, at room temperature), washed with potassium phosphate buffer (50 mM K₂HPO₄, 50 mM KH₂PO₄, pH 6.9) and stored at – 80 °C until further analysis. A detailed description of protein extraction and analysis can be found in the [Supporting information](#) file (Text S1).

2.3. Synechocystis metal resistance analysis

To evaluate the metal resistance capacity of *Synechocystis* wild-type and mutant strains, cells were grown in solid BG11 (lacking sodium thiosulfate) supplemented with different metals, namely boron (463 μM H₃BO₃), cadmium (5 μM CdN₂O₆·4 H₂O), cobalt (5 μM Co(NO₃)₂·6 H₂O), copper (4 μM CuSO₄), iron (229 μM Ferric ammonium citrate), manganese (91.5 μM MnCl₂·4 H₂O), molybdenum (16.1 μM Na₂MoO₄·2 H₂O), nickel (7 μM NiSO₄·6 H₂O), or zinc (14 μM ZnSO₄). *Synechocystis* cultures were grown as indicated in “*Strains and growth conditions*”. Later, cells were harvested by centrifugation (4400 g, 10 min at room temperature) and the cell pellet was suspended in fresh liquid BG11 medium, to a final OD₇₃₀ of 10. Serial dilutions were then prepared, and 15 μL of each cell suspension were spotted onto solid BG11 supplemented with each metal. Petri dishes were incubated for 7 days, under a regimen of 16 h light (25 μmol photons m⁻² s⁻¹)/8 h dark at 28 °C, and cell growth was registered by photographing the dishes. To quantify growth differences, a densitometry analysis was carried out using ImageJ ([Schneider et al., 2012](#)).

2.4. Intracellular metal quantification by atomic absorption spectrometry

The intracellular levels of cobalt, copper, iron and zinc in *Synechocystis* wild-type and *tolC*-mutant strain were quantified by atomic absorption spectrometry (AAS). Briefly, cells were cultivated as described above and collected at an OD₇₃₀ of 0.8–1.0. Then, cells were pelleted by centrifugation at 4400 g for 10 min, and thoroughly washed using extraction buffer (20 mM Tris-HCl, 5 mM EDTA, pH 8.0). Later, cells were transferred to a screw-capped, 2 mL microcentrifuge tube, collected by centrifugation at 16,000 g for 1 min at 4 °C, and washed twice again with extraction buffer for complete removal of BG11 traces. Finally, cell pellets were mixed with 100 μL of 65% HNO₃, and incubated at 75 °C for 12 min, vigorously vortexed every 2 min. Following this procedure, 1 mL of Milli-Q® Type 1 Ultrapure water was added, samples mildly vortexed, and centrifuged at 16,000 g for 5 min at room

temperature. The resulting supernatant was collected for AAS analyses. Copper, iron and zinc levels were determined with an atomic absorption spectrometer coupled to a flame atomization detector (Perkin Elmer), while cobalt levels were determined with a spectrometer with an electrothermal detector (Perkin Elmer). For the various metals, quantifications were performed against calibration curves using specific aqueous pattern solutions. Results are presented in μg of metal L⁻¹ culture OD₇₃₀⁻¹.

2.5. Total RNA extraction and analysis of transcript levels by RT-qPCR

Sample collection, RNA extraction, all the control measurements and PCRs were performed as previously described ([Gonçalves et al., 2018](#)). For cDNA synthesis, 5 μg of total RNA was transcribed with the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad) in a final volume of 20 μL, following the manufacturer's instructions. A control PCR was performed using 1 μL of cDNA as template in reaction mixtures containing: 0.5 U of GoTaq® G2 Flexi DNA Polymerase (Promega), 1x Green GoTaq Flexi buffer, 200 μM of each dNTP, 1.5 mM MgCl₂, 0.25 μM of each *mmpB* primer (Table S1). For the determination of the primers' efficiency, three-fold standard dilutions of the cDNAs were made (1/3, 1/9, 1/27, 1/81 and 1/243) and stored at – 20 °C. RT-qPCRs were performed on Hard-Shell 384-Well PCR Plates (thin wall, skirted, clear/white) covered with Microseal® B adhesive seal (Bio-Rad). Ten microliter reactions were manually assembled and contained: 0.25 μM of each primer (Table S1), 5 μL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and 1 μL of template cDNA (dilution 1/9). The PCR profile was: 3 min at 95 °C followed by 45 cycles of 30 s at 95 °C and 30 s at 60 °C; and in the end a melting curve analysis (10 s cycles between 55 and 95 °C with a 0.5 °C increment per cycle). Primer efficiency standard curves and negative controls (no template cDNA) were included in all assays. RT-qPCRs were performed with three biological replicates and technical triplicates of each cDNA sample in the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). The normalized expression of the target genes was calculated using the Bio-Rad CFX Maestro™ 3.1 software (Bio-Rad), implementing an efficiency-corrected delta-delta Cq method (ΔΔCq). Statistical analysis was performed by means of a one-way ANOVA using the same software, and tests were considered significant if *P* < 0.05. Genes *bfrA*, *slr0083* and *slr0769* were validated as reference genes for data normalization (M value < 0.5), using the reference gene selection tool available in the Maestro™ software. The size of the PCR products for each gene was checked by agarose gel electrophoresis performed by standard protocols. These products were also cloned into the pGEM-T® Easy (Promega) vector according to the manufacturer's instructions. Identity of the amplicons was confirmed by Sanger sequencing (StabVida). These experiments were compliant with the MIQE guidelines ([Bustin et al., 2009](#)) to promote the effort for experimental consistency and transparency, and to increase the reliability and integrity of the results obtained.

2.6. Generation of the Synechocystis strain producing sfGFP-loaded EVs

To construct the *Synechocystis* strain expressing the heterologous protein sfGFP and further targeting it to EVs, the synthetic promoter *P_{trc}*, *x₁laco* ([Ferreira et al., 2018](#)), the signal peptide sequence of the *Synechocystis* native protein FutA2 ([Polyviou et al., 2018](#)), which determines its translocation to the periplasm, and the gene encoding the reporter protein super-folder Green Fluorescent Protein (sfGFP) were amplified by PCR using specific oligonucleotides (Table S1). Assembly of the different DNA fragments was performed by sequential overlap-extension PCR. The 953 bp fragment was digested with *Pst*I and *Xba*I, and ligated to plasmid pSN15KPO previously digested with the same restriction enzymes, rendering plasmid pEV-trc-GFP. Identity of the fragment was determined by Sanger sequencing. Plasmid pSN15KPO is a variant of plasmid pSN15K ([Pinto et al., 2015](#)), in which the kanamycin resistance cassette was replaced by that of plasmid pUC4K (GE

Healthcare). *Synechocystis* wild-type cells were naturally transformed with pEV-trc-GFP, and fully segregated mutants were obtained, rendering strain EV-trc-GFP.

2.7. Total protein extraction, concentration of cell-free extracellular medium and protein analyses

Cells from *Synechocystis* wild-type and mutant strains were grown as described above, and harvested by centrifugation at 4400 g for 10 min at room temperature. Total protein extracts were obtained as described (Lopes Pinto et al., 2011). In brief, cell pellets were suspended in protein extraction buffer [10 mM HEPES, 0.5% (v/v) Triton X-100, 10 mM EDTA, 2 mM DTT, 10% (v/v) glycerol, pH 8.0, supplemented with protease inhibitor cocktail (Roche)] and disrupted by sonication (Branson Sonifier 250). Subsequently, samples were centrifuged at 10,000 g for 10 min at 4 °C, and the resulting supernatant transferred into a new 1.5 mL microcentrifuge tube and stored at – 20 °C until further analysis. Protein concentration was determined using the BCA method (Thermo Fisher Scientific), with bovine serum albumin as standard. For cell-free, extracellular medium concentrated samples, the method described by Oliveira et al. (2015) was followed. Briefly, cells were cultivated as described up to an OD₇₃₀ of approximately 1.0–1.5, and later separated from the extracellular medium by centrifugation (4400 g, 10 min at room temperature). Spent medium was filtered through 0.2 µm pore size filters for cell removal, and further concentrated approximately 1000-fold by ultrafiltration, using Amicon® Ultra-15 Centrifugal Filter Units (Merck) with molecular weight cut-off of 3 kDa. Concentrated samples were either used immediately or stored at – 20 °C. The amount of concentrated spent medium sample analyzed was normalized by cell culture density (OD₇₃₀), volume of cell-free culture medium concentrated, and concentration factor. Total protein and cell-free, concentrated extracellular medium samples were electrophoresed on homogeneous 12 or 16% (w/v) SDS polyacrylamide gels, stained with colloidal Coomassie Brilliant Blue (Sigma), and gels were scanned with a GS-800 Calibrated Densitometer (BioRad).

For Western blotting, after SDS-polyacrylamide gel electrophoresis, protein samples were transferred onto a nitrocellulose membrane (GE Healthcare). Membranes were incubated in Blocking buffer [5% (w/v) fat-free milk powder, and 0.05% (v/v) Tween-20 in TBS (20 mM Tris-base, 0.8% (w/v) NaCl, pH 7.6)] for 2 h before being incubated overnight at 4 °C with rabbit anti-CopM antibody (Giner-Lamia et al., 2015) (1:3000) in fresh blocking buffer. After incubation with the primary antibody, membranes were washed twice in TTBS [0.05% (v/v) Tween-20 in TBS], incubated for 1 h with an anti-rabbit secondary antibody conjugated with horseradish peroxidase (Sigma) (1:10000), washed again twice with TTBS and twice with TBS. For sfGFP detection, the commercial mouse anti-GFP monoclonal antibody (Roche) (1:5000), and the goat-anti-mouse secondary antibody (Invitrogen) (1:5000) were used. Detection was carried out on a ChemiDoc MP System (BioRad), using the Clarity™ Western ECL Substrate (BioRad) reagent according to the manufacturer's instructions.

2.8. Extracellular vesicles isolation and analysis

Synechocystis cultures were grown as presented above ("Strains and growth conditions") and extracellular vesicles (EVs) were isolated as follows: cyanobacterial cells were separated from the extracellular medium by centrifugation at 4400 g for 10 min at room temperature, and further removed from the extracellular medium by filtration (0.2 µm filter pore size). Cell-free extracellular medium was then concentrated by ultrafiltration (4400 g, 16 °C) using Amicon® Ultra-15 Centrifugal Filter Units (Merck) with a molecular weight cut-off of 100 kDa. Later, concentrated samples were diluted with 15 mL of fresh BG11 medium and ultracentrifuged (70 Ti fixed angle rotor, Beckman Coulter Optima L80 XP Ultracentrifuge) using polycarbonate bottles with cap assembly (#355618) for 3 h at 100,000 g at 4 °C. Finally, the resulting pellet was

suspended in sterile BG11 growth medium, and stored at – 80 °C until further analysis.

E. coli cultures were grown as described, and EVs isolated similarly as performed for *Synechocystis* EVs. Briefly, cells were collected by centrifugation at 4400 g for 15 min at room temperature, and further removed from the extracellular medium by filtration (0.45 µm filter pore size). Then, cell-free extracellular medium was concentrated by ultrafiltration (4400 g, 4 °C) with centrifugal filters with a molecular weight cut-off of 100 kDa (Pall). Prior to ultracentrifugation, samples were mixed with 15 mL of PBS (Phosphate Buffered Saline, 1X, pH 7.5) and filtered once again (0.45 µm) to ensure complete removal of any biological contaminant. Ultracentrifugation was performed for 2 h at 174,900 g (70 Ti fixed angle rotor, Beckman Coulter Optima L80 XP Ultracentrifuge) at 4 °C. The resulting pellet was suspended in sterile PBS, and stored at – 80 °C until further analysis.

A similar procedure was performed to isolate EVs from *P. aeruginosa*. Cells were collected by centrifugation at 10,000 g for 30 min at room temperature, and further removed from the extracellular medium by filtration (0.45 µm filter pore size). Then, cell-free extracellular medium was also concentrated by ultrafiltration (4400 g, 4 °C). Prior to ultracentrifugation, samples were treated as mentioned: washed with 15 mL of PBS and filtered once again (0.45 µm). Ultracentrifugation was performed for 3 h at 150,000 g at 4 °C. The resulting pellet was suspended in sterile PBS, and stored at – 80 °C until further analysis.

Various methodologies were performed to evaluate EVs physical features and biochemical composition. Briefly, EVs number and size were analyzed by nanoparticle tracking analysis and their morphological aspect by electron microscopy. Regarding EVs composition, proteins and lipopolysaccharides (LPS) were routinely analyzed by SDS-polyacrylamide gel electrophoresis. Detection of CopM in isolated *Synechocystis* EVs was carried out by Western blot, and vesicular copper levels were quantified by atomic absorption spectrometry (AAS) (see further details about these techniques in Supporting information Text S1).

3. Results

3.1. Inactivation of the TolC-like protein in *Synechocystis* results in several adaptations at the protein level

The TolC-like protein (Slr1270) has been shown in *Synechocystis* to play key roles in protein export and multidrug efflux (Oliveira et al., 2016) as part of the type I secretion system (T1SS) and of the resistance-nodulation-division (RND) efflux pumps (Gonçalves et al., 2018). In addition, a *Synechocystis* tolC-mutant strain was shown to release more EVs than the respective wild-type (Oliveira et al., 2016; Gonçalves et al., 2018). In order to get closer insights into the adaptations occurring in *Synechocystis* cells lacking a functional TolC-like protein, iTRAQ-based quantitative shotgun proteomics was carried out. In this study, a total of 1135 proteins were identified with quantification information [with at least two peptides, and at a false discovery rate (FDR) of less than 1%], corresponding to approximately 32.4% of the total predicted *Synechocystis* proteome. 212 proteins were found to be differentially expressed between the strains analyzed (Table S2). Among the statistically significant differentially expressed proteins, 46 show at least 1.35-fold up- or down-regulation in respect to wild-type protein levels (Table 1), with more than 35% of the hits falling in the "hypothetical" or "functionally uncharacterized" protein categories. Some of the proteins differentially expressed have been previously reported to accumulate differently in the tolC-mutant strain as compared to the wild-type, namely proteins Slr1951 (–3.25-fold), Slr1841 (1.38-fold), and ApcE (1.41-fold) (Oliveira et al., 2016). In addition, proteins related to metal uptake and homeostasis, particularly CopM-Slr6039/Slr0788 (1.75-fold), FutA1 (1.43-fold), FrpC (–2.06-fold), or whose function is dependent on metal cofactors, namely IsiB (3.18-fold) and cytochrome c₆ (–2.55-fold), were found amongst

Table 1

List of proteins identified in the iTRAQ (isobaric tags for relative and absolute quantification) proteomics analysis with differential accumulation (> 1.35 -fold; < -1.35 -fold) in the *Synechocystis* sp. PCC 6803 *tolC*-mutant strain in respect to the wild-type strain cultivated under standard growth conditions.

Uniprot	# peptides	Protein names	Gene names	Fold Changes
P27319	22	Flavodoxin	<i>isiB</i>	3.18
P73742	100	Slr0858 protein	<i>slr0858</i>	2.95
P73238	31	Slr2018 protein	<i>slr2018</i>	2.18
P73354	28	Serine protease HtrA	<i>htrA</i>	2.08
Q6YRW5	2	Slr6039 protein	<i>slr6039</i>	1.75
P72656	2	Ribonuclease E/G-like protein	<i>rne</i>	1.75
Q55604	3	Slr0769 protein	<i>slr0769</i>	1.69
P73066	29	Ycf23 protein	<i>ycf23</i>	1.46
Q55665	58	Glutamate-1-semialdehyde 2,1-aminomutase (GSA)	<i>hemL gsa</i>	1.44
P72827	21	Iron uptake protein A1	<i>slr0017</i>	1.43
P73315	20	50 S ribosomal protein L22	<i>futA1</i>	1.43
P74791	6	PirA (PII interacting protein A)	<i>slr1295</i>	1.42
P37101	163	Phosphoribulokinase (PRK)	<i>rplV rpl22</i>	1.41
Q55544	60	Phycobilliprotein ApcE	<i>slr1803</i>	1.41
P74786	6	UPF0426 protein ssl0294	<i>ssr0692</i>	1.39
P72851	9	50 S ribosomal protein L28	<i>prk pik</i>	1.38
Q55797	14	Phosphoheptose isomerase (EC 5.3.1.28) (Sedoheptulose 7-phosphate isomerase)	<i>slr1525</i>	1.38
P73409	9	Slr1841 protein	<i>apcE</i>	1.38
P74478	16	Slr1926 protein	<i>slr0335</i>	1.38
P73288	40	Aklaviketone reductase	<i>slr1926</i>	1.35
P73644	6	S-adenosyl-methionine: precorrin-2 methyltransferase	<i>slr1825</i>	1.35
Q55770	40	Slr0185 protein	<i>cbiL</i>	-1.35
P52981	22	1,4-alpha-glucan branching enzyme GlgB	<i>slr0185</i>	-1.36
P74722	3	Slr0586 protein	<i>glgB</i>	-1.36
P37277	6	Photosystem I reaction center subunit XI (PSI subunit V) (PSI-L)	<i>slr0158</i>	-1.36
Q55146	9	Slr0064 protein	<i>slr0586</i>	-1.37
Q55162	10	Slr0051 protein	<i>psaL</i>	-1.37
Q55961	8	Slr0699 protein	<i>slr1655</i>	-1.39
P73111	19	Slr1835 protein	<i>slr0064</i>	-1.39
P73477	7	Slr1232 protein	<i>slr0051</i>	-1.39
P74748	10	Slr0602 protein	<i>slr0699</i>	-1.42
P73595	59	Uncharacterized WD repeat-containing protein slr1410	<i>slr1835</i>	-1.42
Q55735	3	Slr0394 protein	<i>slr1232</i>	-1.42
P73559	7	Slr0878 protein	<i>slr0602</i>	-1.44
P73594	62	Uncharacterized WD repeat-containing protein slr1409	<i>slr1410</i>	-1.45
Q55561	10	Slr0167 protein	<i>slr0394</i>	-1.46
P73731	5	Photosystem II reaction center Psb28 protein	<i>slr0878</i>	-1.46
P74485	15	Slr1863 protein	<i>slr1409</i>	-1.46
P74486	2	Slr1862 protein	<i>slr0167</i>	-1.46
Q55549	2	Slr0168 protein	<i>slr0167</i>	-1.52
P74162	24	Slr1380 protein	<i>slr1862</i>	-1.54
Q55776	9	Slr0180 protein	<i>slr0168</i>	-1.54
P73019	5	Iron-regulated protein	<i>slr1380</i>	-1.70
P46445	14	Cytochrome c_6	<i>slr0180</i>	-1.79
Q55747	42	Slr0420 protein	<i>frpC</i>	-2.06
P73817	13	Hemolysin	<i>slr1009</i>	-2.55
			<i>petJ</i>	-2.64
			<i>slr1796</i>	-3.25
			<i>slr0420</i>	
			<i>slr1951</i>	

the most differentially expressed proteins.

3.2. *TolC* is essential for metal homeostasis in *Synechocystis*

Based on these proteomic results, we wondered whether the *tolC*-mutant could be under some sort of metal imbalance. To address this question, the growth of the cyanobacterial strains was tested in the presence of selected metals, namely boron, cadmium, cobalt, copper, iron, manganese, molybdenum, nickel, and zinc (Fig. 1A). No differences in growth could be observed between *Synechocystis* wild-type and *tolC*-mutant in medium supplemented with boron, cadmium, manganese, molybdenum, or nickel. In contrast, significant growth impairment was detected in the mutant strain cultivated in medium supplemented with copper and zinc, and, to a lesser extent, cobalt and iron, when compared to the wild-type. Therefore, we proceeded to determine the intracellular levels of cobalt, copper, iron and zinc by atomic absorption spectrometry in both strains cultivated under standard growth conditions (Figs. 1B and S1). While similar intracellular levels for cobalt, iron and zinc could be detected in both strains (Fig. S1), *tolC*-mutant cells were found to accumulate approximately 3-fold more copper than the wild-type (Fig. 1B).

At this point, we hypothesized that the role of *TolC* in copper homeostasis could be related to: (i) the export of an unknown protein with copper-binding ability, via the T1SS; (ii) the efflux of copper through an RND pump; or (iii) the efflux of copper through a heavy metal efflux (HME) pump. To address these possibilities, we cultivated *Synechocystis* wild-type, and mutant strains $\Delta tolC$, $\Delta hlyB$ (lacking the inner membrane component of the T1SS (Gonçalves et al., 2018)), $\Delta acrA$ (lacking the periplasmic adaptor protein of the main RND pump in *Synechocystis* (Gonçalves et al., 2018)), and $\Delta copB$ (mutant lacking the periplasmic adaptor protein of the copper-specific HME-pump in *Synechocystis* (Giner-Lamia et al., 2012)) in medium supplemented with copper, and the growth of the various strains was analyzed (Fig. 2). All strains grew similarly under standard growth conditions ($0.5 \mu\text{M Cu}^{2+}$), and cells of the *acrA*-mutant strain presented the same growth capacity as the wild-type strain when cultivated in medium supplemented with copper ($4.0 \mu\text{M Cu}^{2+}$). However, while cells of the *hlyB*-mutant could grow slightly better than those of the wild-type strain under copper-challenging conditions, both the *copB*- and *tolC*-mutant strains exhibited growth impairment when compared to the wild-type. Remarkably, the *tolC*-mutant showed a more severe growth impairment as compared to the *copB*-mutant (Fig. 2).

Altogether, these observations suggest that *TolC* plays a key role in metal homeostasis in *Synechocystis*, particularly in keeping an intracellular balance of copper levels, likely working as the outer membrane component of the HME-pump dedicated to copper export.

3.3. Absence of *TolC* in *Synechocystis* triggers upregulation of copper detoxification mechanisms

Two main mechanisms have been described in *Synechocystis* to warrant copper detoxification, both being involved in metal export when cells are challenged with stressfully high copper concentrations: the HME-pump (dependent on the CopBA system), and the periplasmic metallochaperone system (dependent on CopM). In order to evaluate how these systems responded in the *Synechocystis tolC*-mutant strain, Western blotting experiments and/or transcriptional analyses were carried out. On one hand, RT-qPCR experiments revealed that the *tolC*-mutant has significantly higher *copB* transcript levels than the wild-type (Fig. 3A). On the other hand, transcript analysis by Northern blotting showed that the *tolC*-mutant accumulates higher levels of *copM* transcripts than the wild-type (data not shown). In agreement, iTRAQ results (Table 1) also indicated that CopM (Slr6039/Slr0788) is significantly overexpressed in *tolC*-mutant cells. Using a CopM-specific antibody (Giner-Lamia et al., 2015), Western blotting experiments determined that the intracellular CopM levels in the *tolC*-mutant strain are

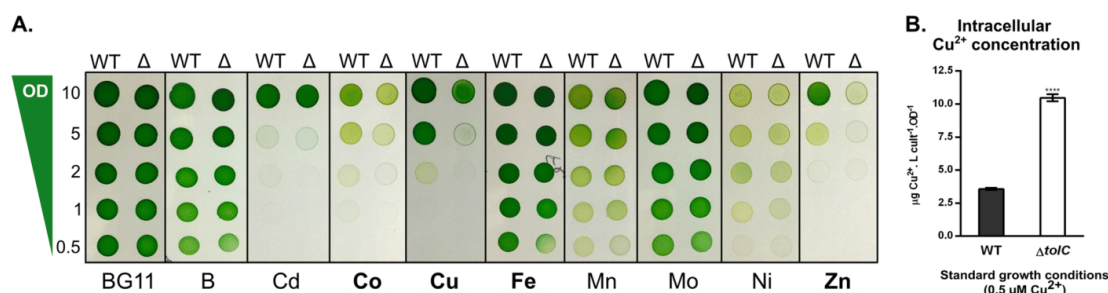


Fig. 1. The *Synechocystis* sp. PCC 6803 *tolC*-mutant shows a growth-sensitive phenotype when cultivated in the presence of elevated concentrations of specific metals. **A.** Cells of *Synechocystis* wild-type (WT) and *tolC*-mutant (Δ) strains were cultivated in standard liquid conditions up to an OD_{730} of approximately 0.8–1.0, concentrated to an OD_{730} of 10, and later diluted (OD_{730} of 5, 2, 1, 0.5). Cells of each dilution were spotted onto standard BG11 agar plates (BG11), or BG11 agar plates supplemented with boron (B - 463 μM), cadmium (Cd - 5 μM), cobalt (Co - 5 μM), copper (Cu - 4 μM), iron (Fe - 229 μM), manganese (Mn - 91.5 μM), molybdenum (Mo - 16.1 μM), nickel (Ni - 7 μM), or zinc (Zn - 14 μM), and cultivated for 7 days. Presented photographs are representative of the results obtained with 3 independent biological replicates. Bold and underlined metal symbols indicate conditions in which the *Synechocystis tolC*-mutant showed growth-sensitive phenotype when compared to the wild-type strain, and further selected for intracellular quantification of the respective metal's concentration. **B.** Intracellular copper concentration determined for *Synechocystis* wild-type (WT) and *tolC*-mutant ($\Delta tolC$) strains cultivated under standard liquid BG11 conditions. Copper levels were determined by atomic absorption spectrometry, and are shown in $\mu g Cu^{2+} \cdot L culture^{-1} \cdot OD_{730}^{-1}$. Error bars represent the standard deviations of at least three independent biological replicates. ****, $P \leq 0.0001$.

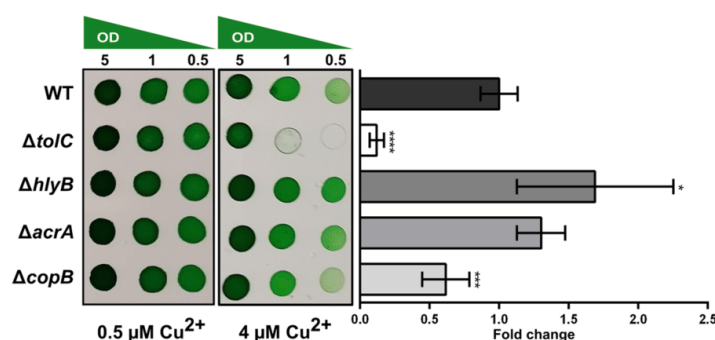


Fig. 2. The *Synechocystis* sp. PCC 6803 *tolC*-mutant copper-sensitive phenotype is not associated to its T1SS- or to its RND-dependent function. Cells of *Synechocystis* wild-type (WT), and mutant strains $\Delta tolC$, $\Delta hlyB$ (T1SS), $\Delta acrA$ (RND), $\Delta copB$ (HME-pump) were cultivated in standard liquid conditions up to an OD_{730} of approximately 0.8–1.0, concentrated to an OD_{730} of 5, and later diluted (OD_{730} of 1 and 0.5). Cells of each dilution were spotted onto standard BG11 agar plates (0.5 $\mu M Cu^{2+}$), or BG11 agar plates supplemented with copper (4 $\mu M Cu^{2+}$), and cultivated for 7 d. Presented photographs are representative of the results obtained with 3 independent biological replicates. The panel to the right shows cell density quantification by densitometry analysis (Image J) determined for each cyanobacterial strain, spotted at initial OD_{730} of 0.5, and cultivated in BG11 agar plates supplemented up to a final concentration of 4 μM copper sulfate. Error bars represent the standard deviations of at least three independent biological replicates. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, each determined against the WT.

$P \leq 0.0001$, each determined against the WT.

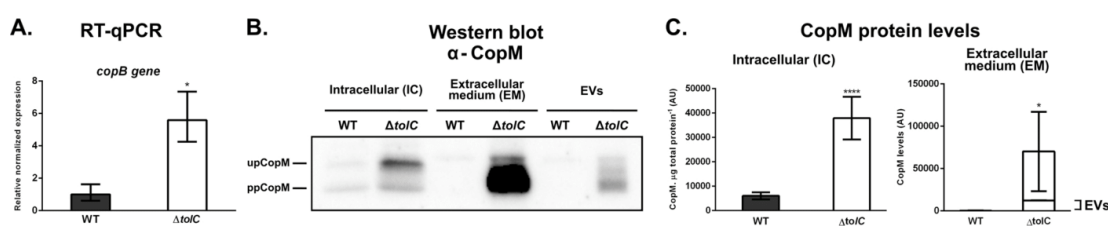


Fig. 3. *Synechocystis* sp. PCC 6803 *tolC*-mutant shows high upregulation of the copper detoxification mechanisms under standard growth conditions. **A.** RT-qPCR analysis of the *copB* transcript levels in *Synechocystis* wild-type (WT) and *tolC*-mutant ($\Delta tolC$) strains cultivated under standard growth conditions. **B.** Western blot analysis of the CopM-protein levels in: 10 μg of total protein extracts (Intracellular - IC); cell-free, concentrated extracellular medium (Extracellular medium - EM); and isolated extracellular vesicles (EVs), of *Synechocystis* wild-type and *tolC*-mutant strains cultivated under standard growth conditions. Concentrated extracellular medium and EVs samples were normalized before loading for initial cell-free culture volume concentrated, final volume of concentrated sample, and culture's OD_{730} . upCopM: unprocessed CopM-protein; ppCopM: processed CopM-protein. **C.** Quantification of CopM protein levels detected by Western blotting in total protein extracts (Intracellular - IC), and in the cell-free extracellular growth medium (Extracellular medium - EM), as determined by densitometry analysis (ImageJ). Contribution of EVs for CopM levels detected in the complete cell-free extracellular medium is highlighted in the graph. Error bars represent the standard deviations of at least three independent biological replicates. *, $P \leq 0.05$; ****, $P \leq 0.0001$.

approximately 6-fold higher than those detected in the wild-type (Fig. 3B and C). Moreover, the two previously described CopM forms could be detected (Fig. 3B): the unprocessed, full-length protein, and the processed CopM, resulting from the signal peptide removal from its N-terminus upon translocation from the cytoplasm to the periplasm (Giner-Lamia et al., 2015). CopM has been described as a periplasmic copper-binding chaperone, but it has also been found to accumulate in the extracellular medium (Giner-Lamia et al., 2015). Accordingly, CopM could also be detected here in cell-free, concentrated extracellular medium samples obtained from both strains, even though in significantly higher amounts in the mutant (Fig. 3B and C). These results suggest that both copper-detoxification mechanisms are activated, and are highly expressed in a *Synechocystis* strain lacking a functional TolC-protein.

3.4. The copper-binding chaperone CopM can be found in EVs isolated from *Synechocystis*

After finding high levels of CopM in extracellular medium samples, it became important to determine whether CopM could be detected in *Synechocystis* EVs. To that end, extracellular medium of both *Synechocystis* wild-type and *tolC*-mutant strain were concentrated by ultrafiltration, and their respective EVs isolated by ultracentrifugation. Western blotting confirmed the presence of CopM protein in EVs, particularly in those isolated from the *tolC*-mutant (Fig. 3B). When normalizing the amount of CopM detected in EVs isolated from wild-type and mutant strain, by the total amount of vesicles recovered in each respective condition as determined by the amount of LPS (molecular marker of EVs released by Gram-negative bacteria), we could estimate that the CopM protein levels are approximately 4-fold higher in *tolC*-mutant EVs as compared to wild-type. Yet, the amount of CopM identified therein corresponded just to a fraction of the total extracellular CopM pool (Fig. 3C).

To investigate if proteins reaching the periplasm of Cyanobacteria could be detected freely available in the growth medium as well as in EVs, we generated a *Synechocystis* mutant expressing the heterologous reporter protein sfGFP (Fig. 4). The gene encoding sfGFP was fused with the Tat-dependent, signal peptide described in the *Synechocystis* periplasmic protein FutA2 (Polyviou et al., 2018), and its expression was controlled by the *P_{trc.x.lacO}* promoter (Ferreira et al., 2018). Confocal microscopy analysis of transformed cells clearly indicated accumulation of sfGFP-protein in the outermost region of the cell, indicating successful targeting of the reporter to the periplasm (Fig. 4A). Moreover, confocal microscopy also showed that sfGFP is packaged in isolated EVs (Fig. 4B). In agreement, Western blotting analyses of isolated EVs confirmed the presence of sfGFP therein, as well as freely available in EVs-free, concentrated extracellular medium (data not shown). The amount of sfGFP freely available in the medium was close to 8-fold higher than that

found in EVs (Fig. 4C). This experiment has now elucidated that soluble proteins reaching the *Synechocystis* periplasm may leave the cell by translocation across the outer membrane or by packaging into EVs, supporting the possibility of CopM being exported by the *Synechocystis* cell by both systems. Moreover, it is also possible that EV-protein cargo is released into the extracellular space by vesicle leakage or breakdown.

3.5. *Synechocystis* wild-type strain produces more CopM-loaded EVs when cultivated in high copper concentrations

In light of these results, the hypervesiculating phenotype observed in the *Synechocystis tolC*-mutant strain could be partly interpreted as a mechanism responding to an intracellular copper accumulation. To evaluate how the *Synechocystis* wild-type strain responded in terms of EVs' production when challenged to grow in the presence of high amounts of copper, the cyanobacterium was cultivated under increasing concentrations of the metal, and the total amount of released EVs was monitored (Figs. 5 and S2). All evidence combined suggests that *Synechocystis* wild-type cells indeed produce more EVs when exposed to high copper concentrations, a response that seems to follow a copper concentration-dependent manner. Interestingly, while regular increments in Cu^{2+} concentration up to 3 μM resulted in modest, but steady increase of EVs' production [as determined by the amount of LPS (Fig. 5A and B) and nanoparticles (Fig. S2)], presence of 4 μM of Cu^{2+} in the medium resulted in a strong boost in vesicle production. In addition, presence of CopM in isolated EVs was also investigated by Western blot: in agreement with the increase in EVs production, the results indicate that the amount of CopM also increases (Fig. 5A). Moreover, detailed analysis of the LPS band profile indicates that EVs released by *Synechocystis* cells challenged with 4 μM of Cu^{2+} present a 5-fold decrease in the amount of high molecular weight, smooth-type LPS relative to the low molecular weight, rough-type LPS (Fig. 5C). This significant difference was also detected in EVs isolated from the *Synechocystis tolC*-mutant strain, though to a lesser extent (Fig. 5C).

3.6. Other model Gram-negative bacteria also release EVs when cultivated in the presence of high copper concentrations

Next, we decided to analyze whether this EVs production phenotype in response to growth-challenging copper concentrations was exclusive of Cyanobacteria or if it could also be observed for other Gram-negative bacteria. Thus, similar experiments as described for *Synechocystis* wild-type were carried out for the enteric *Escherichia coli* [strain BL21 (DE3)], and for the opportunistic pathogen *Pseudomonas aeruginosa* (strain PAO-1). EVs production followed a similar pattern as that observed for *Synechocystis* wild-type, i.e. as copper-concentration in the medium increased, the amount of produced vesicles increased as well

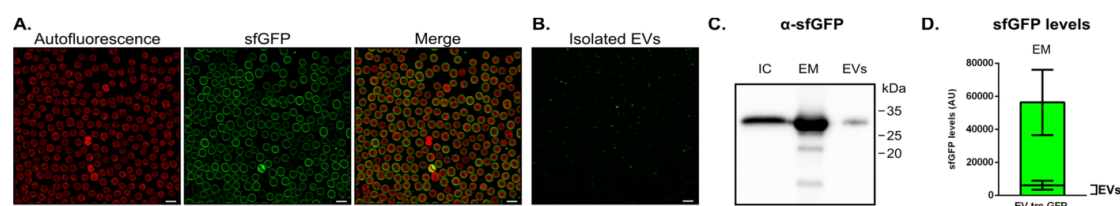


Fig. 4. sfGFP translocated to the *Synechocystis* sp. PCC 6803 periplasm can also be detected in the extracellular medium, both as free protein and in extracellular vesicles. A. Confocal micrographs of EV-trc-GFP cells (*Synechocystis* cells expressing sfGFP fused with the signal peptide of the protein FutA2, and under the control of the *P_{trc.x.lacO}* synthetic promoter) depicting the red autofluorescence signal from the cells' photosynthetic pigments, and the green signal from the sfGFP reporter. A panel showing the result of merging the two micrographs is also presented. Scale bar: 5 μm . B. Confocal micrograph showing green fluorescent foci obtained from EVs isolated from EV-trc-GFP cells. Scale bar: 3 μm . C. Western blot analysis of sfGFP protein in 10 μg of total protein (IC - intracellular), in cell-free concentrated extracellular media (EM) and isolated extracellular vesicles (EVs) samples of EV-trc-GFP cells. EM and EVs samples were normalized for initial cell-free culture volume concentrated, final volume of concentrated sample, and culture's OD_{730} . D. Quantification of sfGFP protein levels detected by Western blotting in the complete cell-free extracellular growth medium (EM), and in EVs only, as determined by densitometry analysis (ImageJ). Error bar represents the standard deviation of at least three independent biological replicates.

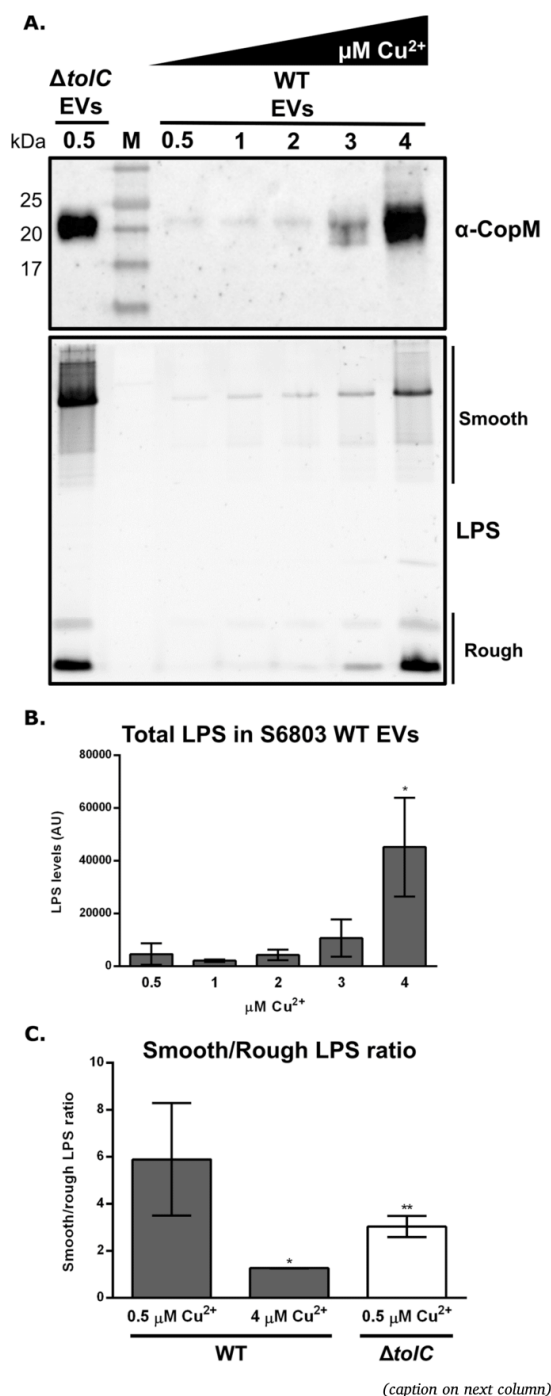


Fig. 5. *Synechocystis* sp. PCC 6803 wild-type produces extracellular vesicles in response to copper supplementation in the medium in a concentration dependent manner. *Synechocystis* wild-type (WT) cells were grown under standard growth conditions (liquid BG11 medium with $0.5 \mu\text{M Cu}^{2+}$), and later distributed into BG11 standard medium ($0.5 \mu\text{M Cu}^{2+}$) or BG11 medium supplemented with copper to a final concentration of 1, 2, 3 and $4 \mu\text{M Cu}^{2+}$. In addition, *Synechocystis tolC*-mutant cells (ΔtolC) were cultivated in standard conditions, and then further grown in fresh BG11 medium. For all conditions, cyanobacterial cells were cultivated for approximately 7 d, and their respective extracellular vesicles (EVs) isolated. **A.** Isolated EVs were analyzed by Western blotting for the detection of CopM-protein levels (upper panel). M, NZYColour Protein Marker II ladder (NZYTech). Molecular weight of selected bands is shown to the left in kDa. As lipopolysaccharides (LPS) are regarded as a marker for the presence and abundance of Gram-negative EVs, EVs samples were separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels and LPS were stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies) (bottom panel). LPS with (Smooth) or without (Rough) O-antigen moieties are indicated. **B.** Total LPS content of *Synechocystis* WT EVs was quantified by densitometry analysis (ImageJ). **C.** Quantification of smooth-to rough-LPS ratio for EVs isolated from *Synechocystis* WT, *Synechocystis* WT cultivated with $4 \mu\text{M Cu}^{2+}$, and *Synechocystis tolC*-mutant cells. Error bars represent the standard deviations of at least three independent biological replicates. **, $P \leq 0.01$; ***, $P \leq 0.001$, each determined against the WT cultivated under standard growth conditions ($0.5 \mu\text{M Cu}^{2+}$).

(Figs. 6A and B, S3). Furthermore, a significant decrease in the relative amount of smooth- to rough-type LPS could be observed in EVs isolated from cells of both strains exposed to high copper concentrations as compared to EVs isolated from cells cultivated under standard growth conditions (Fig. 6C). This result is similar to the response detected in *Synechocystis* wild-type (Fig. 5C). Altogether, this suggests that the selected Gram-negative bacteria, which include members that occupy different ecological niches respond in a similar manner when exposed to high copper concentrations.

3.7. Copper can be detected in isolated cyanobacterial EVs

Finally, to address the possibility of bacterial EVs to work as copper-releasing capsules, the copper amount in vesicles isolated from *Synechocystis* wild-type and *tolC*-mutant strains was analyzed. The choice of strains lies on the fact that the mutant accumulates copper intracellularly already in standard growth conditions (Fig. 1). This means that there is no need to supplement the medium with extra amounts of copper, which could represent a technical challenge when quantifying copper in EVs. Thus, *Synechocystis* wild-type and *tolC*-mutant strains were cultivated in standard growth medium ($0.5 \mu\text{M Cu}^{2+}$), their EVs isolated, thoroughly washed to remove any possible metal binding to the vesicles' surfaces, and later analyzed by atomic absorption spectrometry. Copper could be detected and quantified in EVs samples obtained from both strains. However, the amount of copper quantified in *tolC*-mutant-derived EVs was significantly higher than that of wild-type vesicle fractions, in approximately 3-fold (Fig. 7).

4. Discussion

4.1. TolC is the outer membrane component of the *Synechocystis* copper-efflux pump

A *Synechocystis* strain lacking a functional TolC protein has been shown to be severely impaired in secreting several types of molecules, including proteins and antibiotics (Oliveira et al., 2016; Agarwal et al., 2018), drugs (Gonçalves et al., 2018), and even fatty acids (Bellefleur et al., 2019). This phenotype is related to the fact that TolC works as the outer membrane component in multiple tripartite systems (Gonçalves et al., 2019), in which substrate selectivity is defined by the inner membrane component and the periplasmic adaptor protein (Gonçalves et al., 2021; Russo and Zedler, 2021). Thus, TolC can promiscuously

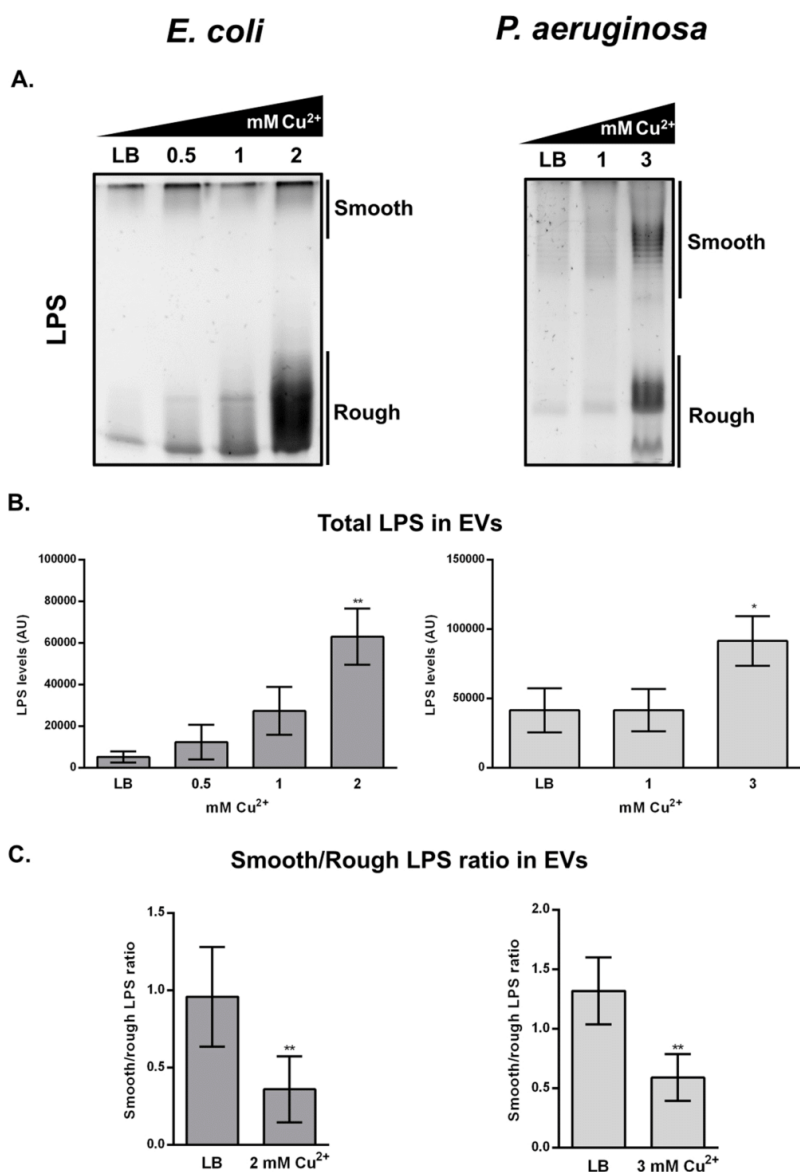


Fig. 6. Model bacterial strains produce extra-cellular vesicles in response to copper supplementation in the medium in a concentration dependent manner. Bacterial strains *Escherichia coli* [strain BL21 (DE3) left-hand panels] and *Pseudomonas aeruginosa* [strain PAO-1 right-hand panels] were cultivated either in LB liquid medium (LB) or in LB liquid medium supplemented with 0.5, 1 and 2 mM CuSO_4 for *E. coli*, or 1 and 3 mM CuSO_4 for *P. aeruginosa*. After overnight cultivation, EVs were isolated. A. EVs samples were separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels and lipopolysaccharides (LPS) were stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies). LPS with (Smooth) or without (Rough) O-antigen moieties are indicated. B. Total LPS content of *E. coli* and *P. aeruginosa* EVs were quantified by densitometry analysis (ImageJ). C. Quantification of smooth- to rough-LPS ratio for EVs isolated from *E. coli* cultivated in LB medium or medium supplemented with 2 mM Cu^{2+} , and *P. aeruginosa* cultivated in LB medium or medium supplemented with 3 mM Cu^{2+} . Error bars represent the standard deviations of at least three independent biological replicates. *, $P \leq 0.05$; **, $P \leq 0.01$, each determined against the respective strain cultivated in standard LB medium (LB).

assemble with either TISS-components or RND-efflux pumps to promote the export of molecules directly from the cytoplasm to the extracellular medium. The observation of an intracellular copper accumulation in *Synechocystis tolC*-mutant, accompanied by adaptations related to such copper-imbalance, including upregulation of *copB* transcripts (Fig. 3), high overexpression of CopM (Table 1 and Fig. 3), moderate overexpression of plastocyanin (Table S2), strong and moderate down-regulation of cytochrome c_6 (PetJ) and of Slr0602, respectively (Table 1), with the latter three proteins part of the PetRP regulon, recently described to be copper-responsive (García-Cañas et al., 2021), highlight the need for the mutant cells to secrete copper.

Taken together, our results support the possibility of TolC working as the outer membrane component of the *Synechocystis* copper-efflux

pump. One of the best described copper efflux systems is the *E. coli* CusCFBA transport system (Franke et al., 2003). In this microorganism, CusA is the inner membrane component, CusB the periplasmic adaptor protein, CusC the outer membrane component, and CusF is a soluble periplasmic protein that transports copper in the periplasm to the pump (Nies et al., 2003). Remarkably, despite low sequence similarity, CusC is structurally very similar to TolC (Kulathila et al., 2011), but TolC is not able to physiologically replace CusC in *E. coli*, in respect to conferring resistance to copper ions in vivo (Franke et al., 2003). The most likely reason for this observation would be an inability of TolC to form a functional pump with CusAB (Kulathila et al., 2011). It has been suggested that the linkage among components maintaining copper homeostasis seems evolutionarily driven by protein-protein interactions

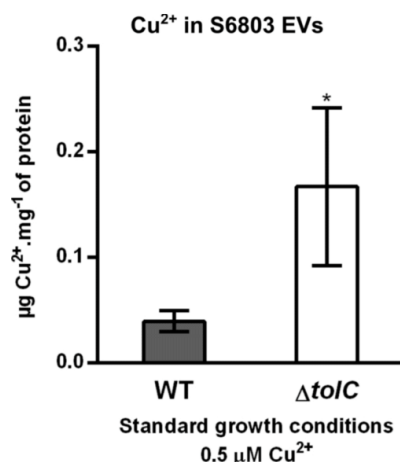


Fig. 7. Extracellular vesicles (EVs) isolated from *Synechocystis* sp. PCC 6803 *tolC*-mutant contain more copper than EVs obtained from the wild-type strain, under standard growth conditions. *Synechocystis* sp. PCC 6803 wild-type (WT) and *tolC*-mutant ($\Delta tolC$) cells were both cultivated under standard growth conditions (liquid BG11 medium with $0.5 \mu\text{M Cu}^{2+}$) up to an OD_{730} of 0.8–1.0, and EVs isolated. Prior to analysis, EVs were thoroughly washed in Tris-HCl-EDTA buffer for removal of copper traces derived from the BG11 medium. Amount of copper in isolated EVs was determined by atomic absorption spectrometry, normalized by the total amount of vesicular protein, and expressed in $\mu\text{g Cu}^{2+} \text{ mg}^{-1}$ of protein. Error bars represent the standard deviations of at least three independent biological replicates. *, $P \leq 0.05$.

rather than by function (Argüello et al., 2013). In fact, a phylogenomic study carried out in γ -proteobacteria showed that copper homeostatic pathways do not behave as evolutionary units, with particular species assembling different combinations of basic functions (Hernández-Montes et al., 2012). This suggests that, depending on the presence or absence of some subunits, bacteria achieve copper homeostasis through the combination of different components and strategies (Hernández-Montes et al., 2012).

In *Synechocystis*, the inner membrane component and the periplasmic adaptor protein of the copper-efflux system have been identified, and are designated as CopA and CopB, respectively (Giner-Lamia et al., 2012). In addition, a gene (*copC*) encoding a small protein of unknown function was found downstream of *copA*, forming the *copBAC* operon. Even though *copC* deletion results in a copper-sensitive phenotype in *Synechocystis*, the role of the encoded protein is not fully understood, but it was proposed to be part of the copper-efflux pump (Giner-Lamia et al., 2012). Nevertheless, the outer membrane component remained unidentified. While CopA and CopB belong to the CusA and to the RND_mfp protein superfamilies, respectively, a search for CusC homologues in selected cyanobacterial genomes retrieves a low number of hits (Ishihara et al., 2020; Nicolaisen et al., 2010), with low sequence similarity. Importantly, the best hits correspond to TolC-like proteins (Nicolaisen et al., 2010), suggesting the absence of CusC in this phylogenetic group. Moreover, these genomic analyses reveal that TolC seems to be the only outer membrane protein in *Synechocystis* capable of interacting with CopAB, and fulfilling the role of exporting copper. Single channel conductance, ion-selectivity studies, and structural modeling have shown that *Synechocystis* TolC protein exhibits different characteristics as compared to the *E. coli* counterpart (Agarwal et al., 2014), which could then be related to its versatile role in mediating the efflux of a wider range of compounds.

4.2. EVs as a copper-efflux mechanism in Gram-negative bacteria

In addition to the secretion of various types of (bio)molecules, the *Synechocystis tolC*-mutant has also been shown to significantly release more EVs than the wild-type strain (Oliveira et al., 2016). Similar results have been reported for the pathogenic Gram-negative bacterium *Actinobacillus pleuropneumoniae* (Li et al., 2019). Nevertheless, the biological reasons underlying these observations remained uncharacterized.

When exposed to increasing concentrations of copper, all the Gram-negative bacteria studied in this work, the freshwater cyanobacterium *Synechocystis*, the enteric *E. coli* and the opportunistic pathogen *P. aeruginosa*, responded with production and release of EVs. One possible explanation for this result could be that, during environmental transitions, EVs production facilitates remodeling of cell envelope components, while promoting outer membrane maintenance (Bonnington and Kuehn, 2016). As exposure to high metal concentrations usually results in generation of oxidative stress, EVs production could rid the cell of damaged structural components, including LPS, lipids and proteins. In fact, recent analyses have shown that the *Synechocystis tolC*-mutant studied in this work accumulates high levels of reactive oxygen species compared to the wild-type (Hewelt-Belka et al., 2020), and that despite high activity of superoxide dismutase and catalase (Oliveira et al., 2016), an increase in approximately 50% could be detected in lipid peroxidation in mutant cells compared to the wild-type (Hewelt-Belka et al., 2020). Another reason for the overproduction of EVs in high metal conditions could be to limit the formation of amorphous metal precipitates on the cell surface, as observed in *Leptospira interrogans* when in high iron conditions (Henry et al., 2013). As suggested by the authors, increased EVs production would result in an overall reduction of the surface iron concentration, which in turn would alleviate some of the associated intracellular iron toxicity (Henry et al., 2013).

Although one cannot exclude these possibilities, here we present evidence that supports that EVs work as a copper-efflux system in bacteria, which could partly explain the phenotype observed for the *Synechocystis tolC*-mutant strain. Metal-binding proteins and siderophores have been detected in isolated EVs of various bacterial species, including e.g. *Neisseria meningitidis* (Lappann et al., 2013), *Mycobacterium tuberculosis* (Prados-Rosales et al., 2014), *Enterococcus faecium* (Wagner et al., 2018), and *Staphylococcus aureus* (Askarian et al., 2018). In particular, laccase, a multi-copper oxidase, has been identified in EVs isolated from the fungus *Cryptococcus neoformans* (Rodrigues Marcio et al., 2008). In this work, we detected the copper-binding protein CopM (Giner-Lamia et al., 2015) in EVs isolated from *Synechocystis* (Figs. 3 and 5). CopM is translocated across the inner membrane to the periplasm via the Sec-translocation pathway (Giner-Lamia et al., 2015), and so, its folding occurs in the periplasm. Consequently, CopM binds copper only in the periplasm or in the extracellular medium. Detection of copper in EVs fractions of the *tolC*-mutant strain (and, to a lesser extent, of the wild-type) is consistent with detection of CopM in the respective vesicles. CopM is able to bind both Cu(I) and Cu(II), but it shows higher affinity toward Cu(I), within the femtomolar range (Giner-Lamia et al., 2015), each protein binding 2 Cu(I) ions (Zhao et al., 2016). Considering that the *Synechocystis tolC*-mutant accumulates more copper than the wild-type strain (Fig. 2), it is likely that the copper detected in EVs came from the intracellular metal pool, with EVs production contributing to its secretion, alleviating the cell from excessive copper accumulation.

Determination of smooth-to-rough relative amount of LPS in EVs isolated from wild-type and *tolC*-mutant strain shows a significant increase in rough-type LPS in EVs isolated from the mutant (Fig. 5A and C). Furthermore, quantification of smooth-to-rough LPS in EVs isolated from *Synechocystis* wild-type (Fig. 5C), and *E. coli* and *P. aeruginosa* cells (Fig. 6C) cultivated in the presence of growth-challenging copper concentrations revealed a similar profile, i.e. a decrease in relative amount of smooth-to-rough LPS as compared to cells maintained under standard growth conditions. In addition to the overall increase in EVs production by bacterial cells in response to copper stress, this result highlights that

reshaping LPS composition in released EVs is a common bacterial reaction to such stressful conditions. Jefferies and Khalid (2020) have recently shown that bacterial EVs with a prevalence of smooth-type LPS retain their spherical shape when they interact with membranes of the recipient cell, whereas EVs with shorter LPS (of the rough-type) distort and spread over the membrane surface. Modulations of these interactions led the authors to suggest that spherical-shaped EVs are more likely to be internalized than low-sphericity EVs, which tend to remain on the surface of the cells (Jefferies and Khalid, 2020). In line with Jefferies and Khalid (2020), our results could therefore indicate that EVs released from copper-stressed bacterial cells are less prone to interact with cyanobacterial cells and to deliver their cargo, making the copper present in EVs to be less accessible.

Our working hypothesis to describe copper homeostasis in bacteria, based on the *Synechocystis* model, is therefore presented in Fig. 8. In standard growth conditions, copper homeostasis is reached by the balance between copper uptake and copper efflux. Although the outer membrane components directly involved in copper uptake in cyanobacteria are not fully identified, recent results have shown that highly-abundant porins in the *Synechocystis* outer membrane are responsible for metal uptake (Cardoso et al., 2021; Qiu et al., 2021). Export of copper is supported both by the heavy metal efflux (HME)-pump

(CopBAC-TolC complex) and by the secretion of the copper-binding protein CopM. When cells are exposed to medium-high copper concentrations, the key mechanisms for maintaining homeostasis seem to be the copper export systems, with an upregulation of the classical HME-pump and of the metallochaperone CopM. In such stressful conditions, EVs seem also to play a role in copper export. However, in spite of EVs being released by stressed cells in a copper concentration dependent manner, their role in copper export does not seem to be relevant until the cells are exposed to higher copper concentrations. In Figs. 5 and 6, it is possible to see a clear trend in EVs production following gradual increments in copper concentration, but the most significant increase in EVs release is observed when bacterial cells are exposed to the highest copper concentrations tested. This may be interpreted as EVs being one of the last mechanisms activated by bacterial cells to cope with extreme copper concentrations, as copper export via the HME-pump and CopM secretion require less components and are likely less energy demanding than EVs release. CopM-loaded EVs may also work as a copper-uptake system, similar to what has been described for *M. tuberculosis* membrane vesicles in iron acquisition (Prados-Rosales et al., 2014). Thus, CopM-loaded EVs may contribute for copper scavenging from the medium, and so work as a copper sink mechanism. This may be particularly relevant when the bacterium is cultivated in

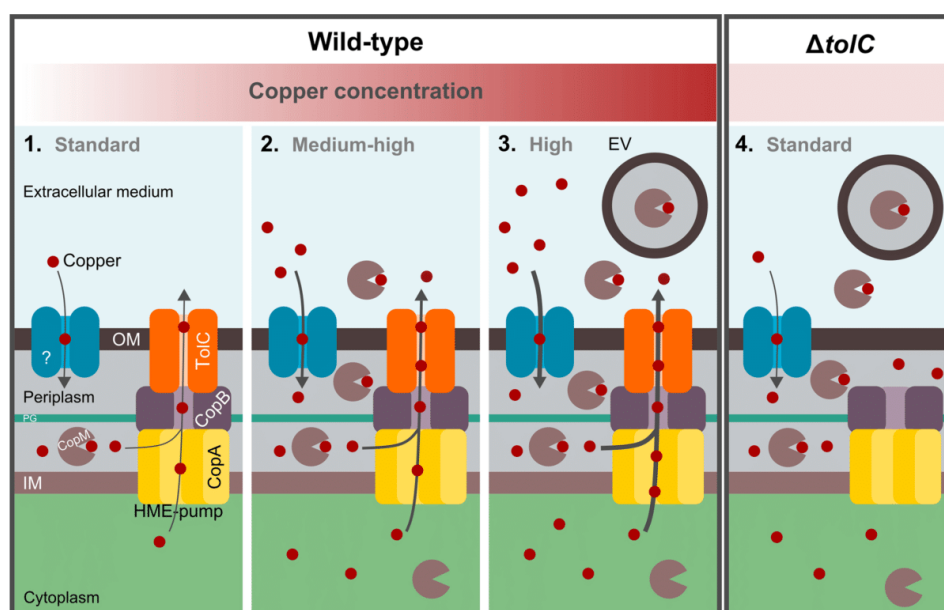


Fig. 8. Schematic representation of the copper detoxification mechanisms in Gram-negative bacteria proposed in this work, with a focus on the cyanobacterium *Synechocystis* sp. PCC 6803. In each panel, a diagram showing the bacterial cell envelope, separating the cytoplasm from the extracellular medium, is presented, composed of the two membrane systems [inner membrane (IM), and outer membrane (OM)] limiting the periplasmic space (or periplasm), where the peptidoglycan layer (PG) is found. The outer membrane protein/porin facilitating uptake of copper (red circles) is represented by "?", as its identity in *Synechocystis* remains to be determined. The heavy metal efflux (HME)-pump composed of the inner membrane component CopA, the periplasmic adaptor protein CopB, and the outer membrane protein TolC is also shown. CopC is not represented as it is not clear yet where it is localized. Arrows indicate direction of the metal flux (inward or outward), and their thickness represent flow rate. The metallochaperone CopM is presented in the cytoplasm, where it is synthesized, in the periplasm and in the extracellular medium, where it has been previously detected (Giner-Lamia et al., 2015), and in extracellular vesicles (EV) (this work). 1. In ordinary conditions, copper homeostasis is achieved by a balanced import and export of copper through the outer membrane porin and the HME-pump system, respectively. In addition, CopM complements the copper export system by binding copper in the periplasm and transporting it to the extracellular space, though it does not represent a major outward route, as indicated by the amount of CopM in the medium (Fig. 3). 2. When cells are exposed to medium-high copper concentrations, the metal enters the cell at a higher rate, triggering upregulation of the export mechanisms to ensure metal homeostasis, namely by expression of CopM and of the CopBA subunits of the HME-pump. 3. In high copper concentrations, bacterial copper detoxification mechanisms (HME-pump and CopM) are fully activated to cope with the excessive copper influx. Nevertheless, in conditions in which these systems are not sufficient to deal with copper imbalance, EVs release represents an alternative copper secretion mechanism. 4. In the *Synechocystis* *tolC*-mutant cultivated under standard conditions, copper homeostasis is compromised, as the HME-pump is functionally inactive due to the lack of the outer membrane protein TolC. Thus, despite the low copper amount in the medium, the cell cannot efficiently balance copper uptake, which leads to activation of the CopM-based secretion system, as well as release of CopM-loaded EVs.

medium supplemented with high copper concentrations: in addition to serving the purpose of exporting copper out of the cell, EVs may also accumulate copper, making it less available for uptake by the cells. Finally, in the *Synechocystis tolC*-mutant, absence of the outer membrane component of the HME-pump severely disrupts copper balance, triggering upregulation of the HME-pump (CopBAC) remaining components and of the CopM system, which seem to be insufficient to deal with an intracellular copper accumulation, even under standard growth conditions. To overcome the stress, CopM-loaded EVs are then released, which help mutant cells to grow similarly to the wild-type (Oliveira et al., 2016).

5. Conclusions

In summary, TolC is presented here as the outer membrane component of the HME-pump in *Synechocystis*. As there are several combinations of copper export components in different bacteria, this result opens the possibility of studying TolC as a metal-efflux mediating protein in other relevant bacterial species. Moreover, EVs are suggested to work as a non-classical secretion mechanism to deal with copper-induced stress, which is relevant in the context of using this metal to promote disinfection of, for example, hospital-based surfaces and equipment. However, many questions remain unanswered: can bacterial EVs work as nanocapsules for secretion of other metals than copper? What mechanisms determine EVs packaging with metal-binding proteins under metal stressful conditions, or is it a stochastic mechanism? How does the cell regulate the proportion of smooth-to-rough type LPS in released EVs depending on the condition? Clear answers to these and other questions will surely increase our knowledge on the role played by EVs in bacterial physiology and survival strategies, and help devising technological solutions targeting EVs release to control unwanted bacterial growth.

Statement of novelty

Copper is an important cofactor in several enzymatic reactions, but when available in high concentrations, it represents a serious risk of causing injury to cells. Here, we present evidence that bacteria use extracellular vesicles (EVs) as copper releasing capsules under copper-induced stressful conditions, representing a breakthrough on the understanding of metal resistance and homeostasis. EVs are discrete, bilayered, nanoscale structures, derived from the bacterial-cell envelope, and released to the extracellular space. The work expands the knowledge on the role played by EVs in bacterial survival strategies, and may help devising technological solutions targeting EVs' release to control unwanted bacterial growth.

CRedit authorship contribution statement

Steeve Lima: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Jorge Matinha-Cardoso:** Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Joaquín Giner-Lamia:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Narciso Couto:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Catarina C. Pacheco:** Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Francisco J. Florêncio:** Supervision, Project administration, Writing – review & editing. **Phillip C. Wright:** Supervision, Funding acquisition, Project administration, Writing – review & editing. **Paula Tamagnini:** Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing – review & editing. **Paulo Oliveira:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Supervision, Resources, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

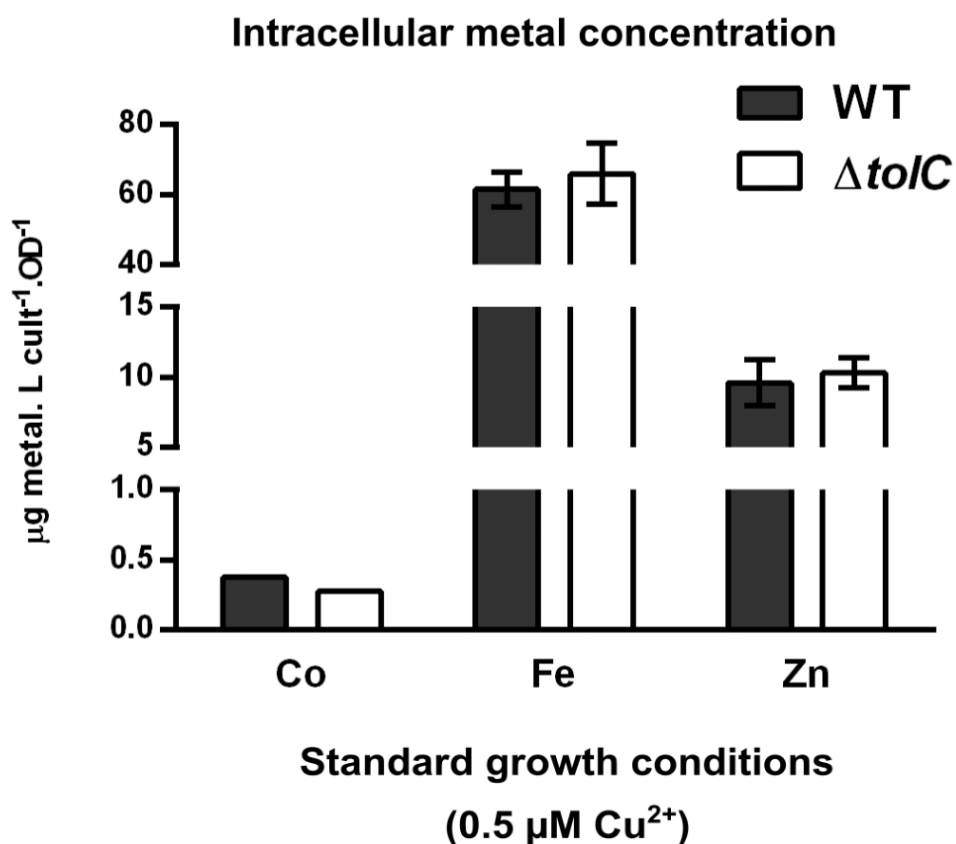
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128594.

References

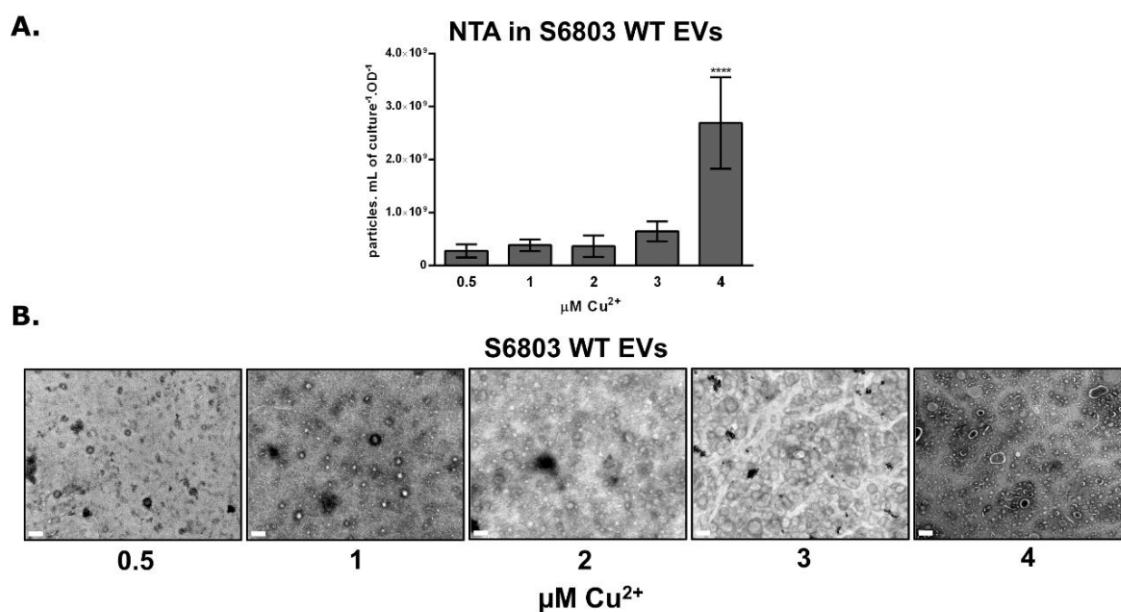
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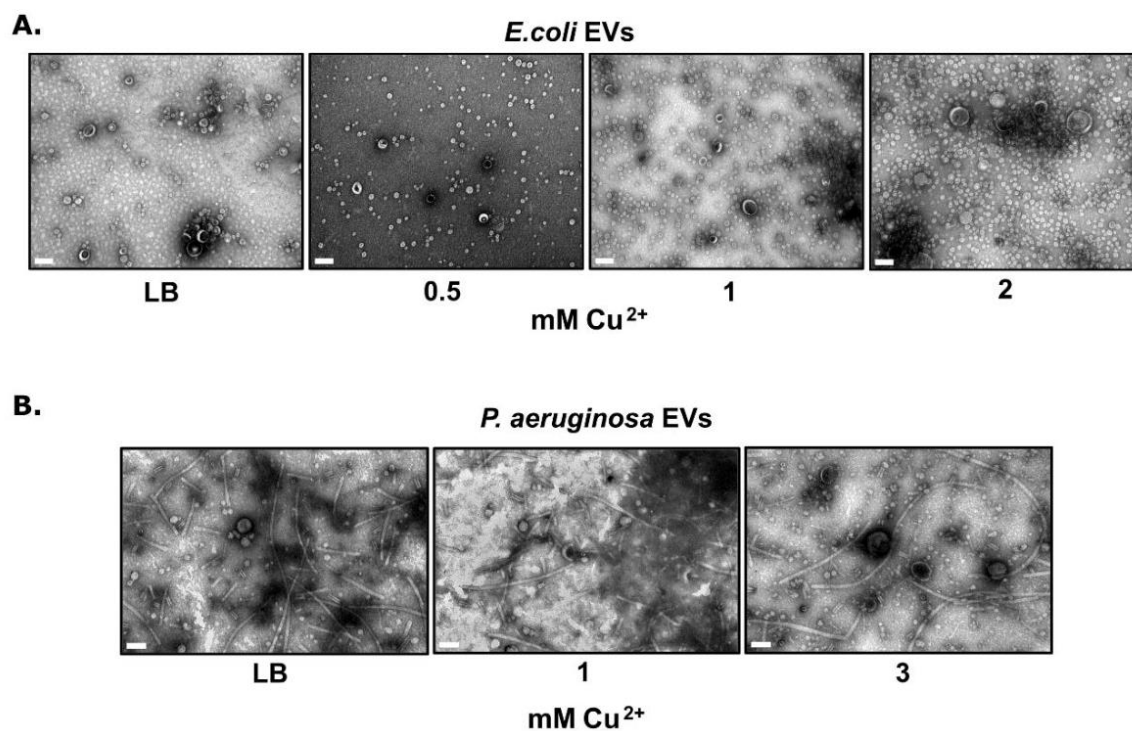
SUPPLEMENTARY FIGURES



Supplementary Figure S1. *Synechocystis* sp. PCC 6803 wild-type and *tolC*-mutant strains do not show differences in intracellular levels of cobalt, iron and zinc. Intracellular concentration of cobalt (Co), iron (Fe) and zinc (Zn) determined for *Synechocystis* wild-type (WT) and *tolC*-mutant (ΔtolC) strains cultivated under standard liquid BG11 conditions. Metals' levels were determined by atomic absorption spectrometry, and are shown in $\mu\text{g metal} \cdot \text{L culture}^{-1} \cdot \text{OD}_{730}^{-1}$. Error bars represent the standard deviations of at least three independent biological replicates. For cobalt, only one biological determination was performed.



Supplementary Figure S2. The amount of extracellular vesicles (EVs) released by *Synechocystis* sp. PCC 6803 (S6803) wild-type increases in response to copper-induced stress. *Synechocystis* wild-type (WT) cells were grown under standard growth conditions (liquid BG11 medium with 0.5 $\mu\text{M Cu}^{2+}$), and later distributed into BG11 standard medium (0.5 $\mu\text{M Cu}^{2+}$ - 0.5) or BG11 medium supplemented with copper to a final concentration of 1, 2, 3 and 4 $\mu\text{M Cu}^{2+}$. Cyanobacterial cells were cultivated for approximately 7 d, and their respective EVs isolated. **A.** Quantification of the number of nanoparticles present in isolated EVs samples as determined by nanoparticle tracking analysis (NTA, Malvern). Results are shown in number of particles . mL culture⁻¹ . OD₇₃₀⁻¹. ****, $P \leq 0.0001$, determined against the WT cultivated under standard growth conditions (0.5 $\mu\text{M Cu}^{2+}$). **B.** Morphological details of the *Synechocystis* WT EVs collected from cells cultivated under the various copper-concentration conditions were analyzed by transmission electron microscopy. Scale bar in the micrographs: 200 nm.



Supplementary Figure S3. Morphological aspects of *Escherichia coli* and *Pseudomonas aeruginosa* extracellular vesicles isolated under standard growth conditions (LB) or in medium supplemented with copper, analyzed by transmission electron microscopy. A. *E. coli* was cultivated either in LB standard medium, or in LB medium supplemented with 0.5, 1 and 2 mM Cu²⁺. **B.** *P. aeruginosa* was cultivated in LB medium, or in LB medium supplemented with 1 and 3 mM Cu²⁺. Scale bar: 100 nm.

Supporting information file Text S1

Extracellular vesicles as an alternative copper-secretion mechanism in bacteria

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MATERIALS AND METHODS

Proteomics analysis by iTRAQ

Cell harvesting and protein extraction

Cell pellets of *Synechocystis* wild-type and *tolC*-mutant were thawed and were resuspended in 400 μ L lysis buffer [200 mM triethylammonium bicarbonate (TEAB), 10 mM dithiothreitol (DTT), 1% (w/v) sodium deoxycholate, 0.1% (v/v) NP40 and 5 μ L protease inhibitor cocktail set II, pH 8.5] and transferred to 2 mL LoBind microcentrifuge tubes (Eppendorf) containing ~400 mg of 200 μ m Zirconium beads. Cell lysis was performed using a cell disruptor (Genie, VWR) using 10 cycles of 1 min with a 1 min cooling step on ice between cycles. Samples were centrifuged at 13,000 *g* for 10 min at 4 °C and the resulting supernatants were combined with their counterparts from the first 10 cycles of cell lysis. The combined supernatants were further clarified by centrifugation at 13 000 *g* for 10 min at 4 °C to ensure that any remaining cell debris was removed from the extracts. The clarified extracts were incubated with 2 μ L of benzonase nuclease (Novagene) for 2 min on ice. Before undertaking protein digestion, RapiGest SF (Waters) was added to a final concentration of 0.1 % (v/v) to increase protein solubility. Protein content in each extract was estimated using the modified Lowry method as previously described (Bensadoun et al, 1976). The protein content in the extracts was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) followed by silver staining (Couto et al., 2011).

Quantitative workflow and iTRAQ labelling

Isobaric tags for relative and absolute quantitation (iTRAQ) 8-plex labelling was performed according to the manufacturer's protocol (8-plex iTRAQ reagent Multiplex kit, ABSciex). Before labelling, protein extracts, 4 independent samples of WT cells and 4 independent samples of *tolC*-mutant cells, were reduced, alkylated and digested as follows: 100 μ g of proteins from each sample was reduced with 2.5 μ L of 10 mM Tris (2-carboxyethyl) phosphine hydrochloride and incubated at 60 °C for 1 h. Samples were then alkylated using 4.5 μ L of 20 mM methyl methanethiosulfonate at room temperature for 30 min. Subsequently, proteins were digested with trypsin (Promega) at a ratio of 1:20 (trypsin:protein) overnight at 37 °C. After digestion, biological replicates from each *Synechocystis* strain were labelled with a specific iTRAQ reagent following manufacturer's recommendations. Labels 113, 114, 115 and 116 were used to label the wild-type samples, labels 117, 118, 119 and 121 were used to label the *tolC*-mutant protein samples. After 2 h incubation at room temperature, labelled peptides were

acidified with trifluoroacetic acid to precipitate RapiGest SF and precipitates were removed by centrifugation at 17 000 *g* for 5 min at 4 °C. iTRAQ-labelled peptides, present in the eight sample supernatants, were combined and concentrated in a vacuum concentrator (Scanvac).

Off-line Hypercarb fractionation

iTRAQ-labelled peptides were resuspended in 100 µL of Hypercarb buffer A [97% (v/v) HPLC water, 3% (v/v) HPLC acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA)] and were fractionated using a Hypercarb porous graphitic column (7 µm particle size, 50 mm length, 2.1 mm diameter and 250 Å pore size), (Thermo Scientific) coupled with an UHPLC Ultimate 3000 RS (Dionex, Thermo Fisher Scientific). Peptides were eluted from the column at a flow rate of 0.2 mL.min⁻¹. Peptides were separated using a 90 min gradient as follows: 2% buffer B [97% (v/v) HPLC acetonitrile, 3% (v/v) HPLC water, 0.1% (v/v) TFA] for 5 min, 2–10% buffer B for 5 min, 10–60% buffer B for 50 min, 60–80% buffer B for 10 min, 80–90% buffer B for 1 min, 90% buffer B for 5 min and 90–2% buffer B for 1 min and 2% buffer B for 13 min. The chromatographic profile of the peptides was monitored at 240 nm through Chromeleon software (Thermo Fisher Scientific). Fractions were collected every 2 min from 10 min to 50 min (20 fractions). Collected fractions were then dried in a vacuum concentrator (Scanvac) and stored at –20 °C until further analysis.

LC MS/MS analysis

Each fraction was resuspended in 10 µL reverse phase (RP) buffer A [97% (v/v) HPLC water, 3% (v/v) HPLC ACN, 0.1% (v/v) FA] and combined to obtain 10 fractions for mass spectrometric analysis. Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific) coupled with an online UHPLC Ultimate 3000 (Thermo Fisher Scientific, Dionex) was used to analyse each fraction. From each fraction, 4 µL were injected two times into the system, peptide separation was performed by PepMap RSLC C18 column (2 µm, 100 Å, 75 µm × 50 cm) (Thermo Fisher Scientific) at a constant flow rate of 300 nL.min⁻¹. A gradient of 135 min was performed using RP buffer B [97% (v/v) HPLC ACN, 3% (v/v) HPLC water, 0.1% (v/v) FA] as follows: 4% buffer B for 5min, 4–40% of buffer B for 100 min, 40–90% of buffer B for 1 min, 90% buffer B for 14 min, ramping buffer B from 90–4% for 1 min and finally 4% of buffer B for 14 min. Mass spectrometry (MS) data was acquired using Xcalibur software v 4.0 (Thermo Scientific) with the following settings. MS scans were acquired with 60,000 resolution, automatic gain control (AGC) target 3e6, maximum injection time (IT) 100 ms. The MS mass range was set to be in the range 100–1500 *m/z*. Tandem mass spectrometry (MS/MS) scans

were acquired using high-energy collision dissociation (HCD), 30,000 resolution, AGC target 5e4, maximum IT 120 ms. In total, 15 MS/MS were acquired per MS scan using normalised collision energy (NCE) of 34% and isolation window of 1.2 m/z.

Data interpretation and protein identification and quantification

The *Synechocystis* sp. PCC 6803 (taxon ID: 1111708) database containing 3507 proteins was downloaded from Uniprot (.fasta) and uploaded on MaxQuant software (version 1.5.4.1). The settings were as follows: for “type the experimental set” MS2 and 8-plex iTRAQ were selected with reporter mass tolerance of 0.01 Da. Enzymatic digestion with trypsin was specified and one missed cleavages was allowed per peptide. Oxidation of methionine and deamidation of asparagine and glutamine were selected as variable modifications and methylthio modification of cysteine was selected as fixed modification. The false discovery rate (FDR) at the peptide spectrum match/protein level was set at 1%. The reporter ions intensities (113, 114, 115, 116, 117, 118, 119, 121) were used for quantification purposes. iTRAQ ratios used for relative quantification were determined by applying an in-house data analysis pipeline (Ow et al, 2009). Isotopic and median corrections were applied to the reporter ion’s intensities to compensate systematic errors between labels. Ultimately, a statistical method, described by Pham *et al.* (2010) was applied to determine which proteins were significantly regulated at $P < 0.05$.

Characterization of isolated extracellular vesicles

Nanosight and nanoparticle size and amount analyses

EVs samples were analyzed on a NanoSight NS300 NTA Dev Build 3.2.16 (Malvern), equipped with an sCMOS camera. Samples were diluted in filtered PBS solution (between 1:1000 and 1:10000), and further analyzed as described (Oliveira et al., 2016). In brief, three movies of 30 s were recorded for each sample (with a threshold of 10 to 50 particles per frame), with the following capturing settings: camera level 14-16, slider shutter 1300, slider gain 512, at 23 °C, and with a syringe pump speed of 40. Data acquisition and processing were performed using the NanoSight NS300 NTA 3.0 software, using a detection threshold of 5.

Electron microscopy

EVs were visualized by negative-staining transmission electron microscopy mainly as described elsewhere (Oliveira et al., 2016). A volume of 20 µL of sample were added on formvar/carbon film coated mesh nickel grids (Electron Microscopy Sciences) and left standing for 1 min. Using filter paper, the liquid in excess was removed, and 7.5 µL of

1% (w/v) uranyl acetate was used to cover the grids and left standing for 10 s, after which liquid in excess was again removed with filter paper. Observation was performed using a Jeol JEM-1400 transmission electron microscope at 80 kV.

Protein quantification

Detection and quantification of proteins was performed by an SDS-PAGE-based method, using bovine serum albumin (BSA) as standard, and stained with colloidal Coomassie Brilliant Blue (Sigma). BSA samples (containing 0 μg , 0.125 μg , 0.5 μg , 1 μg , 2 μg , and 5 μg) were prepared and loaded side by side with isolated EVs samples. A GS-800 Calibrated Densitometer (BioRad) was used to scan the Coomassie-stained gels, and a densitometry analysis was later carried out to determine protein concentration in EVs samples ($\mu\text{g } \mu\text{L}^{-1}$) using ImageJ.

LPS quantification

LPS bands profile was routinely analyzed and used to quantify EVs in isolated samples. Prior to LPS analysis, samples were incubated with a potent protease as described (Oliveira et al., 2016) to remove any contaminating glycoprotein, and further separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels. In brief, samples were heat denatured, incubated with 0.2 U of type XIV protease from *Streptomyces griseus* (Sigma) at 37 °C for 30 min, and then separated by electrophoresis. LPS were stained with Pro-Q® Emerald 300 Lipopolysaccharide gel staining kit (Life Technologies) and visualized on a Gel DocTM XR+ UV transilluminator system (Bio-Rad). Quantitation of LPS was determined by densitometry analysis (ImageJ), using the commercially available *Escherichia coli* serotype 055:B5 LPS (Life Technologies) as standard. Samples were normalized for cell culture density (OD_{730}), volume of cell-free culture medium concentrated and concentration factor (Oliveira et al., 2016).

Copper quantification in Synechocystis EVs samples

Copper levels in EVs isolated from *Synechocystis* wild-type and *tolC*-mutant were quantified by atomic absorption spectrometry. EVs of both strains were isolated from 2 L cultures (collected at an OD_{730} of 0.8-1.0), grown in liquid BG11 medium (0.5 $\mu\text{M } \text{Cu}^{2+}$). Then, to ensure a complete removal of BG11 traces in EVs samples (recovered in approximately 100 μL of sterile BG11), a buffer-exchange to Tris-HCl-EDTA buffer (20 mM Tris-HCl, 5 mM EDTA, pH 8.0). To that end, isolated EVs samples were thoroughly mixed with 15 mL of Tris-HCl-EDTA buffer, and later concentrated using centrifugal filters (Amicon® Ultra-15 Centrifugal Filter Units, Merck) with a MWCO of 3 kDa (4400 g, 16 °C), to a final volume of 500 μL . This step was performed three times, and EVs were

suspended in 250 μL of sterile Tris-HCl-EDTA buffer. Then, EVs were once again mixed with 15 mL of fresh Tris-HCl-EDTA buffer, and subjected to a final ultracentrifugation of 3 hours at 100 000 g at 4 $^{\circ}\text{C}$. The obtained EVs pellets were suspended in 100 μL Tris-HCl-EDTA buffer and stored at -80 $^{\circ}\text{C}$ until further analysis. To obtain EVs extracts, EVs samples were treated with the hot-nitric acid protocol as described for intracellular copper levels quantifications. Copper levels were determined in an atomic absorption spectrometer equipped with an electrothermal atomization detector (Perkin Elmer). Quantification was externally calibrated with standard aqueous solutions. Results are presented in μg of $\text{Cu}^{2+} \cdot \text{mg}^{-1}$ of vesicular protein.

Table S1. Oligonucleotides used in this work.

Primer	Sequence 5' - 3'	Ta (°C)	Source	Purpose
bfrA_fwd	GATCGACCATGAAATGCAGGACG	60	This work	RT-qPCR analysis
bfrA_rev	ACACACGGCCATTGCTTCC			
copB_fwd	CAAGCTGCCTTAAGCGTGG	60		
copB_rev	CATCTTGGCGAGATTCTCCTACC			
slr0769_fwd	CCTAGCAATCCAAC TAGCCAGG	60		
slr0769_rev	CCACACGGGATTTTCCACC			
sll0083_fwd	CTGAATTTGTGCGCCGTTTCC	60		
sll0083_rev	GGTGTGTAAACCTTATCCTTGGC			
rnpBF1	CGTTAGGATAGTGCCACAG	56	Pinto et al. (2012)	
rnpBR1	CGCTCTTACCGCACCTTTG			
lacOF	AAACTGCAGAATTGTGAGCGCTCACAATTTT	63	This work	sfGFP expression and targeting to EVs
lacOR	CGCCGGGAAATCTTAGTTGTCAATTTCTCCTCTTTAATTGTGTGAAAT			
futA2F_ss	ATTTACACAATTAAAGAGGAGAAAATGACAATAAGATTTCCCGGCG	65		
futA2R_ss	TGAACAGCTCTTCGCCTTTACGCATCGTGCGGGACTGGGCA			
sfGFPF	TGCCCAGTCCCGCACGATGCGTAAAGGCGAAGAGCT	63		
sfGFPR	AAATCTAGATCATTTGTACAGTTCATCCATACC			

Underlined nucleotides correspond to restriction enzymes recognition sites.

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CHAPTER VI - Novel protein carrier system based on cyanobacterial nano-sized extracellular vesicles for application in fish

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^{*}Participated in the generation and characterization of *Synechocystis* $\Delta tolC\Delta spy$ and EV-trc-GFP mutant strains, and on the isolation and characterization of cyanobacterial EVs.

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Novel protein carrier system based on cyanobacterial nano-sized extracellular vesicles for application in fish

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Summary

Aquaculture has been one of the fastest-growing food industry sectors, expanding at the pace of consumers' demands. To promote safe and effective fish growth performance strategies, and to stimulate environmentally friendly solutions to protect fish against disease outbreaks, new approaches are needed to safeguard fish welfare, as well as farmers and consumers interests. Here, we tested the use of cyanobacterial extracellular vesicles (EVs) as a novel nanocarrier system of heterologous proteins for applications in fish. We started by incubating

zebrafish larvae with *Synechocystis* sp. PCC6803 EVs, isolated from selected mutant strains with different cell envelope characteristics. Results show that *Synechocystis* EVs are biocompatible with fish larvae, regardless of their structural composition, as EVs neither induced fish mortality nor triggered significant inflammatory responses. We establish also that cyanobacteria are amenable to engineering heterologous protein expression and loading into EVs, for which we used the reporter sfGFP. Moreover, upon immersion treatment, we successfully demonstrate that sfGFP-loaded *Synechocystis* EVs accumulate in the gastrointestinal tract of zebrafish larvae. This work opens the possibility of using cyanobacterial EVs as a novel biotechnological tool in fish, with prospective applications in carrying proteins/enzymes, for example for modulating their nutritional status or stimulating specific adaptive immune responses.

Introduction

With an ever increasing demand for seafood that cannot be met by capture fisheries alone, growing pressure is being placed on aquaculture. According to the Food and Agriculture Organization of the United Nations, aquaculture is probably the fastest growing food-producing sector, now accounting for up to 50% of the world's fish that is used for food (FAO, 2020). However, several constraints limit the expansion of the sector, including fish growth performance in fish farms, disease outbreaks and stressful rearing conditions. Therefore, different strategies are required to maximize the welfare and nutritional status of the fish while minimizing environmental impact and feeding costs, reducing chemicals as prophylactic agents, and decreasing mortalities due to opportunistic pathogens. The use of live microorganisms as probiotics is one of such approaches (Hai, 2015), which has become popular in recent years as it reportedly improves feed value, contributes to enzymatic digestion of nutrients, inhibits the action of pathogenic microorganisms, shows anti-mutagenic and anti-carcinogenic activity, and potentiates immune responses (Pandiyani *et al.*, 2013). With the development of biotechnology, new systems and techniques have been implemented for the expression of heterologous proteins and enzymes in probiotics cells (Yao *et al.*, 2020), further potentiating their use.

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This approach is gaining considerable interest, as these proteins and enzymes can be carefully selected to meet a specific purpose, for example increasing adherence capability, trigger adaptive immune responses or stimulate nutrient digestion (Yao *et al.*, 2020). One example is the use of microbial photosynthetic systems towards a more sustainable development of the aquaculture sector, particularly by the use of microalgae or cyanobacteria as feed or dietary supplements (Abed *et al.*, 2009; Han *et al.*, 2019; Ma *et al.*, 2020; Morais Junior *et al.*, 2020). Recently, through genetic modifications, microalgae have been engineered to express potential antigens for the development of an oral vaccination platform in fish (Kwon *et al.*, 2019). Nevertheless, the choice of whole microorganisms to use in aquaculture remains far from consensual, mainly because the gastrointestinal microbiota of aquatic organisms has been poorly characterized and the effects of the probiotics agents remain to be extensively analysed (Pandiyan *et al.*, 2013). Thus, while the realization of probiotics as protein carrier and molecular display systems is yet to be fulfilled, other alternatives are being investigated.

In the context of carrying and delivering agents with a biological effect (bioactives) (Dezfooli *et al.*, 2019), the use of bacterial extracellular vesicles (EVs) occupies a relevant position. EVs are discrete and non-replicable proteoliposomal nanoparticles (Caruana and Walper, 2020), with a size between 20 and 500 nm in diameter (Zavan *et al.*, 2020). These are bilayered nanostructures, derived from the bacterial cell envelope, containing membrane components as well as soluble products (Lima *et al.*, 2020). Gram-negative bacteria, in particular, have been extensively engineered to release EVs with customized cargo, aiming at fulfilling different purposes, namely for the delivery of chemotherapeutic agents (Kurban *et al.*, 2020), for carrying immunogenic antigens (Fantappiè *et al.*, 2014) and even for performing complex chemical reactions extracellularly otherwise difficult to implement in whole cells (Park *et al.*, 2014). Some key features of the vesicles have encouraged these applications, particularly the fact that cargo properties are maintained in EVs even under harsh conditions, and the capacity of EVs in protecting and trafficking cargo to inaccessible targets (Bonnington and Kuehn, 2014). Despite the progress in employing EVs in biotechnology, administration to higher animals of EVs isolated from Gram-negative bacteria is usually associated with pathological reactions, including systemic inflammatory responses (Park *et al.*, 2010), mainly due to the presence of lipopolysaccharides (LPS). This represents a serious limitation, which has prompted the search for alternatives to minimize the immunogenicity of bacterial EVs, such as: the use of chemical processes to decrease LPS content, the genetic engineering of EVs-

producing bacterial strains for reduced and/or altered LPS content and the choice of bacterial strains containing LPS with low immunogenicity (Jain and Pillai, 2017). Regarding the latter, cyanobacterial derived EVs may represent a good candidate, since there are indications that cyanobacterial LPS are associated with significantly reduced toxicity in mammals, related to low immune reactivity, as compared to LPS from other bacteria (Stewart *et al.*, 2006; Durai *et al.*, 2015; Swanson-Mungerson *et al.*, 2017). Cyanobacteria are a remarkably diverse group of Gram-negative bacteria, with wide ecological distribution, and great metabolic plasticity. They are unique for being the only prokaryotes capable of performing oxygenic photosynthesis, and so, have minimal nutritional requirements. Thus, together with a wide range of genetic engineering tools available, cyanobacteria are increasingly regarded as promising, environmentally friendly and highly sustainable microbial cell factories for the production of added value products (Heidorn *et al.*, 2011). Nevertheless, the study of cyanobacterial EVs is just emerging, and many questions remain unanswered as to their potential in biotechnological applications.

The main goal of this study was, therefore, to understand to what extent are cyanobacterial EVs a viable vehicle for carrying proteins to fish. To that end, the unicellular, freshwater cyanobacterium *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*) was used in this work. Different aspects had to be comprehensively analysed, including determination of toxicity of *Synechocystis* EVs and their LPS in fish, modulation of protein cargo of *Synechocystis* EVs, by packaging them with a reporter protein and evaluation of the reporter protein delivery in fish via *Synechocystis* EVs. Altogether, our results indicate that *Synechocystis* EVs represent an innovative, biocompatible protein carrier system for fish, expanding the molecular toolbox available for the transport of biologically active proteins and enzymes in aquaculture.

Results

Synechocystis wild type and mutant strains release EVs with different surface properties

Extracellular vesicles in cyanobacteria originate mainly from its cell envelope (Lima *et al.*, 2020) (Fig. 1A). In *Synechocystis*, the outermost components of the cell envelope are the S-layer and lipopolysaccharides (LPS) (Fig. 1B), which are also structural components of *Synechocystis* EVs (Fig. 1C). In this work, to evaluate the level of toxicity induced by cyanobacterial-derived EVs in fish, we started by investigating the effect of EVs isolated from *Synechocystis* on the survival and inflammatory response of zebrafish larvae. To get a deeper

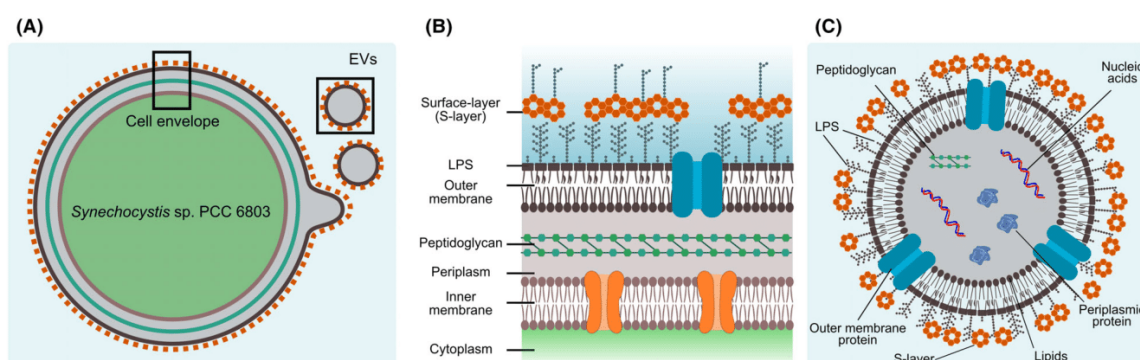


Fig. 1. Schematic representation of the structure and composition of the *Synechocystis* sp. PCC6803 cell wall and extracellular vesicles.

A. Diagram showing the Gram-negative-type cell wall structure of the *Synechocystis* cell, and EVs formation by budding and detachment of the outer membrane. Squares represent close ups shown in sections B and C.

B. Details of the cyanobacterial cell wall. It is worth highlighting that lipopolysaccharides constitute the outer leaflet of the outer membrane, and that the S-layer is the outermost structure of the *Synechocystis* cell.

C. General view of the composition of cyanobacterial EVs.

understanding of the toxic effect of *Synechocystis* EVs components in fish, it became important to use EVs with different surface properties. To that end, different *Synechocystis* mutant strains were selected for their previously reported altered cell envelope characteristics. On one hand, the role of the S-layer was assessed by using the *tolC* mutant, which has been shown to be impaired in the secretion of the S-layer protein (protein Sll1951) (Oliveira *et al.*, 2016) as opposed to the wild-type strain (GT-Kazusa strain) that is known to produce it. On the other hand, the *fucS* mutant was selected for its unique LPS structural characteristics, as it was previously described to produce LPS with truncated O-antigen portions (Fisher *et al.*, 2013). Furthermore, the *tolC* mutant was reported to release more EVs than the wild-type strain (Oliveira *et al.*, 2016), which represented a trait of particular interest for subsequent assays. Another *Synechocystis* mutant strain (*tolC-spy* double mutant, Fig. S1) available in the laboratory showed slightly higher vesiculation capacity than the hypervesiculating *tolC* mutant. Thus, taking advantage of its natural capacity to produce large amounts of EVs, we decided to include the *tolC/spy* double mutant in this study as well.

Lipopolysaccharides from the *Synechocystis* wild type, and mutant strains *tolC*, *tolC/spy* and *fucS* were isolated from cells with a standard hot phenol-water method (Rezania *et al.*, 2011), and EVs were isolated by ultrafiltration and ultracentrifugation of the extracellular medium recovered from cultures of the same strains (Figs 2 and 3). After separation of the isolated samples by electrophoresis on SDS-polyacrylamide gels and subsequent detection with a LPS specific stain, it was possible to observe and confirm that the LPS band profile of the wild-type strain was similar to that of the *tolC* and *tolC-*

spy mutants, while the LPS band pattern of the *fucS* mutant strain was significantly different (Fig. 2 and Fig. S2). LPS from the *fucS* mutant show an accumulation of smooth-type LPS (lipid A, oligosaccharide core and O-antigen) with lower molecular weight than those of the wild-type. Comparing the LPS band profile of isolated LPS and LPS derived from EVs, one could detect a clear absence of low molecular weight, non-polar, rough-type LPS (composed of lipid A and oligosaccharide core) in the samples corresponding to isolated LPS (Fig. 2), likely resulting from the chemical-based LPS extraction protocol (Rezania *et al.*, 2011), which preferentially isolates polar molecules. Furthermore, a substantial difference could also be observed in LPS bands when comparing the various cyanobacterial samples with the commercially available LPS from *Pseudomonas aeruginosa*, used here as reference.

Isolated EVs were also characterized in detail in terms of their abundance, morphological aspect and size (Fig. 3). Determination of the amount of EVs produced by each strain, indicated that *tolC*, *tolC-spy* and *fucS* are hypervesiculating strains (Fig. 3A). This relative EVs production capacity could already be observed during the EVs isolation protocol: after the ultracentrifugation step, EVs pellets from all *Synechocystis* strains showed the typical orange pigmentation, consistent with the presence of carotenoids in the outer membrane of this cyanobacterium, with vesicles from the wild-type corresponding to the smallest pellet (Fig. 3B). Despite the differences in abundance, isolated EVs from the various cyanobacterial strains appeared as spherical nanosized structures when observed by transmission electron microscopy (TEM) of negatively stained samples (Fig. 3B). Based on these transmission electron

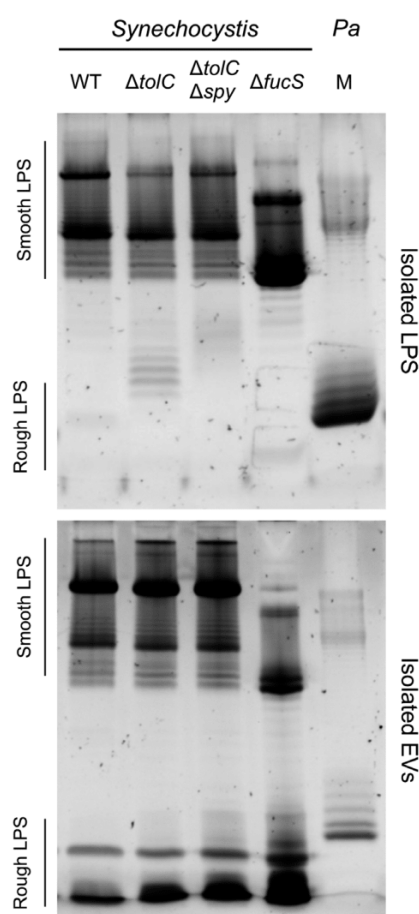


Fig. 2. Lipopolysaccharides (LPS) band profile of isolated LPS and of purified extracellular vesicles obtained from the *Synechocystis* sp. PCC6803 strains studied in this work. LPS from the *Synechocystis* wild-type (WT), and mutant strains $\Delta tolC$, $\Delta tolC/\Delta spy$ and $\Delta fucS$ were isolated with a standard hot phenol-water method (Rezania *et al.*, 2011) (upper panel), while EVs from the same strains were collected from the spent medium by ultrafiltration and ultracentrifugation (bottom panel). LPS present in both sample types were separated on 16% (w/v) SDS-polyacrylamide gels, and stained with the Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies). Commercial LPS from *Pseudomonas aeruginosa* (LPS Pa) was loaded as reference marker (M). High molecular LPS (containing lipid A, oligosaccharide core and O-antigen) are shown as Smooth LPS, and low molecular LPS (containing lipid A, oligosaccharide core) are indicated as Rough LPS. These gels are representative of the results obtained from at least three independent biological replicates.

micrographs, the size of isolated EVs was determined. While EVs from the wild-type and *fucS* mutant strain are more heterogeneous in size distribution, those isolated from the *tolC* and *tolC-spy* mutant strains are enriched in a narrower size range. Approximately 50% of the measured EVs were found to have a diameter between 45

and 60 nm in the *tolC* and *tolC-spy* mutant strains, and between 45 and 70 nm, and 35 and 60 nm for the wild-type and *fucS* mutant respectively (Fig. 3B). Moreover, abnormally large EVs (larger than 130 nm in diameter) could also be observed in the *fucS* mutant strain, which corresponded to more than 10% of all the measured EVs.

Isolated Synechocystis LPS and EVs do not induce zebrafish larvae mortality

We then proceeded to evaluate the biocompatibility of isolated *Synechocystis* LPS and EVs in fish. Three days post-fertilization (dpf) zebrafish (*Danio rerio*) larvae were treated by immersion with different amounts of cyanobacterial LPS or EVs for 5 days, and zebrafish larval survival was monitored daily. In addition, commercially available LPS from *P. aeruginosa* were also included in the test, as these have been reported to induce approximately 50% larval mortality in about 8 h of treatment with $50 \mu\text{g ml}^{-1}$ (Novoa *et al.*, 2009). Altogether, our results showed that neither EVs from any of the cyanobacterial strains used in this work nor isolated cyanobacterial LPS from the wild-type or the *tolC* and *fucS* single mutants induced significant mortality in zebrafish larvae under the tested conditions (Fig. 4; Tables S1 and S2–S5). In contrast, larvae exposed to $500 \mu\text{g ml}^{-1}$ of LPS isolated from the cyanobacterial *tolC-spy*-double mutant, and to a lesser extent $250 \mu\text{g ml}^{-1}$ of LPS, showed a significant reduction in survival rate, although not as severe as that obtained with the *P. aeruginosa* LPS. Thus, while $45 \mu\text{g ml}^{-1}$ *P. aeruginosa* LPS triggered > 80% mortality after 24 h of treatment, isolated EVs concentrations of up to $500 \mu\text{g ml}^{-1}$ from *Synechocystis* may be used without significantly reducing larvae survival.

EVs from the Synechocystis tolC and fucS mutant strains do not trigger high inflammation responses

Taking advantage of the hypervesiculating phenotype of *tolC* and *fucS* single mutants, the innate immune response of zebrafish larvae to EVs isolated from these mutants was evaluated in 3 dpf zebrafish larvae subject to a 24 h immersion trial, against a zebrafish embryo medium control treatment (the *tolC-spy* mutant was not included, as its LPS showed some level of toxicity). The expression of pro-inflammatory cytokine interleukin 1 beta (IL-1 β), tumour necrosis factor (TNF- α) and anti-inflammatory cytokine interleukin 10 (IL-10), was then analysed by RT-qPCR and results normalized by RPL13a mRNA levels (Fig. 5 and Table S6). In contrast to LPS from *P. aeruginosa* that induced significant up-regulation of inflammatory markers, when used at a sub-lethal concentration of $35 \mu\text{g ml}^{-1}$, EVs isolated from the

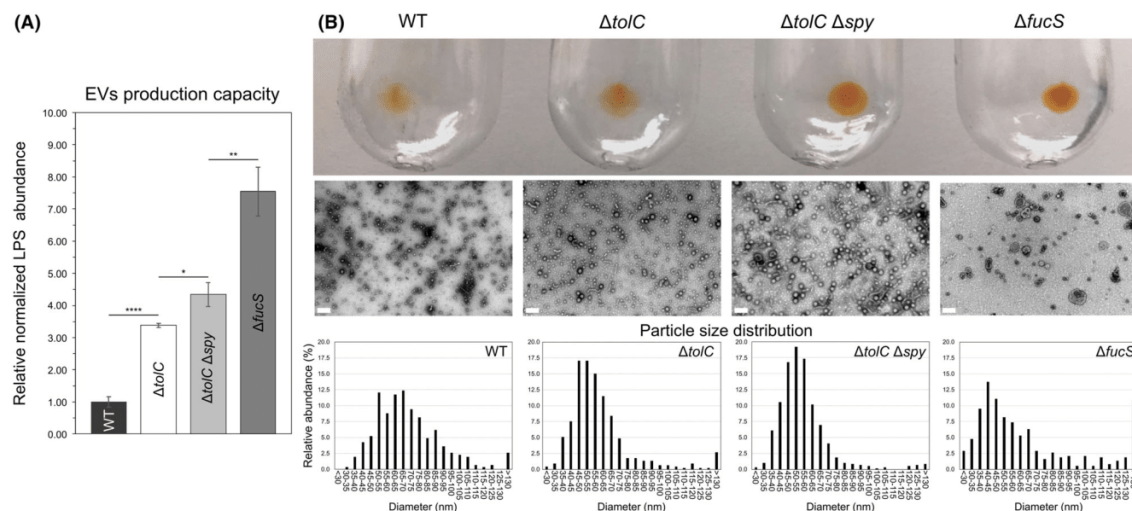


Fig. 3. Characterization of the *Synechocystis* sp. PCC6803 extracellular vesicles isolated from the wild-type and mutant strains. **A.** Relative vesicle production capacity of *Synechocystis* wild type (WT), and *tolC* ($\Delta tolC$), *tolC/spy* ($\Delta tolC/\Delta spy$) and *fucS* ($\Delta fucS$) mutant strains, as determined by densitometry analysis of the outer membrane marker LPS detected on SDS-polyacrylamide gels (Fig. S2). For LPS quantitation, images of stained gels were processed using ImageJ by quantifying pixel intensity values of all bands detected in a given lane, and deducing LPS amount against a calibration curve established with *P. aeruginosa* LPS (Sigma-Aldrich, Burlington, MA, USA) as standard. EVs samples were normalized to culture OD_{730} , culture volume, and final EVs sample volume, prior to separation by electrophoresis. Error bars indicate standard deviations of three independent biological replicates. Statistical analysis was performed by means of a one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. **B.** Pellets resulting from the ultracentrifugation step during EVs isolation from the *Synechocystis* wild-type and mutant strains are shown (top panels). Transmission electron micrographs of negatively stained isolated EVs samples of the various *Synechocystis* strains show details of their size and morphology (middle panels) (scale bar: 200 nm). Note that the relative amount of EVs isolated from each strain cannot be assessed in these micrographs, as different amounts of EVs were loaded onto the grids for optimized visualization. Histograms showing particle size distribution of EVs samples from the different cyanobacterial strains are presented (bottom panels). EVs were separated in 5 nm size groups, distributed between 30 and 130 nm, and in groups comprising those with a size below 30 nm or higher than 130 nm. Vesicle diameter was determined by direct measurement from electron micrographs of three independent biological replicates. Abundance of each size group is presented as relative figures, that is number of vesicles with a given diameter range relative to the total number of vesicles measured. More than 300 vesicles were measured for the wild-type, 450 for the *tolC* mutant, 550 for the double *tolC-spy* mutant, and 350 for the *fucS* mutant.

tolC and *fucS* single mutants were shown not to induce a significant inflammatory response even at a concentration of $500 \mu\text{g ml}^{-1}$. The observation that *Synechocystis* EVs harbouring LPS with different O-antigen length and characteristics do not trigger acute inflammation in fish, further supports that *Synechocystis* EVs structural components are biocompatible with fish even at high concentrations.

Modulation of *Synechocystis* EVs protein content

To evaluate the capacity of customizing the protein content of *Synechocystis* EVs, the super-folder green fluorescent protein (sfGFP) was used as reporter, as recently described (Lima *et al.*, 2022). Hence, sfGFP was expressed in *Synechocystis* cells under the control of the well-characterized, strong promoter $P_{trc.x.lacO}$ (Ferreira *et al.*, 2018) and further targeted to the periplasm by the signal peptide of the *Synechocystis* periplasmic protein FutA2, a substrate of the twin-arginine translocation (Tat) pathway (Waldron *et al.*, 2007; Badarau *et al.*,

2008) (Fig. 6). Plasmids pEV-trc-GFP(S) were constructed for this purpose, and despite various transformation attempts in the hypervesiculating *Synechocystis* *tolC* mutant strain, sfGFP expressing cyanobacteria were only possible to obtain with wild-type cells as background strain. Fully segregated cells (EV-trc-GFP) were cultivated in liquid medium, and a yellowish/greenish coloration of the extracellular medium could be observed (Fig. 6A). Cells were studied by confocal microscopy to evaluate sfGFP expression and cellular localization of the fluorescent signal (Fig. 6B). The sfGFP-dependent fluorescent signal was localized outside of the autofluorescence signal, which derives from the photosynthetic pigments located in the thylakoid membranes, indicating that most of the sfGFP protein is in the periplasm of cyanobacterial cells (Fig. 6C). Occasionally, it was also possible to observe small fluorescent foci in the extracellular medium. Thus, the extracellular medium of EV-trc-GFP cells was collected, and the respective EVs were isolated. The EV-free extracellular medium was also further concentrated and analysed. Western blotting analysis of

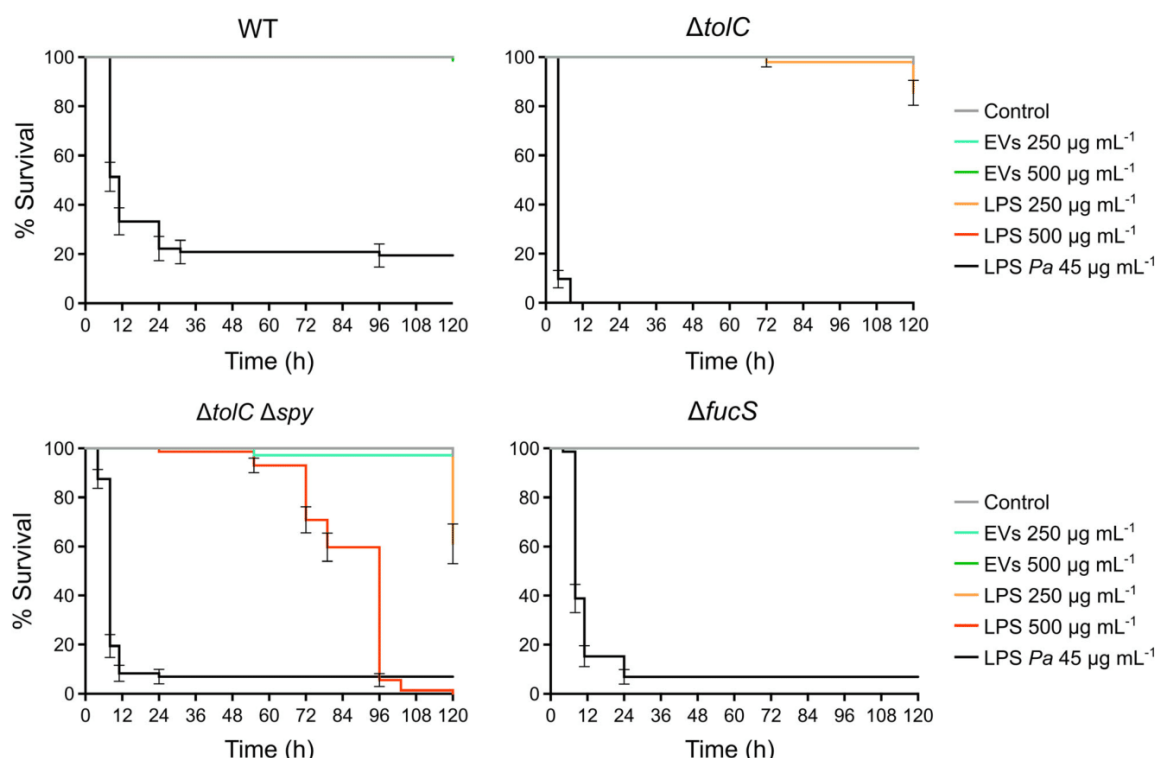
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Fig. 4. Effect of isolated lipopolysaccharides and extracellular vesicles from the *Synechocystis* sp. PCC6803 strains studied in this work on zebrafish larvae survival. Kaplan-Meier survival curves of 3 days post-fertilization (dpf) zebrafish larvae subjected to zebrafish embryo medium containing 250 or 500 $\mu\text{g mL}^{-1}$ of isolated LPS or EVs from *Synechocystis* sp. PCC6803 wild-type (WT), and mutant strains ΔtolC , $\Delta\text{tolC}/\Delta\text{spy}$ and ΔfucS . Survival tests also included two control treatments, which were carried out separately for each set of experiments corresponding to each *Synechocystis* strain. Control treatments received either zebrafish embryo medium only (Control) or zebrafish embryo medium containing commercial LPS from *Pseudomonas aeruginosa* (LPS *Pa*) at 45 $\mu\text{g mL}^{-1}$. Survival was monitored for 5 days (120 h) of immersion challenge. Data are represented as mean $\pm$ standard deviation of two independent biological experiments, each condition tested in triplicates of 12 larvae, making a total of 72 larvae analysed within each experimental condition. Survival percentages data points are listed in Table S1.

the various fractions, using a GFP-specific antibody, determined that EV-trc-GFP cells indeed express sfGFP, in agreement with confocal microscopy results and that the reporter successfully accumulates in the extracellular medium, both in isolated EVs fractions and in concentrated EV-free extracellular medium (Fig. 6D). Moreover, it was possible to quantify that approximately 90% of the sfGFP protein present in the extracellular medium was found soluble in the medium, with the remaining part present in the isolated EVs fraction (Fig. 6D).

Characterization of EVs obtained from the EV-trc-GFP strain was carried out by different microscopic methods. Confocal microscopy observations of isolated EVs preparations determined the existence of numerous, highly fluorescent foci in the sample (Fig. 6E), consistent with possible sfGFP-packed EVs. TEM analysis of negatively stained samples indicated the presence of many spherical nanostructures, morphologically similar to EVs from the *Synechocystis* wild-type and mutant strains studied in this work (Fig. S3A). Moreover, TEM analysis of

immunogold labelled EVs samples demonstrated the presence of sfGFP inside EVs (Fig. 6E). To complement the characterization of sfGFP-packed EVs, isolated samples were investigated by LPS analysis upon electrophoretic separation on SDS-polyacrylamide gels, and by determining their size from the analysis of TEM micrographs (Fig. S3B and C). The results indicate that strain EV-trc-GFP releases approximately 3.5-fold more EVs than the wild-type strain, and that sfGFP-containing EVs are slightly smaller than EVs from any other strain investigated in this study (50% of the analysed EVs had a diameter between 35 and 55 nm).

sfGFP-loaded Synechocystis EVs reach the gastrointestinal tract of zebrafish larvae upon immersion treatment

To understand whether cyanobacterial EVs can work as nanocarriers of custom proteins into fish, 5 dpf zebrafish larvae were treated by immersion with isolated sfGFP-

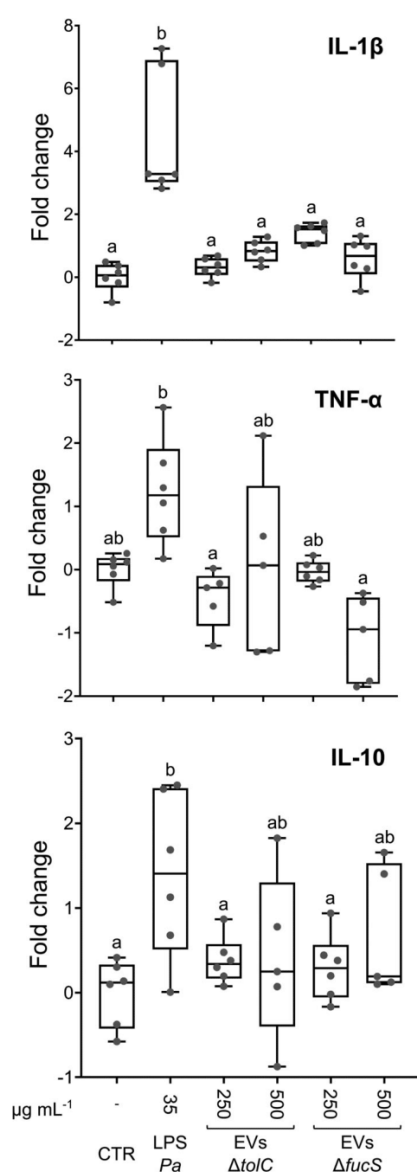


Fig. 5. Effect of isolated extracellular vesicles from the *Synechocystis* sp. PCC6803 mutant strains $\Delta tolC$ and $\Delta fucS$ on the expression of fish inflammation markers. Quantitative expression analysed by RT-qPCR of the genes encoding Interleukin 1 beta (IL-1 β), Tumor necrosis factor alpha (TNF- α), and Interleukin 10 (IL-10) in zebrafish larvae immersed in zebrafish embryo medium only (CTR – control), zebrafish embryo medium containing 250 or 500 $\mu g LPS ml^{-1}$ of *P. aeruginosa* (LPS *Pa*), or in zebrafish embryo medium containing 250 or 500 $\mu g LPS ml^{-1}$ of EVs from *Synechocystis* mutants $\Delta tolC$ (EVs $\Delta tolC$) and $\Delta fucS$ (EVs $\Delta fucS$), or in zebrafish embryo medium containing 35 $\mu g LPS ml^{-1}$ of *P. aeruginosa* (LPS *Pa*). For each treatment, 6 pools of 20 larvae each were analysed (individual data points are shown). Extreme outliers (for details, see Experimental procedures) were removed from the analysis. Treatments effects were analysed by one-way ANOVA and significant differences among means ($P < 0.05$) were determined by the Tukey's multiple range test (for details, see Table S6).

loaded *Synechocystis* EVs for 24 h. In agreement with earlier observations (Fig. 4), zebrafish larvae treated with EVs did not show signs of morbidity, and did not die. Upon treatment, larvae were euthanized and observed by confocal microscopy. Under the microscope, while a clear fluorescence signal could be observed in the ventral region of zebrafish larvae treated with sfGFP-loaded EVs, control larvae (in which no EVs were added) did not show fluorescence signal (Fig. 7). Closer inspection of the signal in respect to the zebrafish larvae anatomy indicated that it originates mainly from the gastrointestinal (GI) tract of the animal, particularly the intestine. This observation establishes that *Synechocystis* EVs with customized protein cargo: (i) can be suspended in the medium where zebrafish larvae are maintained; (ii) are ingested by the animals; (iii) accumulate in the GI tract; and (iv) can help to maintain structure and activity of the heterologous protein, as sfGFP retained its fluorescence throughout the whole process.

Discussion

One of the main objectives of this work was to determine to what extent are cyanobacterial-derived EVs toxic to fish. To meet that goal, we decided to use the well-characterized unicellular cyanobacterium *Synechocystis*, and zebrafish (*Danio rerio*) as model organisms. On one hand, as EVs are nanostructures that result from the release of material from the bacterial cell envelope (Figs 1–3), we decided to include various *Synechocystis* mutant strains with different cell envelope/surface properties, and differential vesiculation capacity. On the other hand, analysis of immune responses in zebrafish are simplified with the use of individuals in their larval stage, as they are transparent, easy to cultivate and only the innate immune system is present, allowing the assessment of physiological responses with the whole organism (Novoa *et al.*, 2009).

Immersion trials using 3 dpf zebrafish larvae and isolated LPS or EVs from the various genetically engineered *Synechocystis* strains did not show severe mortality as compared to the control condition (*P. aeruginosa* LPS (Novoa *et al.*, 2009)), except for LPS isolated from the *tolC-spy* mutant strain (Fig. 4). In previous work, we have found that absence of a functional TolC protein in *Synechocystis* results in high intracellular oxidative stress, as indicated by the accumulation of reactive oxygen species (ROS) in the cytoplasm and high level of lipid peroxidation (Hewelt-Belka *et al.*, 2020), with the consequent activation of ROS detoxifying enzymes (Oliveira *et al.*, 2016). Moreover, we have also found that cell envelope stress pathways are upregulated in the *tolC* mutant, as indicated by upregulation of transcript levels of genes (*spy*, *degQ*) and

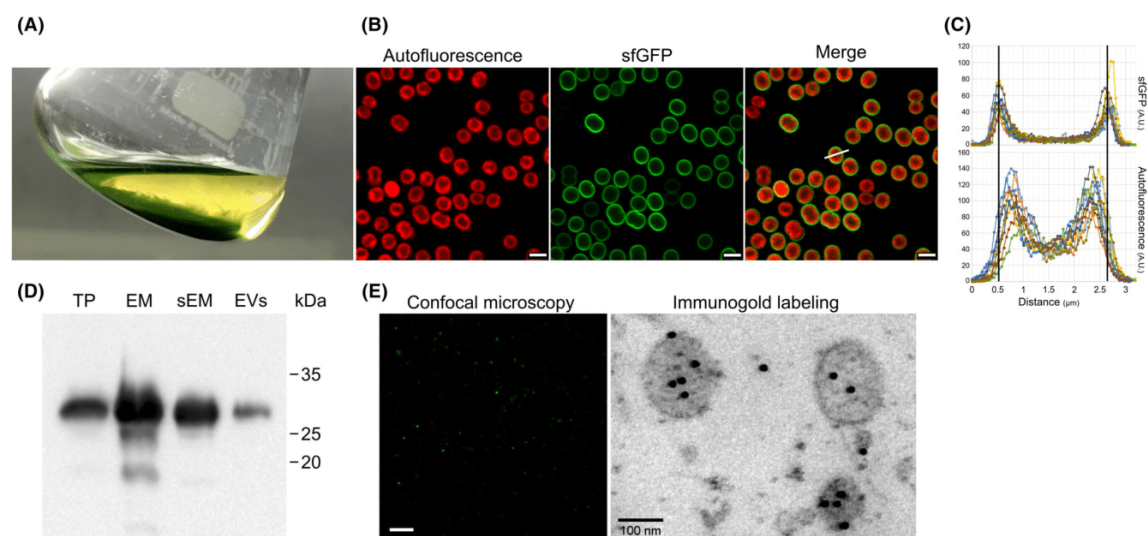
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Fig. 6. *Synechocystis* sp. PCC6803 extracellular vesicles loaded with the reporter sfGFP.

A. Photograph of a liquid culture of EV-trc-GFP (*Synechocystis* cells expressing sfGFP fused with the signal peptide of the FutA2 protein, under the control of the $P_{trcX:lacO}$ promoter), showing details of the yellow/green colour found in the extracellular medium.

B. Confocal micrographs of EV-trc-GFP cells depicting the red autofluorescence signal from the photosynthetic pigments (Autofluorescence), and the green signal from the sfGFP reporter (sfGFP). A panel showing the result of merging the two micrographs is also presented (Merge). Scale bar: 3 μm .

C. sfGFP (upper panel) and autofluorescence (bottom panel) fluorescence intensity plots determined for 10 different EV-trc-GFP cells (a.u., arbitrary units). Fluorescence intensity was quantified across the chosen cells as indicated by the white line drawn over one of the EV-trc-GFP cells in the merged fluorescence signal confocal micrograph, and plotted against line distance (in μm).

D. Western blot analysis for the detection of sfGFP protein in 10 μg of total cellular protein (TP), and cell free concentrated extracellular media (EM) samples of EV-trc-GFP cells. From the whole extracellular medium sample, extracellular vesicles (EVs) were separated from the soluble part (sEM), and also included for analysis. EM, sEM and EVs samples were normalized for initial cell-free culture volume concentrated, final volume of concentrated sample, and culture OD_{730} . This blot is representative of the results obtained from three independent biological replicates.

E. Microscopic analyses of EVs isolated from EV-trc-GFP cells, including a confocal micrograph showing green fluorescent foci (scale bar: 5 μm), and a transmission electron micrograph of immunogold labelled EVs. Black dots indicate the presence of sfGFP epitopes.

overexpression of proteins (Spy, DegP) (Gonçalves *et al.*, 2018) related to envelope stress responses. These results highlight that the *tolC* mutant is under stress, with undergoing adaptations at the periplasmic level. Spy is an important periplasmic chaperone, contributing to protein folding in the periplasm (Quan *et al.*, 2011). Deletion of *spy* in the *tolC* mutant background resulted in higher vesiculation (Fig. 3), which could be interpreted as a sign of further stress. Thus, it is possible that *tolC-spy* double mutant cells are under higher oxidative stress than *tolC* cells, which could lead to an increased level of LPS oxidation. Consequently, we hypothesize that LPS from the *tolC-spy* mutant are more toxic than LPS isolated from other *Synechocystis* strains used in this work, which could account for the differences observed in the zebrafish survival tests. However, despite the higher toxicity of its LPS, EVs isolated from *tolC-spy* mutant did not induce significant mortality in zebrafish larvae. This may be related to the bioavailability of isolated LPS versus LPS in vesicles to fish cells and tissues under the tested conditions. Still, the mortality observed with the

LPS isolated from the double mutant strain seems to confirm that LPS are the most immunogenic structural components of EVs.

Expression analysis of molecular markers associated with inflammatory responses in fish, namely IL-1 β , TNF- α and IL-10, showed their significant upregulation in zebrafish larvae exposed to sub-lethal concentrations of *P. aeruginosa* LPS, but not to isolated *Synechocystis* EVs of the *tolC* and *fucS* mutant strains, even when high amounts of EVs were used (Fig. 5). Altogether, these results indicate that zebrafish larvae can withstand high dosages of *Synechocystis* LPS and EVs, without triggering systemic inflammatory responses. This is in agreement with earlier works reporting a significantly reduced toxicity of LPS isolated from various cyanobacterial strains (*Anacystis nidulans*, *Oscillatoria* sp. and species of the genera *Anabaena*, *Aphanizomenon*, *Microcystis* and *Nodularia*) compared to LPS from other bacteria (*Escherichia coli*, *Salmonella* sp.) (Stewart *et al.*, 2006; Durai *et al.*, 2015; Swanson-Mungerson *et al.*, 2017). This low reactivity against cyanobacterial LPS can be

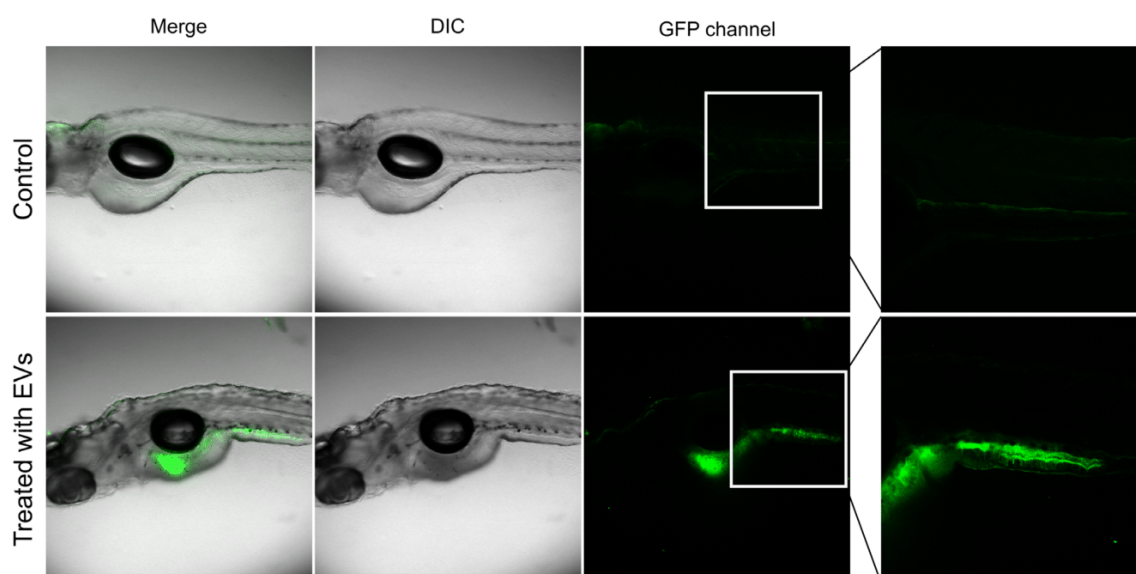


Fig. 7. *Synechocystis* extracellular vesicles loaded with sfGFP accumulate in the gastrointestinal tract of zebrafish larvae. Confocal micrographs of 5 dpf zebrafish larvae subjected for 24 h to immersion trials without (Control – upper panels) or with $1000 \mu\text{g ml}^{-1}$ sfGFP-loaded EVs isolated from the cyanobacterial strain EV-trc-GFP (lower panels). The differential interference contrast (DIC) micrographs are shown in the middle panels, while the fluorescence signal detected in the GFP channel (excitation: 488 nm; emission: 495–540 nm) are shown on the right hand panels. The result of merging the DIC and GFP channel micrographs is presented on the left-hand side (Merge). The white square in each 'GFP channel' micrograph indicates the position where a larger magnification of the anatomic region corresponding to the gastrointestinal tract was obtained (right-hand side).

explained by structural differences detected in the cyanobacterial lipid A backbone, as compared to other Gram-negative bacteria (Hahn and Schleiff, 2014). In particular, a missing phosphorylation at position 1-4' of the glucosamine disaccharide in the cyanobacterial lipid A has been suggested to represent the key factor associated with cyanobacterial LPS-reduced toxicity (Hahn and Schleiff, 2014). Additional biochemical and structural studies are needed to fully elucidate this effect.

In addition to showing that high amounts of *Synechocystis*-derived EVs are not toxic to zebrafish larvae, in this study we also present evidence supporting the capacity of customizing protein cargo of *Synechocystis* EVs (Fig. 6). By fusing the reporter sfGFP with the Tat-dependent signal peptide of protein FutA2 (Waldron *et al.*, 2007; Badarau *et al.*, 2008) as previously described (Polyviou *et al.*, 2018), we were able to show expression and translocation of sfGFP to the periplasm and, ultimately, to EVs. Loading of EVs with proteins of interest has long been shown for example for *E. coli* (Kesty and Kuehn, 2004), and here, we demonstrate that the protein content of *Synechocystis* EVs can also be customized with a heterologous protein. Curiously, strain EV-trc-GFP showed an increased vesiculation capacity as compared to the wild-type strain (Fig. S3). This observation is consistent with the possibility of an

increased 'periplasmic pressure' resulting from an accumulation of protein in the periplasm, as suggested by Schwechheimer *et al.* (2014). In fact, this pressure could also account for the difficulty in transforming the sfGFP construct into the *tolC* mutant background: as discussed above, this hypervesiculating mutant strain is under oxidative stress, with several adaptations at the periplasmic level. With this construct, protein expression is under the control of the constitutive, relatively strong promoter $P_{trc.x.lacO}$ (Ferreira *et al.*, 2018), and translocation to the periplasm seems to be rather efficient, likely recruiting several Tat transporters, as indicated by the accumulation of sfGFP signal in the periplasm as compared to that in the cytoplasm (Fig. 6C). Thus, over accumulation of protein in the periplasm may lead to an increase in periplasmic pressure, which in turn leads to hypervesiculation in the wild-type strain, but impairs growth in the *tolC* mutant. In the future, expression of sfGFP with a weak promoter in the *tolC* mutant will help in clarifying whether the strain can cope with low sfGFP abundance in the periplasm and release EVs packed with the reporter.

Several aspects require optimization towards an efficient loading of cyanobacterial EVs with proteins of interest, including fine-tuned expression of the protein, translocation to the periplasm, protein-folding and

-stability in the periplasm, and efficient targeting to EVs. Adjusting these aspects brings the prospect of making cyanobacteria sustainable microbial factories for the production of safe EVs, suitable for various biotechnological applications. In that line of thinking, cyanobacterial EVs have already been used with success to stimulate cutaneous wound healing in mice, by promoting angiogenesis (Yin *et al.*, 2019).

Here we also established that sfGFP-loaded EVs from *Synechocystis* can successfully carry the customized protein cargo to fish, as illustrated by the detection of sfGFP signal in the GI tract of zebrafish larvae upon immersion treatments. The uptake and biodistribution of nanoparticles (in the range of 25–100 nm) in zebrafish larvae have been described before, but the focus has been mainly on nanoplastics in the context of environmental pollution (van Pomeroy *et al.*, 2017; Pitt *et al.*, 2018; Sendra *et al.*, 2021). Curiously, these nanoparticles could also be detected in the GI tract of the animals analysed, which further supports the notion that nano-sized EVs in suspension will follow the same internalization route towards the fish gut, naturally accumulating in the intestine. The fact that sfGFP signal is detected in a concentrated manner (Fig. 7) suggests prolonged residence time of the protein in the gut environment. However, several questions remain to be addressed: are the EVs degraded, and, if so, is the protein cargo released to the gut environment? Can the EVs be internalized integrally by gut cells? Can EVs even cross the gut-blood barrier and reach the blood stream? Previous work using unicellular microalgae expressing GFP also showed accumulation of fluorescence signal in zebrafish intestinal tissues (Kwon *et al.*, 2019). The authors hypothesized that the microalgal cell wall is broken down in the hind gut by cellulolytic commensal bacteria, and, consequently, the recombinant protein is released and absorbed by intestinal cells (Kwon *et al.*, 2019). In the case of *Synechocystis* EVs, this may also occur. However, it is also possible that these nano-sized structures are internalized by gut cells, releasing their cargo therein. In fact, several cellular entry pathways of bacterial EVs have already been described in eukaryotic hosts (O'Donoghue and Krachler, 2016). In this context, if the goal is to promote the absorption of a protein of interest, cellular uptake of EVs may be an advantage over the use of whole-cell microbes. For other biotechnological applications, however, the release of protein cargo from EVs may not even be required. If the protein is an enzyme, it could benefit its activity to be shielded inside the vesicle against proteolytic activity (Alves *et al.*, 2016). In this case, the enzyme substrate could cross the membrane of the vesicle, enabling the reaction to ensue (Billar *et al.*, 2022a). Future work should address these questions in order to explore the full potential of

Synechocystis EVs as effective carrier systems for different types of applications in fish.

In summary, this work presents an innovative protein carrier system to address several challenges in the fish production sector. Whether one envisions fine-tuning metabolic functions in fish towards an improved nutritional status, or modulating its immune system to generate specific immune responses against selected antigens, stimulating protection against a given pathogenic agent, customized cyanobacterial EVs have the prospect of becoming serious nanocarriers for the targeted delivery of specific proteins to fish. Future studies will clarify how cyanobacterial EVs can be best administered to fish (e.g. immersion, oral or injection) without compromising delivery efficacy and maximizing protein/enzyme activity.

Experimental procedures

Cyanobacterial strains and routine cultivation conditions

The cyanobacterium *Synechocystis* sp. PCC6803 wild-type (sub-strain GT-Kazusa; glucose tolerant, with S-layer and non-motile) and mutant strains *tolC* (Oliveira *et al.*, 2016), and *tolC-spy*, *fucS* and EV-trc-GFP (this work) (list of strains is available in Table S7) were maintained in liquid BG11 medium (Rippka *et al.*, 1979) in 100 ml Erlenmeyer flasks, kept on an orbital shaker (100 r.p.m.), under a 16 h light (OSRAM Lumilux, 18W/865, Cool daylight) (30–40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)/8 h dark regimen, at 28°C. In the case of the mutant strains, medium was supplemented with antibiotics: 100 $\mu\text{g kanamycin ml}^{-1}$ for *tolC*, *fucS* and EV-trc-GFP; and 50 $\mu\text{g kanamycin ml}^{-1}$, and 2 $\mu\text{g spectinomycin ml}^{-1}$ and 2 $\mu\text{g streptomycin ml}^{-1}$ for the double mutant *tolC-spy*. Growth was monitored spectrophotometrically by analysing optical density at 730 nm (OD_{730}). Routinely, cyanobacterial strains were streaked on solid BG11 medium plates to assess the axenic state of the cultures.

Generation of Synechocystis deletion mutant strains $\Delta\text{tolC}/\Delta\text{spy}$ and ΔfucS

To generate the deletion mutant strains used in this work, the *Synechocystis* natural transformation capability and gene replacement by double homologous recombination were used as previously described (Heidorn *et al.*, 2011). Briefly, two DNA fragments of approximately 600 bp, in the vicinity of both the *spy* gene (*sl10858*) and the *fucS* gene (*sl1213*) were amplified by PCR using specific oligonucleotides (see Table S8). For both cases, the DNA fragment closer to the 5' region of the gene of interest was digested with the restriction enzymes XhoI and PstI, while the fragment closer to the 3' region of

the gene of interest was digested with PstI and BamHI. All the restriction sites were included in the sequence of the oligonucleotides (Table S8). The two DNA fragments for each gene deletion were then ligated simultaneously to the plasmid pSKII + (Stratagene), previously digested with XhoI and BamHI, rendering plasmids pSpy and pFucS, for deletion of the *spy* and *fucS* genes respectively. The identity of the fragments was determined by Sanger sequencing (StabVida). Then, DNA fragments containing the determinants for conferring antibiotic resistance, either for spectinomycin/streptomycin or for kanamycin, were amplified by PCR with specific oligonucleotides, containing the recognition site for the restriction enzyme PstI. While the spectinomycin/streptomycin resistance cassette was used to replace the nucleotide sequence of the *spy* gene between positions 127 and 549 relative to the first nucleotide of the gene, the kanamycin resistance cassette was used to replace the nucleotide sequence of the *fucS* gene between positions 131 and 678. Thus, plasmids pSpy and pFucS were linearized with PstI, and the antibiotic resistance cassettes previously digested with PstI were ligated to the respective plasmids, rendering plasmids pSpy-SpSm and pFucS-Km respectively.

Synechocystis wild type and *tolC* mutant were then cultivated up to an OD₇₃₀ of about 0.5, and naturally transformed with plasmids pFucS-Km and pSpy-SpSm, respectively, as described elsewhere (Pinto *et al.*, 2015). Fully segregated mutants were selected by increasing concentration of the respective antibiotic, and segregation was assessed by PCR using specific oligonucleotides.

Engineering of *Synechocystis* heterologous protein expression and targeting to EVs

To construct the *Synechocystis* strain expressing the heterologous protein sfGFP and further targeting it to extracellular vesicles (Lima *et al.*, 2022), the synthetic promoter P_{trc.x.lacO} (Ferreira *et al.*, 2018) the signal peptide sequence of the *Synechocystis* native protein FutA2 (amino acids 1 to 35) as previously used by Polyviou *et al.* (2018), which determines its translocation to the periplasm, and the gene encoding the reporter protein super-folder Green Fluorescent Protein (sfGFP) were amplified by PCR using specific oligonucleotides (Table S8). Assembly of the different DNA fragments was performed by sequential overlap-extension PCR. The 953 bp fragment was digested with PstI and XbaI, and ligated either to plasmid pSN15KPO or to plasmid pSN15S previously digested with the same restriction enzymes, rendering plasmids pEV-trc-GFP and pEV-trc-GFPS. Identity of the fragment in each plasmid was determined by Sanger sequencing. Plasmids pSN15KPO

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and pSN15S are variants of plasmid pSN15K (Pinto *et al.*, 2015), in which the original kanamycin resistance cassette was replaced by that present in plasmid pUC4K (GE Healthcare), or by the spectinomycin/streptomycin resistance cassette from plasmid pRL278 (Cai and Wolk, 1990) respectively. *Synechocystis* wild-type cells were naturally transformed with pEV-trc-GFP, and fully segregated mutants were obtained, while *Synechocystis tolC* mutant cells were transformed with pEV-trc-GFPS, but without resulting in transformed colonies.

Isolation of *Synechocystis* lipopolysaccharides and extracellular vesicles

To isolate cyanobacterial LPS and EVs, cells from *Synechocystis* wild-type and mutant strains *tolC*, *tolC/spy*, *fucS* and EV-trc-GFP were cultivated in glass gas washing bottles with aeration (1L air min⁻¹), under a 16h light (30–40 μmol photons m⁻² s⁻¹)/8 h dark regime, at 28°C. Cultures were grown until an OD₇₃₀ of approximately 1.5. LPS were isolated by the hot phenol-water method as previously described (Rezania *et al.*, 2011), recovering LPS from the aqueous phase (LPS in the organic phase were discarded, as it was difficult to remove contaminating phenol even after several rounds of dialysis), and storing them at –20°C until further analysis. Regarding EVs' isolation, *Synechocystis* cultures were centrifuged at 4400 g for 10 min at room temperature to separate cells from the extracellular medium. A total volume of 3.6 l of extracellular medium, unless stated otherwise, was filtered through 0.45 μm pore size filters, and further concentrated by ultrafiltration using centrifugal filters with a molecular weight cut-off of 100 kDa (Millipore, Burlington, MA, USA). A final volume of approximately 15 ml of concentrated extracellular medium was then centrifuged at 100 000 g for 3 h, at 4°C, as previously described (Biller *et al.*, 2022b). The resulting EVs pellets were then suspended in sterile BG11 medium and stored at –80°C until further analysis.

For both isolated LPS and isolated EVs, the amount and band profile of LPS were routinely analysed. LPS and EVs samples were prepared as described (Oliveira *et al.*, 2016) and further separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels. After separation, LPS were detected by the Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies, Carlsbad, CA, USA), and visualized by UV-light radiation on a GelDoc™ XR+ system (Bio-Rad, Hercules, CA, USA). Quantitation of LPS was determined by densitometry analysis using the ImageJ software (Abramoff *et al.*, 2004). In brief, images of stained gels were processed by quantifying pixel intensity values of all bands detected in a given lane as previously described (Peterson,

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2010), and LPS amount was determined against a calibration curve established with *P. aeruginosa* LPS (Sigma-Aldrich, Burlington, MA, USA) as standard, whose samples were separated in the same gel.

Characterization of isolated Synechocystis extracellular vesicles

To characterize *Synechocystis* EVs, various methods were used to complement the SDS-PAGE analysis described above, namely by determining their amount and size (by nanoparticle tracking analysis, and TEM), and their morphological aspect (by TEM). Thus, EVs samples from *Synechocystis* wild type and mutant strains were analysed on a NanoSight NS300 (Malvern, Malvern, UK), equipped with an sCMOS camera. EVs samples were diluted in filtered PBS solution (between 1:1000 and 1:10 000), and further analysed as described (Oliveira *et al.*, 2016). In brief, five movies of 30 s were recorded for each sample (with a threshold of 10–50 particles per frame), with the following capturing settings: camera level 14, slider shutter 800–1300, slider gain 350–500, at 25°C and with a syringe pump speed of 40. Data acquisition and processing were performed using the NanoSight NS300 NTA 3.0 software (Malvern), using a detection threshold of 5.

Furthermore, for negative staining transmission electron microscopy, 10 µl of EVs preparations from the various cyanobacterial strains were mounted on formvar/carbon film coated mesh nickel grids (EMS, Hatfield, PA, USA) and left standing for 2 min. The liquid in excess was removed with filter paper, and 10 µl of 1% uranyl acetate was added on to the grids and left standing for 10 s, after which liquid in excess was removed with filter paper. Visualization was carried out on a Jeol JEM-1400 transmission electron microscope at 80 kV. Vesicle size was determined by direct measurement from electron micrographs using the open source software ImageJ (Abramoff *et al.*, 2004). Several micrographs obtained from three biological replicates were used, and the diameter of more than 300 (WT), 450 ($\Delta tolC$), 550 ($\Delta tolC/\Delta spy$), 350 ($\Delta fucS$) and 370 (EV-trc-GFP) particles was measured.

For immunogold labelling of sfGFP-loaded EVs, vesicles were isolated as described above. EVs were fixed in 2% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde prepared in 0.1 M sodium cacodylate buffer for 2 h, and then washed twice in the same buffer, without the fixing agents (30 min per incubation step). Later, the EVs sample was dehydrated according to the following scheme: 15 min in 50% (v/v) ethanol; twice in 70% (v/v) ethanol (30 min each incubation); twice in 100% (v/v) ethanol (30 min each incubation). For resin infiltration, the EVs sample was treated for 1 h with a mix of LR

White resin and 100% (v/v) ethanol in a proportion of 2:1, twice for 1 h with LR White resin, once overnight in fresh LR White resin, and, finally, 1 h in fresh LR White resin. Resin polymerization was achieved by incubating samples at 60°C for 24 h. Sections were then washed in Tris-buffered saline solution (TBS: 50 mM of Tris-HCl, 150 mM of NaCl, pH 7.6) for 10 min, and incubated in 14.4% (w/v) sodium metaperiodate (antigen retrieval) for 1 h. Later, sections were washed in TBS for 10 min and in 20 mM of glycine for 5 min, and incubated with blocking solution (2% (w/v) BSA in TBS) for 30 min, before being incubated overnight, at 4°C, with a GFP specific monoclonal mouse antibody (Roche), diluted 1:100 in blocking solution supplemented with 3% (w/v) NaCl. Samples were washed three times in 0.1% (w/v) BSA in TBS (4 min each wash), blocked for 20 min in blocking solution and incubated with a goat anti-mouse IgG (H + L) conjugated with gold nanoparticles (15 nm) (BBI International) for 1 h. Then, samples were washed four times in TBS (2 min each), treated with 1% (w/v) glutaraldehyde in TBS for 5 min, washed again 6 times with distilled water (10 min each wash) and finally contrasted with uranyl acetate (5 min) and lead citrate (5 min). Visualization was carried out on a Jeol JEM-1400 transmission electron microscope at 80 kV.

Confocal microscopy analysis of Synechocystis cells

Synechocystis cells from the mutant strain EV-trc-GFP, and its derived EVs, were visualized on a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). EV-trc-GFP cells were cultivated in glass gas washing bottles as described above to an OD₇₃₀ of 1.5. Ten micro litres of cell suspension or 2 µl of EVs suspension were laid onto glass slides covered with solid BG11 medium supplemented with 1% low-melting agarose, and later visualized. GFP emission (collected between 500 and 540 nm) was observed when samples were exposed to a laser beam at 488 nm, while cyanobacterial cell autofluorescence was collected between 610 and 680 nm after excitation at 488 nm.

Protein extraction and analysis

To obtain total protein extracts, *Synechocystis* wild type or EV-trc-GFP cells were cultivated to an OD₇₃₀ of approximately 1.5, collected by centrifugation (4400 g, 10 min, at room temperature), suspended in protein extraction buffer (Lopes Pinto *et al.*, 2011) and sonicated as described (Gonçalves *et al.*, 2018). Cell debris was collected by centrifugation (10 000 g, 10 min, 4°C), and the cell free, total protein extract was stored at –20°C until further analysis. Total protein quantification was determined using the Bradford assay (BioRad). A total

volume of 600 ml of extracellular medium recovered after culture centrifugation was filtered through 0.45 µm pore size filters to remove any contaminating cells, and EVs were isolated as described above. Soluble extracellular proteins were recovered by concentrating the EV-free extracellular medium by ultrafiltration using centrifugal filters with a molecular weight cut-off of 3 kDa (Millipore). Concentrated samples were stored at -20°C until further analysis.

For protein analyses, samples were separated by standard electrophoresis on SDS-polyacrylamide gels, and gels were stained with Coomassie brilliant blue G (Sigma-Aldrich) for visualization of the peptide profile. For Western blotting, after electrophoretic separation, proteins were transferred from the gels to nitrocellulose membrane filters. Membranes were blocked for 3 h with 5% (w/v) milk powder in TBS-T [TBS with 0.05% (v/v) Tween 20] and then incubated overnight at 4°C with GFP-specific monoclonal mouse antibody (Roche, Basel, Switzerland) in fresh blocking buffer. After washing with TBS-T, blots were incubated for 1 h with goat anti-mouse IgG (Invitrogen, Waltham, MA, USA) linked to horseradish peroxidase. Membranes were washed with TBS-T before immunodetection, which was performed by chemiluminescence using ECL Western Blotting Analysis System detection reagents. Quantitation of relative band intensity was determined by densitometry analysis, using the ImageJ software as previously described (Peterson, 2010).

Ethics statement

Animal experiments were approved by the Animal Welfare and Ethics Body (ORBEA) committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR). This study was directed by accredited scientists (following FELASA category C recommendations) and all animal procedures were conducted in compliance with the guidelines provided by the International Guiding Principles for Research Involving Animals (EU 2010/63).

Zebrafish husbandry and breeding

Wild-type zebrafish (*Danio rerio*) were maintained in a recirculating water system under standard husbandry conditions (water temperature of $28 \pm 1^\circ\text{C}$; oxygen between $7.5\text{--}8\text{ mg l}^{-1}$; ammonia and nitrite levels kept around 0 mg l^{-1} ; and photoperiod of 14 h light/10 h dark). Fish were fed TetraMin tropical flakes (Tetra) twice a day. Adult fish were maintained at sex ratio of two males to one female, and group in-tank breeding was achieved with the addition of artificial nests to aquaria on the day prior to embryo collection. Embryos were collected 1 h after light onset, cleaned, separated from

unfertilized eggs and incubated in sterile Petri dishes with zebrafish embryo medium [egg water: 60 mg l^{-1} of Sea Salt (Instant Ocean, Blacksburg, VA, USA) in deionized water], at pH 7.2, containing 0.38 mg l^{-1} methylene blue (Sigma-Aldrich) at 28°C .

For confocal microscopy analyses, fish were fed with SPAROS thrice a day. Adult fish were maintained in breeding cages at sex ratio two females to one male on the night prior to embryo collection. Next morning, upon light onset, the divider was removed, and fish started to reproduce. Breeding tanks are equipped with a net at the bottom, so the eggs can fall through the net, avoiding to be eaten by adult fish. Eggs were collected, cleaned and incubated at 28°C in embryo medium (E3 – NaCl, KCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), with 0.2 mM of 1-phenyl-2-thiourea to avoid pigmentation development.

Zebrafish challenge with Synechocystis LPS and EVs

The effects of LPS and EVs from *Synechocystis* wild-type and mutant strains *tolC*, *tolC/spy*, *fucS* on the survival of zebrafish larvae with 3 days post-fertilization were assessed by immersion trials performed in 6-well flat-bottom plates (Sarstedt, Nümbrecht, Germany) incubated at 28°C , in a final volume of 6 ml, for 5 days. The LPS or EVs of each *Synechocystis* strain were tested at 250 and $500\text{ }\mu\text{g LPS ml}^{-1}$ in egg water, against a zebrafish embryo medium control treatment and a positive control treatment of $45\text{ }\mu\text{g ml}^{-1}$ of commercial *Pseudomonas aeruginosa* LPS (Sigma) in zebrafish embryo medium. Treatments were tested in triplicates of 12 larvae, and each experiment was performed twice (two biological replicates), making a total of 72 larvae analysed within each experimental condition.

To assess how EVs from *Synechocystis* mutant strains *tolC* and *fucS* affect gene expression of inflammation associated markers, 3 dpf zebrafish larvae were subject to 250 and $500\text{ }\mu\text{g LPS ml}^{-1}$ of EVs from each strain, against a zebrafish embryo medium control and $35\text{ }\mu\text{g ml}^{-1}$ of commercial *P. aeruginosa* LPS (sub-lethal concentration) in zebrafish embryo medium, used as positive control. All treatments were tested six times, in 6-well flat-bottom plates, with 20 larvae and 10 ml of solution per well. At the end of the trials, larvae from each well were pooled and stored in RNeasy lysis buffer for gene expression analysis.

RNA extraction, cDNA synthesis and Gene expression analysis

Total RNA was extracted from 20 larvae pools, using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), according to manufacturer's

recommendations. Prior to extraction, larvae pools were homogenized with 1 ml of TRIzol reagent using 1.4 mm ceramic beads, and three cycles of 10 s at 7200 rpm in a Precellys Evolution homogenizer (Bertin Technologies, Montigny-le Bretonneux, France). RNA was eluted in 50 μ l of DEPC-treated water, its integrity confirmed by agarose gel electrophoresis and its quality measured with a Multiskan GO Spectrophotometer equipped with a μ Drop™ Plate (Thermo Fisher Scientific, Waltham, MA, USA).

cDNA was generated from 1 μ g of total RNA using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal), and stored at -20°C until further analysis.

Real-time PCR was performed with a CFX Connect Real-Time PCR system (BioRad) using SsoAdvanced Universal SYBR Green Supermix (BioRad), according to the manufacturer's protocol. PCR reactions were set up using 0.5 μ l of 10 μ M of each primer (Table S8) and 0.5 μ l of 2-fold diluted cDNA in a reaction volume of 10 μ l. Reactions specificity was verified using melting curve analysis and absence of primer dimer. Standard curves were obtained for each cDNA template by plotting C_t values against the $\log_{10}$ of six different dilutions of a cDNA mix solution from all samples analysed. Real-time PCR efficiency (E) was calculated from a standard curve according to the equation $E = 10(-1/\text{slope})$. Rpl13a was selected as the endogenous control gene (Table S8) and gene expression of the control group was used as calibrator.

Statistical analysis

Survival curves were plotted using the Kaplan–Meier method and pairwise comparisons between treatments were performed with nonparametric log-rank test, at 0.05 significance level, in GraphPad Prism version 9 (GraphPad Software Inc., San Diego, CA, USA). Gene expression data was checked for normal distribution and homogeneity of variances and normalized when appropriate. When found, extreme outliers, that is data values which lie more than three times the interquartile range below the first quartile or above the third quartile, were removed from the analysis. Treatments effects were analysed by one-way ANOVA and significant differences among means ($P < 0.05$) were determined by the Tukey's multiple range test, using SPSS for Windows version 26 software package (IBM SPSS Statistics, Armonk, NY, USA).

Confocal microscopy analysis of zebrafish larvae treated with sfGFP-loaded *Synechocystis* EVs

To study the possibility of carrying sfGFP to zebrafish via *Synechocystis* EVs, 5 dpf zebrafish larvae were

subjected to immersion trials performed in 24-well flat-bottom plates (Sarstedt) incubated at 28°C , in a final volume of 2 ml, for 24 h. Three zebrafish larvae were treated with 0 (control) or 1000 $\mu\text{g ml}^{-1}$ of sfGFP-loaded EVs in zebrafish embryo medium. Each experiment was performed three times. After the incubation period, zebrafish larvae were euthanized by subjecting the larvae to a lethal dose of 300 mg l^{-1} of tricaine methanesulfonate (MS222), immediately mounted on a glass slide, with 50% glycerol in PBS solution as mounting medium, and visualized by confocal microscopy (Leica TCS SP5 II). The sfGFP signal was collected between 495 and 540 nm upon exposing the specimens to a laser beam of 488 nm, using the same acquisition settings for all tested conditions. In addition, differential interference contrast (DIC) images were also acquired.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Survival percentages of 3 days post-fertilization (dpf) zebrafish larvae subjected to zebrafish embryo medium containing 250 or 500 $\mu\text{g mL}^{-1}$ of LPS or EVs from *Synechocystis* sp. PCC 6803 wild-type (WT), and mutant strains ΔtolC , $\Delta\text{tolC}/\Delta\text{spy}$ and ΔfucS . Survival was monitored for 5 days (120 h) of immersion challenge. Control treatments received zebrafish embryo medium only (CTR) or zebrafish embryo medium containing commercial LPS from *Pseudomonas aeruginosa* (LPS Pa) at 45 $\mu\text{g mL}^{-1}$.

Table S2. *P*-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 wild-type extracellular vesicles (EVs WT), LPS, and *Pseudomonas aeruginosa* LPS (LPS Pa), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

Table S3. *P*-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 ΔtolC mutant extracellular vesicles (EVs), LPS, and *Pseudomonas aeruginosa* LPS (LPS Pa), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

Table S4. *P*-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 $\Delta\text{tolC}/\Delta\text{spy}$ mutant extracellular vesicles (EVs), LPS, and *Pseudomonas aeruginosa* LPS (LPS Pa), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

Table S5. *P*-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 ΔfucS mutant extracellular vesicles (EVs), LPS, and *Pseudomonas aeruginosa* LPS (LPS Pa), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

Table S6. Results of *post hoc* Tukey's tests after one-way ANOVA of quantitative gene expression of the genes encoding Interleukin 1 beta (IL-1 β), Tumor necrosis factor alpha (TNF- α), and Interleukin 10 (IL-10) in zebrafish larvae immersed in zebrafish embryo medium only (CTR – control), zebrafish embryo medium containing 250 or 500 μg LPS mL^{-1} of EVs from *Synechocystis* mutants ΔtolC (EVs ΔtolC)

and $\Delta fucS$ (EVs $\Delta fucS$), or in zebrafish embryo medium containing 35 $\mu\text{g LPS mL}^{-1}$ of *P. aeruginosa* (LPS *Pa*). Data obtained from $n=6$, with each replicate representing a pool of 20 larvae. Statistical differences in gene expression by ANOVA ($p < 0.05$) are represented in bold.

Table S7. List of *Synechocystis* sp. PCC 6803 strains used in this work.

Table S8. Oligonucleotide primers used in this study.

Fig. S1. Generation of the *Synechocystis* sp. PCC 6803 *fucS*- and *tolC*-*spy*-mutant strains. Green-safe stained agarose gels showing the result of a PCR testing the extent of chromosome segregation of *Synechocystis* cells in which the genes *fucS* (left-hand side) or *spy* (right-hand side) were partially deleted in the wild-type or *tolC*-mutant backgrounds, respectively. $\Delta fucS$: lanes 1 and 2 correspond to two different clones; Δspy : lanes 1 and 2 correspond to two different clones. Genomic DNA from the *Synechocystis* wild-type (gWT) or *tolC*-mutant (*gtolC*) strains was used as the PCR positive control, while a reaction in which the DNA template was replaced by water (C) was used as negative control. M, O'GeneRuler DNA ladder mix (Thermo Fisher Scientific). Selected sizes of the marker, and the exact size of the bands resulting from PCR amplification are shown in base pairs (bp).

Fig. S2. The *Synechocystis* sp. PCC 6803 strains used in this strain show different vesiculation capacities. The *Synechocystis* wild-type (WT), and the *tolC*- ($\Delta tolC$), *tolC*-*spy*- ($\Delta tolC \Delta spy$) and *fucS*- ($\Delta fucS$) mutant strains were cultivated under standard conditions and their vesicles isolated. Prior to loading, EVs samples were normalized for cell culture density (OD_{730}), volume of cell-free culture medium concentrated and concentration factor. EVs samples were then separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels, and stained with the Pro-Q™

Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies) for the detection of LPS as an outer membrane marker. The gel is representative of 3 independent biological replicates, and the densitometry analysis and quantification resulted in the graph presented in Figure 3A. On the left-hand side, schematic representation of the smooth-LPS (lipid A, oligosaccharide core, and O-antigen), and rough-LPS (lipid A and oligosaccharide core) that are found in *Synechocystis*.

Fig. S3. Characterization of the vesicles released by the *Synechocystis* sp. PCC 6803 strain EV-trc-GFP. A, Transmission electron micrograph representative of the morphological aspect of the vesicles released by EV-trc-GFP strain. Scale bar: 200 nm. B, The *Synechocystis* wild-type (WT) and EV-trc-GFP strains were cultivated under standard conditions and their EVs isolated. Prior to loading, samples were normalized for cell culture density (OD_{730}), volume of cell-free culture medium concentrated and concentration factor. EVs samples were then separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels, and stained with the Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies) for the detection of LPS. The gel is representative of 5 independent biological replicates, and the densitometry analysis and quantification is shown on to the right. ***, $p < 0.001$. C, Histogram showing particle size distribution of EVs samples from the EV-trc-GFP strain. EVs were separated in 5 nm size groups, distributed between 30 and 130 nm, and in groups comprising those with a size below 30 nm or higher than 130 nm. Vesicle diameter was determined by direct measurement from electron micrographs of three independent biological replicates. Abundance of each size group is presented as relative figures, i.e. number of vesicles with a given diameter range relative to the total number of vesicles measured.

SUPPLEMENTARY MATERIAL FOR

Novel protein carrier system based on cyanobacterial nano-sized extracellular vesicles for application in fish

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SUPPLEMENTARY TABLES

Supplementary Table S1. Survival percentages of 3 days post-fertilization (dpf) zebrafish larvae subjected to zebrafish embryo medium containing 250 or 500 $\mu\text{g mL}^{-1}$ of LPS or EVs from *Synechocystis* sp. PCC 6803 wild-type (WT), and mutant strains ΔtolC , $\Delta\text{tolC}/\Delta\text{spy}$ and ΔfucS . Survival was monitored for 5 days (120 h) of immersion challenge. Control treatments received zebrafish embryo medium only (CTR) or zebrafish embryo medium containing commercial LPS from *Pseudomonas aeruginosa* (LPS Pa) at 45 $\mu\text{g mL}^{-1}$.

	Time (hours)	CTR	EVs 250	EVs 500	LPS 250	LPS 500	LPS Pa 45
WT	0	100.0	100.0	100.0	100.0	100.0	100.0
	2	100.0	100.0	100.0	100.0	100.0	100.0
	4	100.0	100.0	100.0	100.0	100.0	100.0
	8	100.0	100.0	100.0	100.0	100.0	51.4
	11	100.0	100.0	100.0	100.0	100.0	33.3
	24	100.0	100.0	100.0	100.0	100.0	22.2
	31	100.0	100.0	100.0	100.0	100.0	20.8
	48	100.0	100.0	100.0	100.0	100.0	20.8
	55	100.0	100.0	100.0	100.0	100.0	20.8
	72	100.0	100.0	100.0	100.0	100.0	20.8
	79	100.0	100.0	100.0	100.0	100.0	20.8
	96	100.0	100.0	100.0	100.0	100.0	19.4
	103	100.0	100.0	100.0	100.0	100.0	19.4
	120	100.0	100.0	98.6	100.0	100.0	19.4
ΔtolC	0	100.0	100.0	100.0	0.0	100.0	100.0
	2	100.0	100.0	100.0	0.0	100.0	100.0
	4	100.0	100.0	100.0	0.0	100.0	9.7
	8	100.0	100.0	100.0	0.0	100.0	0.0
	11	100.0	100.0	100.0	0.0	100.0	0.0
	24	100.0	100.0	100.0	0.0	100.0	0.0
	31	100.0	100.0	100.0	0.0	100.0	0.0
	48	100.0	100.0	100.0	0.0	100.0	0.0
	55	100.0	100.0	100.0	0.0	100.0	0.0
	72	100.0	100.0	100.0	0.0	97.9	0.0
	79	100.0	100.0	100.0	0.0	97.9	0.0
	96	100.0	100.0	100.0	0.0	97.9	0.0
	103	100.0	100.0	100.0	0.0	97.9	0.0
	120	100.0	100.0	100.0	0.0	85.4	0.0

Supplementary Table S1 (continued). Survival percentages of 3 days post-fertilization (dpf) zebrafish larvae subjected to zebrafish embryo medium containing 250 or 500 $\mu\text{g mL}^{-1}$ of LPS or EVs from *Synechocystis* sp. PCC 6803 wild-type (WT), and mutant strains ΔtolC , $\Delta\text{tolC}/\Delta\text{spy}$ and ΔfucS . Survival was monitored for 5 days (120 h) of immersion challenge. Control treatments received zebrafish embryo medium only (CTR) or zebrafish embryo medium containing commercial LPS from *Pseudomonas aeruginosa* (LPS Pa) at 45 $\mu\text{g mL}^{-1}$.

	Time (hours)	CTR	EVs 250	EVs 500	LPS 250	LPS 500	LPS Pa 45
ΔfucS	0	100.0	100.0	100.0	100.0	100.0	100.0
	2	100.0	100.0	100.0	100.0	100.0	100.0
	4	100.0	100.0	100.0	100.0	100.0	98.6
	8	100.0	100.0	100.0	100.0	100.0	38.9
	11	100.0	100.0	100.0	100.0	100.0	15.3
	24	100.0	100.0	100.0	100.0	100.0	6.9
	31	100.0	100.0	100.0	100.0	100.0	6.9
	48	100.0	100.0	100.0	100.0	100.0	6.9
	55	100.0	100.0	100.0	100.0	100.0	6.9
	72	100.0	100.0	100.0	100.0	100.0	6.9
	79	100.0	100.0	100.0	100.0	100.0	6.9
	96	100.0	100.0	100.0	100.0	100.0	6.9
	103	100.0	100.0	100.0	100.0	100.0	6.9
	120	100.0	100.0	100.0	100.0	100.0	6.9
ΔtolC Δspy	0	100.0	100.0	100.0	100.0	100.0	100.0
	2	100.0	100.0	100.0	100.0	100.0	100.0
	4	100.0	100.0	100.0	100.0	100.0	87.5
	8	100.0	100.0	100.0	100.0	100.0	19.4
	11	100.0	100.0	100.0	100.0	100.0	8.3
	24	100.0	100.0	100.0	100.0	98.6	5.6
	31	100.0	100.0	100.0	100.0	98.6	5.6
	48	100.0	100.0	100.0	100.0	98.6	5.6
	55	100.0	97.2	100.0	95.8	93.1	5.6
	72	100.0	97.2	100.0	91.7	70.8	5.6
	79	100.0	97.2	100.0	91.7	59.7	5.6
	96	100.0	97.2	100.0	91.7	5.6	5.6
	103	100.0	97.2	100.0	90.3	1.4	5.6
	120	97.2	97.2	100.0	51.4	0.0	5.6

Supplementary Table S2. P-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 wild-type extracellular vesicles (EVs WT), LPS, and *Pseudomonas aeruginosa* LPS (LPS *Pa*), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

WT	Control	EVs 250	EVs 500	LPS 250	LPS 500
Control	-	-	-	-	-
EVs 250	>0.9999	-	-	-	-
EVs 500	0.3173	0.3173	-	-	-
LPS 250	>0.9999	>0.9999	0.3173	-	-
LPS 500	>0.9999	>0.9999	0.3173	>0.9999	-
LPS <i>Pa</i> 45	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Supplementary Table S3. P-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 ΔtolC mutant extracellular vesicles (EVs), LPS, and *Pseudomonas aeruginosa* LPS (LPS *Pa*), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

ΔtolC	Control	EVs 250	EVs 500	LPS 500
Control	-	-	-	-
EVs 250	0.1558	-	-	-
EVs 500	0.1558	>0.9999	-	-
LPS 500	0.016	0.0009	0.0009	-
LPS <i>Pa</i> 45	<0.0001	<0.0001	<0.0001	<0.0001

Supplementary Table S4. P-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 $\Delta tolC/\Delta spy$ mutant extracellular vesicles (EVs), LPS, and *Pseudomonas aeruginosa* LPS (LPS *Pa*), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

$\Delta tolC/\Delta spy$	Control	EVs 250	EVs 500	LPS 250	LPS 500
Control	-	-	-	-	-
EVs 250	0.9887	-	-	-	-
EVs 500	0.1558	0.1558	-	-	-
LPS 250	<0.0001	<0.0001	<0.0001	-	-
LPS 500	<0.0001	<0.0001	<0.0001	<0.0001	-
LPS <i>Pa</i> 45	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Supplementary Table S5. P-values for pairwise comparisons with the log-rank test between the survival curves of zebrafish larvae subjected to *Synechocystis* sp. PCC 6803 $\Delta fucS$ mutant extracellular vesicles (EVs), LPS, and *Pseudomonas aeruginosa* LPS (LPS *Pa*), at the indicated concentrations ($\mu\text{g mL}^{-1}$). Control refers to larvae exposed to Egg water only.

$\Delta fucS$	Control	EVs 250	EVs 500	LPS 250	LPS 500
Control	-	-	-	-	-
EVs 250	>0.9999	-	-	-	-
EVs 500	>0.9999	>0.9999	-	-	-
LPS 250	>0.9999	>0.9999	>0.9999	-	-
LPS 500	>0.9999	>0.9999	>0.9999	>0.9999	-
LPS <i>Pa</i> 45	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Supplementary Table S6. Results of *post hoc* Tukey's tests after one-way ANOVA of quantitative gene expression of the genes encoding Interleukin 1 beta (IL-1 β), Tumor necrosis factor alpha (TNF- α), and Interleukin 10 (IL-10) in zebrafish larvae immersed in zebrafish embryo medium only (CTR – control), zebrafish embryo medium containing 250 or 500 μg LPS mL^{-1} of EVs from *Synechocystis* mutants ΔtolC (EVs ΔtolC) and ΔfucS (EVs ΔfucS), or in zebrafish embryo medium containing 35 μg LPS mL^{-1} of *P. aeruginosa* (LPS *Pa*). Data obtained from $n=6$, with each replicate representing a pool of 20 larvae. Statistical differences in gene expression by ANOVA ($p < 0.05$) are represented in bold.

		CTR	LPS Pa 35	ΔtolC EVs 250	ΔtolC EVs 500	ΔfucS EVs 250	ΔfucS EVs 500
IL-1β							
ANOVA	Tukey						
0.017	CTR	-	-	-	-	-	-
	LPS Pa 35	0.037	-	-	-	-	-
	ΔtolC EVs 250	1.000	0.038	-	-	-	-
	ΔtolC EVs 500	1.000	0.041	1.000	-	-	-
	ΔfucS EVs 250	1.000	0.047	1.000	1.000	-	-
	ΔfucS EVs 500	1.000	0.040	1.000	1.000	1.000	-
TNF-α							
ANOVA	Tukey						
0.011	CTR	-	-	-	-	-	-
	LPS Pa 35	0.057	-	-	-	-	-
	ΔtolC EVs 250	0.998	0.030	-	-	-	-
	ΔtolC EVs 500	0.952	0.367	0.816	-	-	-
	ΔfucS EVs 250	1.000	0.050	0.999	0.939	-	-
	ΔfucS EVs 500	0.957	0.011	0.999	0.585	0.967	-
IL-10							
ANOVA	Tukey						
0.018	CTR	-	-	-	-	-	-
	LPS Pa 35	0.014	-	-	-	-	-
	ΔtolC EVs 250	0.996	0.046	-	-	-	-
	ΔtolC EVs 500	0.931	0.164	0.997	-	-	-
	ΔfucS EVs 250	0.998	0.037	1.000	0.993	-	-
	ΔfucS EVs 500	0.788	0.298	0.964	0.999	0.943	-

Supplementary Table S7. List of *Synechocystis* sp. PCC 6803 strains used in this work.

Strains	Antibiotic resistance	Deleted gene function	Characteristics	Plasmid used for mutant strain generation	Reference
<i>Synechocystis</i> sp. PCC 6803	-	-	Wild-type strain	-	GT-Kazusa
<i>Synechocystis</i> $\Delta toIC$	Km ¹	<i>toIC</i> : encodes an outer membrane protein involved in secretory functions	Without S-layer; hypervesiculating strain	pBSMN1K	Oliveira <i>et al.</i> , 2016
<i>Synechocystis</i> $\Delta toIC \Delta spy$	Km; Sp/Sm ²	<i>toIC</i> : see above; <i>spy</i> : encodes a periplasmic protein with a role in protein folding	Deletion of <i>spy</i> carried out in the <i>Synechocystis</i> $\Delta toIC$ background; hypervesiculating strain	pSpy-SpSm	This work
<i>Synechocystis</i> $\Delta fucS$	Km	<i>fucS</i> : encodes a fucose synthetase; fucose is a structural sugar of <i>Synechocystis</i> LPS	LPS with truncated O-antigen moieties; hypervesiculating strain	pFucS-Km	This work
<i>Synechocystis</i> EV-trc-sfGFP	Km	-	Expresses sfGFP and targets it to the periplasm; produces vesicles packaged with sfGFP	pEV-trc-GFP	This work

¹ Km – kanamycin, ² Sp/Sm – spectinomycin/ streptomycin

Supplementary Table S8. Oligonucleotide primers used in this study

Target	Primer Name	Primer Sequence	Size (bp)	Reference
<i>fucS</i>	sII1213_5F	AA ACTCGAG CCAAGGACTATGTACGGGCC	681	This work
	sII1213_5R	AA ACTGCAG AGTCTTTGGAGCGGGGAATG		
<i>sII1213</i>	sII1213_3F	AA ACTGCAG GGAACCCAAGCCTACGACAA	708	This work
	sII1213_3R	AAAGGATCCTTTCCCGTCAATGTGGTCC		
<i>spy</i>	sII0858_5F	AA ACTCGAG GTCCTCGATCTGTTGCAACTC	681	This work
	sII0858_5R	AA ACTGCAG TAAACCGCTAAGCAAACCGG		
<i>sII0858</i>	sII0858_3F	AA ACTGCAG CGCAATAGTCGTCAAGGTCG	708	This work
	sII0858_3R	AAAGGATCCAGTGCCGCTCTCAAATTTGG		
IL-1 β	IL-1 β - F	TTCCCCAAGTGCTGCTTATT	149	Saraceni <i>et al.</i> , 2016
	IL-1 β - R	AAGTTAAAACCGCTGTGGTCA		
TNF- α	TNF- α - F	ACCAGGCCTTTTCTTCAGGT	148	Saraceni <i>et al.</i> , 2016
	TNF- α - R	GCATGGCTCATAAGCACTTGTT		
IL-10	IL-10 - F	ATTTGTGGAGGGCTTTCCTT	198	Wang <i>et al.</i> 2015
	IL-10 - R	AGAGCTGTTGGCAGAATGGT		
Rpl13 α	Rpl13 α - F	TCTGGAGGACTGTAAGAGGTATGC	148	Weir <i>et al.</i> 2015
	Rpl13 α - R	AGACGCACAATCTTGAGAGCAG		
<i>P_{trc.x.lacI}</i>	lacOF	AA ACTGCAGA ATTGTGAGCGCTCACAATTTT	145	This work
	lacOR	CGCCGGGAAATCTTAGTTGTCAATTTCTCCTC TTTAATTGTGTGAAAT		
<i>futA2</i> SP	futA2F_ss	ATTTACACACAATTAAGAGGAGAAAAATGACAA CTAAGATTTCCTGGCG	155	This work
	futA2R_ss	TGAACAGCTCTTCGCCTTTACGCATCGTGCGG GACTGGGCA		
<i>sfGFP</i>	sfGFPF	TGCCAGTCCCGCACGATGCGTAAAGGCGAA GAGCT	742	This work
	sfGFPR	AA ACTAGAT CATTTGTACAGTTCATCCATACC		

Highlighted nucleotides correspond to restriction enzymes recognition sites (**bold**: XhoI; underlined: PstI; *italic*: BamHI; **bold and underlined**: XbaI)

SUPPLEMENTARY FIGURES

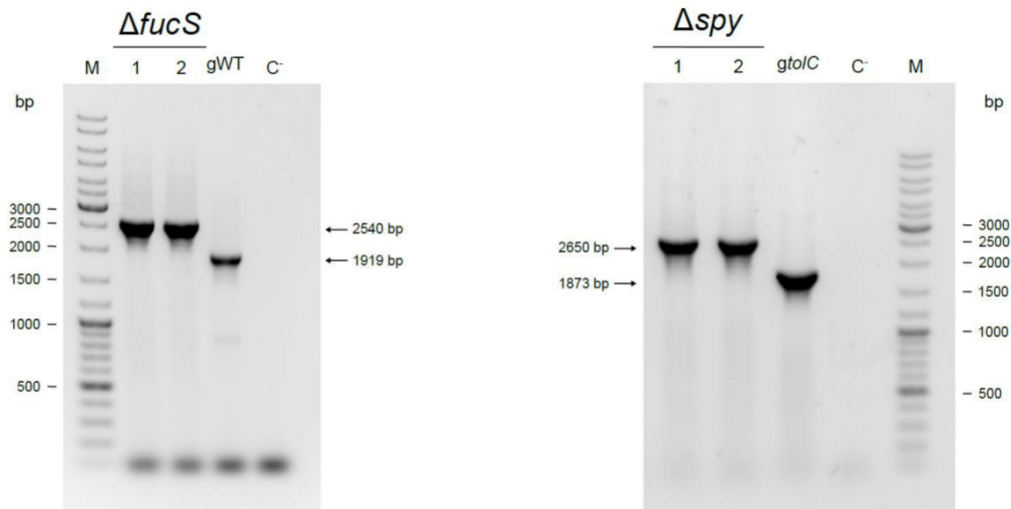


Figure S1. Generation of the *Synechocystis* sp. PCC 6803 *fucS*- and *tolC-spy*-mutant strains. Green-safe stained agarose gels showing the result of a PCR testing the extent of chromosome segregation of *Synechocystis* cells in which the genes *fucS* (left-hand side) or *spy* (right-hand side) were partially deleted in the wild-type or *tolC*-mutant backgrounds, respectively. $\Delta fucS$: lanes 1 and 2 correspond to two different clones; Δspy : lanes 1 and 2 correspond to two different clones. Genomic DNA from the *Synechocystis* wild-type (gWT) or *tolC*-mutant (gto/C) strains was used as the PCR positive control, while a reaction in which the DNA template was replaced by water (C-) was used as negative control. M, O'GeneRuler DNA ladder mix (Thermo Fisher Scientific). Selected sizes of the marker, and the exact size of the bands resulting from PCR amplification are shown in base pairs (bp).

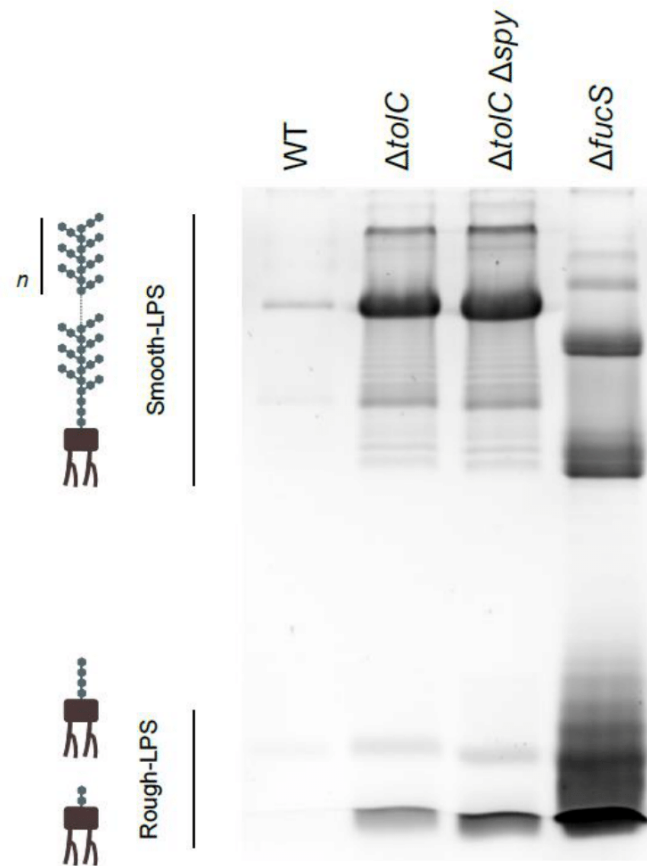


Figure S2. The *Synechocystis* sp. PCC 6803 strains used in this strain show different vesiculation capacities. The *Synechocystis* wild-type (WT), and the *tolC*- ($\Delta tolC$), *tolC-spy*- ($\Delta tolC \Delta spy$) and *fucS*- ($\Delta fucS$) mutant strains were cultivated under standard conditions and their vesicles isolated. Prior to loading, EVs samples were normalized for cell culture density (OD_{730}), volume of cell-free culture medium concentrated and concentration factor. EVs samples were then separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels, and stained with the Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies) for the detection of LPS as an outer membrane marker. The gel is representative of 3 independent biological replicates, and the densitometry analysis and quantification resulted in the graph presented in Figure 3A. On the left-hand side, schematic representation of the smooth-LPS (lipid A, oligosaccharide core, and *O*-antigen), and rough-LPS (lipid A and oligosaccharide core) that are found in *Synechocystis*.

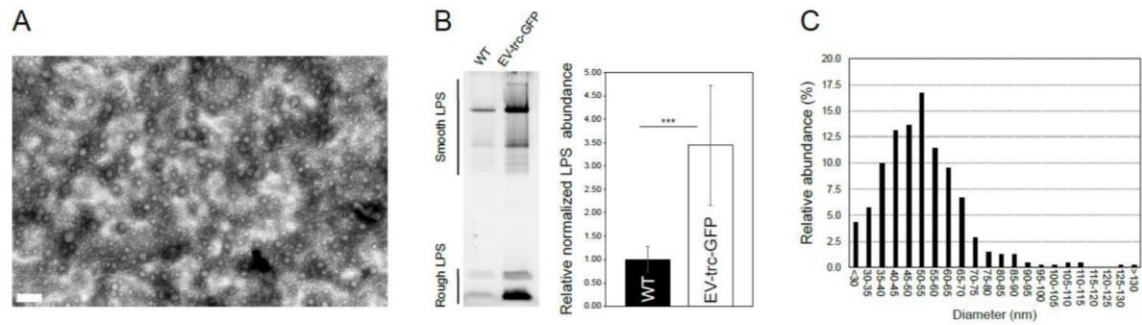


Figure S3. Characterization of the vesicles released by the *Synechocystis* sp. PCC 6803 strain EV-trc-GFP. **A**, Transmission electron micrograph representative of the morphological aspect of the vesicles released by EV-trc-GFP strain. Scale bar: 200 nm. **B**, The *Synechocystis* wild-type (WT) and EV-trc-GFP strains were cultivated under standard conditions and their EVs isolated. Prior to loading, samples were normalized for cell culture density (OD_{730}), volume of cell-free culture medium concentrated and concentration factor. EVs samples were then separated by electrophoresis on 16% (w/v) SDS-polyacrylamide gels, and stained with the Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit (Life Technologies) for the detection of LPS. The gel is representative of 5 independent biological replicates, and the densitometry analysis and quantification is shown on to the right. ***, $p < 0.001$. **C**, Histogram showing particle size distribution of EVs samples from the EV-trc-GFP strain. EVs were separated in 5 nm size groups, distributed between 30 and 130 nm, and in groups comprising those with a size below 30 nm or higher than 130 nm. Vesicle diameter was determined by direct measurement from electron micrographs of three independent biological replicates. Abundance of each size group is presented as relative figures, i.e. number of vesicles with a given diameter range relative to the total number of vesicles measured.

CHAPTER VII - General discussion, Conclusions and Future Perspectives



1. General discussion

Cyanobacteria can be encountered in almost all known land and aquatic based habitats. Some of them are extreme in terms of physical and biochemical conditions. A common consequence of exposure to harmful stresses is the generation of reactive oxygen species (ROS), highly noxious to all organisms (Singh, 2018). Therefore, cyanobacteria evolved several adaptation survival strategies (Agostoni & Montgomery, 2014). One of these biological tactics addresses the ultimate frontier between what is considered life and inertia, the cell envelope. Surroundings can shift at any moment, and so, cells are constantly receiving, integrating and responding to stimuli from it. Accordingly, cyanobacteria developed uptake and secretion mechanisms, tightly coordinated with the cell wall architecture plasticity. Due to their photoautotrophic lifestyle, cyanobacteria do not depend as much as other bacteria on the import of organic and inorganic nutrients, which is also a defence against toxic molecules (Gonçalves *et al.*, 2018; Kowata *et al.*, 2017). *Synechocystis* sp. PCC 6803 has 20% lower cell permeability to exogenous small carbohydrates, amino acids, and inorganic ions, than *E. coli* (Kowata *et al.*, 2017). Cyanobacterial secretion, on the other hand, is essential. It occurs either through secretory portals embedded in the cell wall, the so called “classical secretion systems”, or mediated by EVs, an overlooked “non-classical secretion system” (Gonçalves *et al.*, 2018) (*Chapter II*).

Here, an integration of the main results achieved within the scope of this thesis regarding cyanobacterial EVs is presented. Initially, general recommendations on isolation and characterization of bacterial EVs are delivered, namely concerning cyanobacterial vesicles, focusing on *Synechocystis*. Then, a comprehensive discussion addresses the contribution of this work to the state-of-the-art on cyanobacterial EVs’ biogenesis, composition, biological roles, and biotechnological applicability.

1.1 Cyanobacterial EVs

1.1.1 Isolation and characterization

To recover and analyse relatively pure samples of EVs from bacterial cultures is a challenging process. In a laboratory setting, the isolation workflow usually includes bacterial cultivation, culture filtrate collection, concentration and, if possible and needed, further purification (Klimentová & Stulík, 2015). Each of these steps are important and deserves careful evaluation as they affect the outcome.

Regarding bacterial cultivation, the type of media used (defined or not), and the exact physical conditions for growth, need to be meticulously reported (Théry *et al.*, 2018). To recover the cell-free extracellular medium, low speed centrifugation and filtration are usually performed. Main differences on the workflow usually relate to downstream steps, namely EVs concentration and recovery. Several approaches can be employed, and even combined. The most common is ultracentrifugation, being ultrafiltration also normally applied. If required and possible, the vesicle fraction is further purified via e.g. density gradient ultracentrifugation (DGU) and size-exclusion chromatography (SEC) (Klimentová & Stulík, 2015; Watson *et al.*, 2021).

Subsequent analyses include both physical and biochemical aspects of EVs. Morphological data is commonly retrieved from transmission electron microscopy (TEM) techniques, nanoparticle tracking analysis (NTA) and/or dynamic light scattering (DLS) (Théry *et al.*, 2018). While the three enable the analysis of vesicles size, NTA and/or DLS give additional information about particle concentration. However, parameter variations occur between these techniques. For instance, the size measured by NTA and DLS are related to the hydrodynamic diameter of EVs, whereas TEM results are related to their dry state (Singh *et al.*, 2019; Wang *et al.*, 2010). Zeta potential data reported by DLS estimates the surface charge, which are relevant to understand EVs interactions (Tashiro *et al.*, 2017). Regarding their composition, general lipopolysaccharides (LPS) and protein content of EVs can be evaluated by electrophoresis on denaturing SDS-polyacrylamide gels, followed of respective staining (Dauros Singorenko *et al.*, 2017; Gonçalves *et al.*, 2018; Oliveira *et al.*, 2016). As LPS are Gram-negative EVs molecular markers, if normalized and quantified, they can serve also as an EV quantity indicator (Gonçalves *et al.*, 2018).

Overall, it should be highlighted that there are no universal, one fits-all protocols to isolate and analyse bacterial vesicles. All of them present advantages and disadvantages, and depend on the bacteria studied, as each have their own particularities and environmental dependencies. Consistent report of the workflow process is the key factor, as defended by MISEV recommendations (Théry *et al.*, 2018). Finally, yet importantly, downstream applications need to be considered in advance to understand the level of purity required (Klimentová & Stulík, 2015).

All these considerations were taken into account when publishing guidelines to isolate and characterize cyanobacterial EVs (*Chapter III*). These guidelines include not only the freshwater cyanobacterium *Synechocystis*, but also marine cyanobacteria, such as *Prochlorococcus* and *Synechococcus*. Differences between recovering EVs from cyanobacterial cultures and field samples are also addressed. One of the major challenges

faced when recovering EVs from cyanobacterial cultures is their lower concentrations compared to other bacteria, e.g. *E. coli* (Biller *et al.*, 2014; Oliveira *et al.*, 2016; Reimer *et al.*, 2021). Optimization of a protocol to harvest vesicles from *Synechocystis* using supernatant filtration, followed by ultrafiltration and ultracentrifugation (*Chapter III*), revealed to be time-consuming. One should highlight the initial benefit of using a *Synechocystis* hyper-vesiculating mutant strain, $\Delta toI/C$ (Oliveira *et al.*, 2016), to ease these trial and error processes. Briefly, two types of sterile filters (0.2 μm and 0.45 μm) were tested, several concentrators with distinct MWCOs (Molecular Weight Cut-Offs) capacities (3, 30 and 100 kDa MWCO) tried, different ultracentrifugation running times applied and distinct combinations of washes on EVs fractions performed. Mode of cultivation (e.g. orbital shaking vs continuous bubbling aeration; media composition) also affected the isolation output. For instance, tweaking the copper media concentration affected vesiculation (*Chapter V*).

The *Synechocystis* vesicle fraction obtained with the final established protocol (*Chapter III*) was considered adequate and with the necessary yield to proceed with intended downstream applications. For future '-omics' style high-throughput analyses, DGU or SEC may be performed to further purify *Synechocystis* EVs fractions. In summary, the published guidelines were followed, unless stated otherwise, throughout this thesis. Moreover, all these recommendations can function for researchers potentially interested in EVs from other cyanobacterial species.

1.1.2 Biogenesis

In Gram-negative bacteria, vesicle formation and release is a complex process (Schwechheimer & Kuehn, 2015; Toyofuku *et al.*, 2019). Among the EVs biogenesis pathways, some are relatively well described. Two of them essentially focus on i) the modulation of outer membrane-peptidoglycan links, and on ii) the existence of nanoterritories, where a confined build-up pressure of cellular components occurs promoting vesiculation. Regarding cyanobacterial EVs production routes, apart from the fact that membrane blebbing has been observed on the cell wall of diverse cyanobacterial strains [e.g. (Biller *et al.*, 2014; Brito *et al.*, 2017; Oliveira *et al.*, 2016; Zarantonello *et al.*, 2018)], nothing more was reported.

Here, upon analysis on the protein content of isolated *Synechocystis* EVs, a group of proteins was identified (Slr1841, Slr1908 and Sll1550). These proteins are outer membrane porins involved in inorganic ions uptake, harbouring simultaneously the S-layer homology (SLH) domain, which binds the peptidoglycan layer (Kojima *et al.*, 2016), and the

carbohydrate selective porin OprB (OprB) domain, which forms a β -barrel structure, anchoring it in the outer membrane (Kojima *et al.*, 2016; Schätzle *et al.*, 2021). Given their composition, and high abundance both in the outer membrane and vesicle preparations, a hypothesis was raised where these porins, which establish links between the outer membrane and the peptidoglycan, could have a role in outer membrane overall stability, and potentially in vesiculation (*Chapter IV*). *In silico* analysis identified in the genome of *Synechocystis* a total of six genes encoding outer membrane porins harboring SLH/OprB-domains (*slr1841*, *slr1908*, *sll1550*, *slr0042*, *sll1271* and *sll0772*). They are not simple duplicates of each other, as the predicted proteins have differences in size and domain length. Using a genetic-based approach, it was possible to generate fully segregated single deletion mutants for five of them, whereas gene *slr1841* was found to be essential for *Synechocystis*, under the tested conditions.

Several attempts to create a single deletion mutant for gene *slr1841* were performed. Other approaches were tried, such as the generation of a *Synechocystis* strain in which Slr1841 was replaced by the chimeric Slr1841::super folder Green Fluorescent Protein (sfGFP) protein, but resulted invariably in a non-fully segregated mutant. In agreement, Qiu *et al.* (Qiu *et al.*, 2021) also failed in generating a full knockout mutant of gene *slr1841*. Thus, these results strongly suggest that Slr1841 is essential under the tested conditions, and that its deletion or modification are deleterious to the cell. One should highlight that Slr1841 is the most abundant protein in the outer membrane of *Synechocystis* (Kowata *et al.*, 2017). Remarkably, using a CRISPRi (interference) approach, Kojima and Okumura recently reported a mutant with a *slr1841* downregulation (*slr1841i*) (Kojima & Okumura, 2020). Upon repression, *Synechocystis slr1841i* cells lost approximately 70% of their outer membrane, validating the significance of Slr1841 for *Synechocystis* outer membrane stability, and possibly in vesiculation. Indeed, observations of transmission electron micrographs of these cells suggest the release of nanostructures that resemble EVs (Kojima & Okumura, 2020).

Among the five genes successfully deleted, vesicle production was monitored in the respective mutants. It was found that a mutant in the gene encoding the second most abundant SLH/OprB protein in *Synechocystis* outer membrane (Slr1908) produces more vesicles than any of the other tested strains. Curiously, Δ *slr1908* EVs are smaller than those released by the wild-type (WT) strain. Physiological relevance of such aspect remains to be clarified. It is possible that the potential instability created through the absence of Slr1908 in the outer membrane could promote the production of smaller sized EVs. Furthermore, upon deletion of gene *slr1908*, it was possible to identify another porin, Sll1550, in the OM preparation of this mutant. Its appearance denotes an outer membrane protein content

adjustment provoked by the absence of Slr1908. Altogether, these results suggest that proteins containing the SLH/OprB-domains, on top of their role in inorganic ions uptake, are related to the outer membrane stability and vesiculation.

In Gram-negative bacteria the envelope integrity is suggested to be maintained by OmpA porins and Braun's Lpp, which bind to the peptidoglycan layer, and through the Tol-Pal complex, a multi-protein system that spans the entire periplasm, bridging the inner and outer membranes (Schwechheimer & Kuehn, 2015). Observations showed that decreasing the number of these envelope connections augmented the vesiculation (Bernadac *et al.*, 1998; Deatherage *et al.*, 2009; Park *et al.*, 2012; Schwechheimer & Kuehn, 2013; Schwechheimer *et al.*, 2015; Turner *et al.*, 2015). However, not all bacteria harbor the complete set of these anchor-proteins. For example, Lpp are absent in cyanobacteria (Hoiczky & Hansel, 2000). This is not the last peculiarity of the cyanobacterial cell envelope. In terms of their peptidoglycan thickness and crosslinking, they share striking aspects with their Gram-positive counterparts (Hoiczky & Hansel, 2000). Interestingly, in *Bacillus anthracis*, a Gram-positive bacteria, it was shown that pyruvylation of the peptidoglycan-associated polysaccharide is essential for the proper binding of the SLH-protein domain (Mesnage *et al.*, 2000). Altogether, modulation of links between the outer membrane and peptidoglycan, such as those mediated by SLH/OprB porins, are suggested to be involved in cyanobacterial vesicle biogenesis, as similarly described for other bacteria (Schwechheimer & Kuehn, 2015).

Attempts to target a heterologous protein highly expressed to the periplasm of *Synechocystis*, in order to augment the probability of its encapsulation, resulted in an increased vesiculation (Chapter VI). As a proof of concept, the sfGFP reporter was used. This allowed not only the visualization of its presence in the periplasm, but as well to detect highly fluorescent foci in the extracellular medium, consistent with sfGFP-packed EVs. One can hypothesize that by targeting sfGFP to the *Synechocystis* periplasm, the reporter protein accumulates therein, creating a turgor pressure that forces the cell to bleb and vesiculate. Interestingly, in a mutant strain of *Leptolyngbya boryana* deleted in a gene involved in one of the last stages of chlorophyll biosynthesis, a cyanobacterial protein named protochlorophyllide (Pchl_{ide}) was suggested to be secreted to the medium by EVs (Usui *et al.*, 2022). Authors speculated that an abnormal Pchl_{ide} periplasmic accumulation might cause a rise in the periplasm pressure, weakening locally the interaction between the outer membrane and peptidoglycan layer, and thus provoking vesiculation (Usui *et al.*, 2022). Reasoning supporting these examples are partially in line with the theory of nanoterritories described for other Gram-negative bacteria, in which an augment of cellular

components, like proteins, promote periplasmic pressure and EV formation (Schwechheimer & Kuehn, 2015; Schwechheimer *et al.*, 2014).

Relevant progresses regarding vesicle biogenesis in cyanobacteria were performed. Outer membrane porins harboring SLH/OprB-domains were suggested as the first players to be involved in *Synechocystis* vesiculation (*Chapter IV*). Due to their common distribution in cyanobacteria, it will be interesting to unveil if their involvement in vesiculation is transversal. Also, the insight that overloading the *Synechocystis* periplasm with proteins results in increased EV formation (*Chapter VI*) cracks opens a new avenue to fine tune protein encapsulation of vesicles. Nevertheless, more efforts are required to understand all the steps implicated in these processes. Designing strategies to identify players involved in the regulation, formation and release of EVs will certainly reveal a more detailed picture of this secretory mechanism. For that purpose, a comprehensive compositional characterization of cyanobacterial EVs would be important.

1.1.3 Composition

Vesicle composition is tightly regulated with its formation pathway (McMillan & Kuehn, 2021). As EVs derive mainly from the outer membrane, several reports show the presence of periplasmic and OM proteins, and LPS, in vesicles of Gram-negative bacteria (Lee *et al.*, 2016). Nevertheless, cytoplasmic proteins, nucleic acids and metabolites have also been observed therein (Kulp & Kuehn, 2010; Schwechheimer & Kuehn, 2015).

There is very limited information available concerning the exact composition of cyanobacterial EVs. However, publications by Biller *et al.* (Biller *et al.*, 2021; Biller *et al.*, 2017; Biller *et al.*, 2014) give an extensive compositional characterization of *Prochlorococcus* EVs. One should remember that marine cyanobacterium *Prochlorococcus* sp. is one of the most abundant microorganisms on Earth, being at the root of the food chain in open oceans (Flombaum *et al.*, 2013). Proteome, lipidome, and metabolome approaches were recently performed to compare vesicles of two ecologically distinct *Prochlorococcus* – MIT9312, a high-light adapted strain, and MIT9313, a low-light adapted strain (Biller *et al.*, 2021). Both strains displayed a distinctive vesicle profile, suggesting that no universal vesicle composition exist among cyanobacteria. Fascinatingly, the proteome analysis revealed that several of the most abundant proteins in the vesicles were putative enzymes. *In vitro* functional assays showed the presence of catalytically active enzymes in *Prochlorococcus* EVs, such as lipases, phosphatases, proteases, and sulfatases. Lipid analyses showed primarily the presence of sulfolipids - monoglycosyl diacylglycerol (MGDG), diglycosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) - mirroring *Prochlorococcus* cellular membrane adaptations to the low phosphorous

oligotrophic environment they inhabit (Van Mooy *et al.*, 2006). However, hydroxylated forms of all these major intact polar lipids were relatively more abundant in EVs than cells. Metabolomics uncovered the existence of non-polar molecules (e.g. oxidized carotenoids) in vesicles of both strains. Interestingly, throughout this thesis work (e.g. *Chapters III and VI*), *Synechocystis* vesicle fractions showed a typical orange pigmentation, consistent with the presence of carotenoids, an outer membrane component (Jürgens & Weckesser, 1985), as reported by Pardo *et al.* (Pardo *et al.*, 2015).

Regarding *Synechocystis* EVs, it was possible to detect compositional adjustments, namely related to alterations in protein (e.g. OM porins containing the SLH/OprB-domains – *Chapter IV*) and LPS profiles (*Chapters IV, V and VI*). An array of different *Synechocystis* mutant strains aided in this respect. For instance, Western Blot analysis of EVs isolated from the TolC-deficient mutant revealed the presence of CopM, mirroring this protein's cellular overexpression. As this metallochaperone binds copper, the presence of this element in vesicles was later confirmed by atomic absorption spectrometry in *Synechocystis* vesicles (*Chapter V*). Furthermore, vesicles isolated from the WT strain and from hyper-vesiculating mutants $\Delta tolC$ and $\Delta tolC/spy$ displayed a similar LPS profile in terms of smooth-type LPS (composed of lipid A, oligosaccharide core and O-antigen) and rough-type LPS (composed of lipid A and oligosaccharide core). However, EVs from $\Delta fucS$ strain, holding LPS truncated O-antigen portions as reported (Fisher *et al.*, 2013), presented a LPS band pattern significantly different, namely in respect to the smooth-type LPS (*Chapter VI*). These differences in terms of LPS composition of EVs are physiologically relevant, as discussed in the next sub-section. Notwithstanding these valuable compositional indications, many aspects remain unaddressed, and it will be interesting to perform '-omics' style high-throughput techniques on *Synechocystis* vesicles to start gaining further insights and details into their composition.

1.1.4 Biological roles

Topics related with the formation pathway and compositional aspect of EVs are ultimately relevant to grasp their physiological relevance in an *in vivo* context (McMillan & Kuehn, 2021). In this work, one major role in metal homeostasis was proposed to occur in *Synechocystis* and other Gram-negative bacteria, like *E. coli* and *P. aeruginosa* (*Chapter V*). In addition, some evidences were collected that suggest the involvement of EVs in cyanobacteria in alleviating periplasmic pressure (*Chapter VI*) and as a prompt cell envelope remodelling process, enabling a rapid adaptation towards an environmental shift (e.g. metal induced stress). Moreover, contribution of *Synechocystis* EVs in iron acquisition was suggested (*Chapter IV*).

A hyper-vesiculating mutant strain, $\Delta toIC$, was investigated to further understand the basis of its vesiculation phenotype (*Chapter V*). TolC is an outer membrane protein that can combine with multiple protein partners to secrete a wide range of biological molecules, being the substrate selectivity defined by the remaining inner membrane and periplasmic adaptor protein components (Gonçalves *et al.*, 2018; Oliveira *et al.*, 2016; Russo *et al.*, 2021). The over-release of vesicles by the $\Delta toIC$ mutant was initially interpreted as a balancing response resulting from a strong secretion impairment (Oliveira *et al.*, 2016). In addition, TolC-deficient mutant was described to lack a surface layer (S-layer) (Oliveira *et al.*, 2016), the outermost cell-wall structure of *Synechocystis*, composed of a network of glycoproteins that contributes to the integrity of the cell envelope (Fagan & Fairweather, 2014). Later, Gonçalves *et al.* (Gonçalves *et al.*, 2018) reported other strains, impaired in several secretory functions, also lacking the S-layer, which consistently presented a higher vesiculation capacity comparing to the wild-type strain. Yet, none of these analysed mutants reached the vesiculation capacity of the $\Delta toIC$ strain (Gonçalves *et al.*, 2018). Thus, beyond the secretion impairment of substrates (proteins and antimicrobial compounds) and the absence of a biological frontier such as the S-layer, other factors were contributing to the hyper-vesiculating phenotype in the *toIC*-deletion mutant.

A combination of metal resistance tests and intracellular metal quantifications, revealed that *Synechocystis toIC*-mutant accumulates 3-fold more copper intracellularly than the WT (*Chapter V*). Copper is an essential micronutrient. Several enzymes depend on this element as a redox co-factor in their catalytic centres (Arguello *et al.*, 2013; Imlay, 2015). However, increased levels trigger the transition of copper that is protein-associated to its free form, which can turn into a potential hazard because of its high chemical reactivity (Arguello *et al.*, 2013). Free copper triggers redox reactions and generate highly reactive hydroxyl radicals (Gaetke & Chow, 2003). These in their turn attack and destroy iron-sulfur protein clusters, thereby releasing iron and further promoting oxidative stress (Rensing & McDevitt, 2013). Thus, copper intracellular balance is pivotal to prevent severe cellular damage. Biochemical and *in silico* evidences (Agarwal *et al.*, 2014; Kulathila *et al.*, 2011) suggest that TolC could be the outer membrane component of the heavy metal efflux (HME) pump to secrete copper in *Synechocystis*. In agreement, a recent publication demonstrated that TolC is key in mitigating copper sensitivity even in *E. coli* (Gugala *et al.*, 2021), an organism that present other pathways dedicated to copper efflux (Rensing & Grass, 2003). Remarkably, a periplasmic metallochaperone that binds copper, CopM, was found to be overexpressed in the *toIC*-deletion mutant. Even more intriguing, vesicles isolated from this mutant were enriched in CopM comparing to WT vesicles, under standard growth conditions. Still under normal cultivating settings, EVs isolated from the mutant showed

approximately 3-4 fold more copper comparing to EVs of the wild-type strain. Altogether, it was suggested that *Synechocystis* EVs could have a role in copper homeostasis, working as nanocapsules for the secretion of copper. Observations that both *Synechocystis* wild-type and other Gram-negative bacteria (*E. coli* and *P. aeruginosa*) released more EVs when subjected to increasing concentrations of copper, led to propose that EVs could fulfil a general role in metal homeostasis in bacteria (*Chapter V*). Remarkably, Bellingham *et al.* (Bellingham *et al.*, 2015) suggested that eukaryotic EVs may also fulfil this role in higher organisms.

EVs loaded with CopM may also work as a copper chelating system, making it less available for uptake by the *Synechocystis* copper stressed cells. It was curious to note that bacterial cells exposed to more copper released vesicles with a significant increase in less complex LPS (belonging to the rough-type) (*Chapter V*). Using simplified molecular dynamics simulations, it was recently proposed that vesicles with more rough LPS would distort and spread over the membrane surface, not being assimilated by cells. Inversely, bacterial EVs enriched in smooth LPS would preserve their spherical shape when interacting with cellular membranes, promoting internalization (Jefferies & Khalid, 2020). Copper-stressed *Synechocystis* cells secrete EVs enriched in rough LPS that are potentially less prone to be captured by cells, making less accessible the amount of copper present in solution. A similar bias to rough LPS was observed in *E. coli* and *P. aeruginosa* EVs when subjected to increasing but still sub-lethal concentrations of copper. Hence, on top of serving the purpose of exporting copper out of the cell, bacterial EVs may also accumulate copper extracellularly, working as a sink mechanism for copper.

It was described elsewhere that, during environmental shifts, secreting EVs is the only mechanism known to locally remodel LPS of the cell wall, promoting rapid outer membrane maintenance and thereby bacterial adaptation (Bonnington & Kuehn, 2016; McMillan & Kuehn, 2021). Moreover, high metal concentrations can trigger the production of ROS (Imlay, 2015). *Synechocystis tolC*-mutant show signs of high oxidative stress compared to the wild type, as testified by the increase in lipid peroxidation (Hewelt-Belka *et al.*, 2020). Even with cellular antioxidant defences upregulated (Oliveira *et al.*, 2016), EVs release could aid in the detoxification of periplasmic and cell wall oxidized components. Evaluation of the $\Delta tolC$ cell envelope stress pathways revealed an upregulation of transcript levels of genes (*spy*, *degQ*) and overexpression of proteins (Spy, DegP) related to envelope stress responses (Gonçalves *et al.*, 2018). Spy is an important periplasmic chaperone, contributing to protein folding in the periplasm (Quan *et al.*, 2011). Here, the generation of a double mutant, $\Delta tolC/spy$, revealed not only a higher sensitivity to copper (data not shown) but also an increment in vesiculation (*Chapter VI*). Thus, higher cell wall stress provokes an increase

in vesiculation, plausibly with the purpose of secreting copper and restructuring the outer membrane landscape, particularly in terms of oxidized OM components.

Based on this work (*Chapter V*), it can be therefore proposed that *Synechocystis* mutant strain ΔtolC accumulates copper intracellularly due to the absence of the outer membrane component (TolC) of the HME pump for copper. This leads to a dangerous intracellular accumulation of this metal, being EVs used as an alternative secretion mechanism to secrete copper. When subjected to high but still sub-lethal amounts of copper, *Synechocystis* WT, *E. coli* and *P. aeruginosa*, use this non-classical secretion route to balance intracellular levels of copper and survive. Taking all into consideration, it should be relevant to consider EVs as a novel but still overlooked form of bacterial metal resistance.

Other metal binding proteins and siderophores were detected in several bacterial EVs [e.g. (Biller *et al.*, 2021; Lappann *et al.*, 2013; Prados-Rosales *et al.*, 2014)], but it has always been interpreted as a means of metal acquisition for the microbial community. A classic example is the case of *P. aeruginosa*, which communicate through a quorum-sensing signal that promotes also EVs release, being those in their turn able to capture iron, which are later internalized (Bredenbruch *et al.*, 2006; Diggle *et al.*, 2007). A similar biological strategy has been described for the pathogen *Mycobacterium tuberculosis*. Under iron deficiency, *M. tuberculosis* secrete EVs containing high-affinity iron chelators or siderophores named mycobactins, which could then be uptaken by cells (Prados-Rosales *et al.*, 2014). Interestingly, $\Delta\text{slr1908}$, the hyper-vesiculating *Synechocystis* mutant where the second most abundant outer membrane porin (Slr1908) was deleted, presented indications of being under iron limitation (*Chapter IV*). Cells from $\Delta\text{slr1908}$ registered a reduction of 40% in the intracellular iron content. Moreover, a component of the iron uptake system, the periplasmic iron uptake protein A2 (FutA2), was detected in higher amounts in the mutant's exoproteome as compared to that of the WT. This corroborates a physiological adjustment to the absence of Slr1908, which was already suggested to work as an iron-selective porin (Qiu *et al.*, 2021). Hence, it would be necessary to evaluate the $\Delta\text{slr1908}$ EVs content to understand why the strain releases more vesicles than the parental strain, and so, to shed some light about their biological role. One can recall that marine cyanobacterial vesicles have been proposed to work as authentic public goods, by transporting nutrient binding proteins or scavenging molecules. Substrate-binding proteins, e.g. for iron and manganese, and even ATP, have been detected in EVs of *Prochlorococcus* sp. (Biller *et al.*, 2021). Through vesicles prolonged permanence in seawater, other microbes can eventually use them as energizing nutrients snacks (Biller, 2020; Biller *et al.*, 2021). Indeed, *in vitro* experiments demonstrated that *Prochlorococcus*-derived EVs were

able to sustain the growth of heterotrophic bacteria, without adding any exogenous carbon sources (Biller *et al.*, 2014).

Taken together, this contributed to unveil a novel role for bacterial EVs, namely as an alternative copper (and plausibly, in a more general manner, metal) secretion mechanism, thereby being implicated in metal homeostasis (*Chapter V*). However, several open questions remain: the suggestion that EVs could function as sinks for copper, ultimately avoiding cellular recapture by modulating LPS vesicular content, deserves further studies. Moreover, the capacity of *Synechocystis* vesicles of playing a role in nutrient acquisition, namely for iron, needs also to be addressed (*Chapter IV*).

1.1.5 Biotechnological applicability

Efforts are under way to transform cyanobacteria into efficient and sustainable microbial cell factories (Zahra *et al.*, 2020). The notion that secretion could play a part to fulfil this vision is recent, even more when considering cyanobacterial EVs (Gonçalves *et al.*, 2019). Here, the targeting of proteins of interest to cyanobacterial vesicles was tested and implemented (*Chapter V* and *VI*), opening an array of technological applications. The idea of customizing *Synechocystis* vesicles as specific protein carrier platforms for fish in aquaculture is discussed (*Chapter VI*). Moreover, the possibility of using *Synechocystis* vesicles for bioremediation purposes is also presented.

Significant progress was made to tweak cyanobacteria to load a protein of interest into EVs by passive diffusion. First, a construct harbouring a relatively strong heterologous promoter, $P_{trc.x.lacO}$ (Ferreira *et al.*, 2018), and the native signal peptide of protein FutA2 (Polyviou *et al.*, 2018), fused with the coding sequence of *sfgfp* gene, was assembled. Promoter $P_{trc.x.lacO}$, is a well-characterized synthetic part containing two *lacO* operators and a Bba_B0030 RBS (ribosome binding site) (Ferreira *et al.*, 2018). Protein FutA2 is the most abundant protein in the periplasm of *Synechocystis*, holding a twin-arginine translocation (Tat)-dependent signal peptide to be efficiently translocated therein (Badarau *et al.*, 2008; Polyviou *et al.*, 2018; Waldron *et al.*, 2007). Construct was then integrated in the *Synechocystis* genome by double-homologous recombination in the genomic neutral site 15 (Pinto *et al.*, 2015). Genomic neutral sites refer to portions of the genome used for the stable integration of ectopic DNA without compromising cellular fitness (Pinto *et al.*, 2015). Fully segregated cells revealed that sfGFP was indeed targeted to the periplasm of *Synechocystis*, where it accumulated (*Chapter V* and *VI*). It was proposed that protein accumulation creates a turgor pressure in this inter-membrane space that forces the cell to bleb more, releasing sfGFP packed EVs. Among the various tests performed, the one where sectioned immunogold labeled EVs are analysed by TEM, revealing the presence of sfGFP

in their lumen, is descriptive (*Chapter VI*). Customization of bacterial EVs with proteins of interest have been described, for example, in *E. coli* (Kesty & Kuehn, 2004), but it is rather novel in cyanobacteria.

Several attempts to transform *Synechocystis tolC*-mutant were performed using the *sfgfp*-assembled construct, with the goal of taking advantage of its hyper-vesiculating phenotype to produce more sfGFP-loaded vesicles. However, efforts of transformation were unsuccessful. One should remember that $\Delta tolC$ is still naturally transformable, as shown by the generation of the double mutant $\Delta tolC/spy$ (*Chapter VI*). As already discussed, *tolC*-deletion mutant is under stress, particularly at the periplasmic level (Gonçalves *et al.*, 2018). The strong and constitutive expression of sfGFP promoted by $P_{trc.x.lacO}$, together with an efficient translocation to the periplasm given by FutA2 signal peptide, probably recruiting many Tat transporters, are additional traits that could impair $\Delta tolC$ cells to be transformed with the sfGFP-harboring construct developed in this work.

Holding a *Synechocystis* strain capable of secreting sfGFP packed EVs, it was important to address the efficacy of the encapsulation process. Unfortunately, only about 10% of the total sfGFP secreted to the extracellular medium was shown to be bound to vesicles, being the remaining protein soluble in the medium (*Chapters V and VI*). Interestingly, a similar occurrence was observed when analysing CopM in the $\Delta tolC$ mutant (*Chapter V*). These results suggest that soluble proteins reaching the *Synechocystis* periplasm can leave the cell either by classical translocation systems located in the outer membrane, or packaged into EVs. Thus, it would be important to discover the transporter(s) involved in the secretion of soluble periplasmic proteins through the outer membrane. One can imagine that by blocking these OM exit routes, the periplasmic pressure would increase even more, leading to an increment in encapsulation and vesiculation. Another alternative would be to transform an already hyper-vesiculating strain, other than $\Delta tolC$ mutant, as background. By doing so, one could foresee the isolation of more sfGFP packed EVs, even though the issue of packaging efficiency would remain unsolved. Additionally, another level of optimization addresses the choice of promoter and signal peptide used. Regarding the promoter, one can think about its origin (native vs heterologous), regulation (constitutive vs inducible) and expression strength (strong vs medium vs weak). The nature of the signal peptide, mediating the passage from the cytoplasm through the inner membrane, can rely either on sequences identified from the general secretory (Sec) pathway or derived from the Tat pathway (Freudl, 2018). Protein folding in the cytoplasm is a requirement for Tat-targeted proteins to be translocated to the periplasm, whereas Sec-secreted proteins are transported unfolded (Freudl, 2018). Proteins like sfGFP can only be secreted after cytoplasmic folding, thereby being unsuitable for translocation through the Sec pathway (Feilmeier *et al.*, 2000;

Thomas *et al.*, 2001). Thus, an important matter to consider when pretending to encapsulate a protein of interest into cyanobacterial EVs is their mode of translocation to the periplasm. Signal peptides affect the biosynthesis, the folding kinetics, and the stability of the respective target protein (Freudl, 2018). Moreover, deletion of native genes encoding proteins competing for the Sec or Tat transporters could be another strategy to improve protein of interest transportation to the periplasm (Russo *et al.*, 2021), ultimately favouring vesicle packaging. Overall, addressing several and distinct degrees of the vesiculation process will allow the development of a *Synechocystis* strain capable of secreting more efficiently EVs packaged with a protein of interest.

The use of *Synechocystis* EVs as novel protein carrier systems for fish in aquaculture was tested (*Chapter VI*). Major problems arise in aquaculture farms regarding fish growth. Overcrowding in fish tanks result invariably in nutritional deficiencies and disease outbreaks. These are tackled with chemicals and antibiotics to keep the purpose of commercial production. Sometimes these compounds are inappropriately and dangerously applied, particularly when considering one of the end goals of aquaculture: human consumption. An ingenious form to protect consumers, as well as farmers interests, would be to develop environmentally-friendly solutions to address these occurrences in fish farming (Hai, 2015). Here, *Synechocystis* EVs were studied as potential nanocarriers of proteins of interests, envisioning different applications, ranging from fish dietary supplementation to modulation of their immune system against selected antigens. For that purpose, an evaluation of their potential toxicity against zebrafish larvae was performed. Bacterial EVs toxicity derive essentially from their LPS content (Gnopo *et al.*, 2017). Various *Synechocystis* strains possessing distinct cell envelope/surface properties, and differential vesiculation capacities were used: WT, $\Delta tolC$, $\Delta tolC/spy$ and $\Delta fucS$. Overall, *Synechocystis* EVs were safe compared to the control condition [*P. aeruginosa* LPS (Novoa *et al.*, 2009)]. Notably, when subjecting zebrafish larvae to high dosages of *Synechocystis* EVs, no systemic inflammatory responses were observed. These results are in agreement with earlier observations showing a reduced toxicity of cyanobacterial LPS (Durai *et al.*, 2015; Stewart *et al.*, 2006; Swanson-Mungerson *et al.*, 2017), which can be explained by structural differences detected in the cyanobacterial lipid A backbone, as compared to other Gram-negative bacteria (Hahn & Schleiff, 2014). Then, the recognition that *Synechocystis* EVs could be loaded with a protein of interest, led to perform immersion tests of zebrafish larvae with sfGFP packed EVs. Remarkably, sfGFP accumulated in the gastrointestinal tract, suggesting prolonged residence time of the protein in this environment (*Chapter VI*). Upon oral administration of microalgae expressing GFP, it was also described an accumulation of this protein in the fish intestinal tissues, being its presence further detected

in the blood, as determined by ELISA (Kwon *et al.*, 2019). Authors speculated that microalgae cells are lysed in the hind gut by cellulolytic commensal bacteria, being the protein locally released and absorbed by intestinal cells (Kwon *et al.*, 2019). In the case of EVs, this reasoning might also be valid. However, it is also likely that EVs are internalized by gut cells, releasing their cargo therein. Several cellular entry pathways of bacterial EVs have already been described in eukaryotic hosts (O'Donoghue & Krachler, 2016). It should be pointed out that cellular uptake of EVs packed with the protein of interest, shielding it from intestinal proteases, is beneficial to maximize protein delivery. Moreover, other biotechnological applications may not even require the release of EVs content. In the case of packing an enzyme, the periplasm-like microenvironment of a vesicle lumen and the protection against gut proteolytic activity, benefits its stability (Bonnington & Kuehn, 2014). In this scenario, substrates cross the vesicle envelope, enabling enzymatic activity (Biller *et al.*, 2021). All in all, *Synechocystis* EVs have the prospect of becoming a valid alternative of carrying specific proteins, opening a wide range of technological applications, namely for aquaculture.

Realization that *Synechocystis* EVs can function as an alternative mechanism to secrete metals and concentrate them extracellularly (*Chapter V*), could open the door to further explore cyanobacterial EVs in the context of bioremediation, namely in freshwater polluted sites, as proposed elsewhere (Alves *et al.*, 2018; Alves *et al.*, 2015; Caruana *et al.*, 2020). If EVs are packed with metal binding proteins and dispersed into a determined metal contaminated site, they could act as sinking agents, reducing metal bioavailability. This reasoning could be applied for copper polluted locations, using CopM enriched vesicles. This approach holds drawbacks, as it would only reduce metal availability for organisms in a given environment, instead of a total and definite cleanse of it.

Overall, cyanobacterial EVs, in particular *Synechocystis* EVs, demonstrate biotechnological potential. On top of being produced by a group of bacteria with increasing commercial attractiveness, the cyanobacterium used throughout this work is genetically tractable. *Synechocystis* holds an already solid molecular toolbox, expected to develop even more due to their relation with synthetic biology (Pacheco *et al.*, 2021). These qualities allowed the generation of several mutants with hyper-vesiculation capacity, relevant when considering a large scale EVs production. Moreover, recognition that *Synechocystis* EVs and their LPS content appear safe without further bioengineering is a significant asset, namely for fish and possibly other organisms. Efforts to streamline EVs modulation and encapsulation with protein(s) of interest(s) will benefit greatly these end goals. Connecting all these dots in one single approach will possibly contribute to use *Synechocystis* EVs as an environmental-friendly and sustainable solution for biotechnological applications.

2. Conclusions and Future Perspectives

Overall, this work contributed to pave the way to systematically study extracellular vesicles in cyanobacteria. A solid protocol for reproducibly isolating and characterizing cyanobacterial EVs was developed, identifying a number of critical steps and aspects (*Chapter III*). Then, using *Synechocystis* as model strain, the first players and mechanisms involved in EVs biogenesis were revealed, including the involvement of outer-membrane porins (*Chapter IV*), but also owing to periplasmic protein pressure (*Chapter VI*). In addition, cyanobacterial EVs physiological relevance was uncovered, through their use as metal-efflux nanocapsules, revealing an unknown role for bacterial EVs and metal homeostasis (*Chapter V*). Interestingly, this ability concerning EVs was suggested to occur in bacteria inhabiting completely different ecological niches. Moreover, initial steps towards the protein content customization of cyanobacterial EVs were taken, envisioning their use as biotechnological tools, applied here in the field of aquaculture but with the prospect of expanding to other topics (*Chapter VI*). As EVs constitute an exciting biological aspect in the 21st century, these results could trigger the curiosity towards the study of vesicles from other cyanobacterial species and address other applications.

Although this work contributed to improve the knowledge on cyanobacterial EVs, particularly in *Synechocystis*, several questions remain unanswered. One can divide these interrogations into distinct categories, namely *Synechocystis* EVs biogenesis, composition, biological roles and biotechnological applicability.

Regarding their formation process:

What are the molecular mechanisms behind the binding between the outer membrane porins harbouring the SLH/OprB domains and the peptidoglycan layer in *Synechocystis*? Can the players involved in this process be identified and tweaked to modulate vesiculation?

Addressing their compositional aspect:

What are *Synechocystis* EVs made of? Will a comprehensive proteome, lipidome, metabolome and nucleic acid content analysis reveal novel clues and players significant to vesiculation, uncovering possible biological roles?

Focusing on results achieved within the biological significance of *Synechocystis* EVs:

Can EVs work as nanocapsules contributing to the homeostasis of other metals than copper? What mechanisms determine EVs packaging with metal-binding proteins under metal stressful conditions? How does the cell regulate the proportion of smooth-to-rough type LPS in released EVs depending on the condition? Can *Synechocystis* EVs work as

metal sinks when subjected to high amounts of a particular metal? And under metal deficiency, can they function to acquire it? Is TolC a metal-efflux mediating protein in other relevant bacterial species beyond *Synechocystis* (and *E. coli*)?

Bioengineering *Synechocystis* EVs will require to explore the following:

How can the regulation and encapsulation yield of vesicles be improved? Can another promoter and signal peptide perform best? Will the transformation of an already hyper-vesiculating strain (e.g. $\Delta slr1908$) with the construct assembled to target sfGFP to the periplasm improve the yield of packed EVs? If EVs reach the fish gut environment, are they degraded and the protein cargo released? Or are they internalized integrally by gut cells? Can EVs even cross the gut-blood barrier and reach the blood stream? Can EVs be packed with a selected fish antigen to trigger a specific immune response?

The reality is that these are all intertwined aspects. Like dominoes that can topple in both directions, breakthroughs in one given sub-topic can also become a novelty for the entire topic. The goal is to focus in detail in every feature of *Synechocystis* EVs, envisioning one day to zoom out and observe the full landscape concealed by these about to fall dominoes.

Throughout this work, distinct aspects of cyanobacterial EVs biology were addressed, particularly focusing on their biological functions and possible biotechnological applications. However, the impact of studying cyanobacterial EVs does not end here. Given cyanobacteria's evolutionary history and current important role in ecology, the study of cyanobacterial EVs has the prospect of elucidating some of the greatest questions in Biology. For example, an intriguing but poorly addressed matter is the relation of EVs with intra/inter-kingdom communication and exchange of genetic information. Have cyanobacterial vesicles any relevance for this topic?

Regarding intra-kingdom communication, cyanobacterial vesicles have been proposed as mediators of horizontal gene transfer (HGT) in oceans (Biller *et al.*, 2014). Phages are also implicated in this process, but they exhibit closer taxa ranges compared to EVs (Nazarian *et al.*, 2018). Meaning, while a given class of bacteriophages only infects a narrow group of bacteria, limiting the range of gene transfer, EVs released by one group of bacteria can easily be incorporated by phylogenetically unrelated prokaryotes. Allowing vesicles abundance in oceans, namely of cyanobacterial origin, to the fact that they shuttle diverse mobile genetic elements, it was recently suggested that cyanobacterial EVs participate(d) in the genetic modeling of marine microbial populations (Hackl *et al.*, 2020)

When thinking of vertical interactions between evolution levels, one can recall that EVs may be regarded as fossil signatures of primordial lipid structures that once participated in

life genesis (*Chapter I – Introduction, 1.1 EVs as fossils of the origin of life*). All organisms on Earth should then have a trace of this phenomenon, and EVs release is ubiquitous to all domains of life (Deatherage & Cookson, 2012). Thus, EVs could be considered as nano-lifts to study inter-kingdom communication. Vesicles have already been implicated with key biological questions related to evolution, such as the appearance of the eukaryotic endomembrane system (Gould *et al.*, 2016). Based on endosymbiotic theories, eukaryotes originated from an archaeal host that engulfed an α -proteobacterium (the mitochondrial ancestor) (Ku *et al.*, 2015). One theory supports that, once entrapped within the cytosol of the host, the mitochondrial ancestor continued to vesiculate. Vesicles could then accumulate and fuse with each other, forming organelles, or merge with the plasma membrane, shaping the cell envelope (Gould *et al.*, 2016). Interestingly, mitochondria are still known to secrete vesicles (Neuspiel *et al.*, 2008). In plants, chloroplasts have a well described internal vesicular transport system (Lindquist & Aronsson, 2018). However, it remains to be elucidated if chloroplasts liberate vesicles to the cytosol of vegetal cells. Chloroplasts ancestors, cyanobacteria, releases EVs. In this line of thinking, it is worth highlighting that there are cases where cyanobacteria live in symbiosis, protected inside their hosts and exchanging biological material with them, including through vesicles. In the cyanobacteria–water fern *Azolla microphylla* symbiotic association of perpetual nature, the nitrogen-fixing cyanobiont releases EVs, in which DNA was detected (Zheng *et al.*, 2009). This could suggest lateral gene transfer between the two symbiotic partners. More recently, in a diatom–cyanobacteria symbiosis, it was observed what appear to be the detachment of OIMVs from the cyanobacterium envelope (Flores *et al.*, 2022). It was hypothesized that these vesicles could represent a vehicle for the transfer of fixed nitrogen to the cytoplasm of the diatom. There are indications that this solid partnership led the cyanobiont to adapt to an intracellular lifestyle, reorganizing its genome (Hilton *et al.*, 2013) and morphology (Flores *et al.*, 2022). Even the use of cyanobacterial EVs for biotechnological applications in higher organisms could be regarded as an one-way imposed informational exchange. These cyanobacterial vesicles' cross-kingdom communication examples could drive the way to study their impact on the evolution between prokaryotes and eukaryotes, namely on the flux of cyanobacterial genes into plant nuclear genomes (Deutsch *et al.*, 2008).

Dissecting fundamental aspects of vesicle biology in cyanobacteria could then breach and elucidate frontiers within and between kingdoms, deciphering some of the ecological and evolutionary roles of cyanobacterial EVs.

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