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MÁSTER EN BIOLOGÍA MOLECULAR Y BIOMEDICINA

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Characterization of OMVs from *Brucella abortus* 2308

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Content

Abstract.....	3
Resumen	4
Introduction	5
1. Composition of outer membrane vesicles.....	5
2. Formation of OMVs	7
3. Functions of OMV	10
4. Possible roles of RNA contained within OMVs	12
<i>S. vesiculosa</i> M7 ^T	12
<i>Brucella</i> sp.	12
Hypothesis and objectives	14
Materials and methods.....	15
Bacterial strains and growth conditions.....	15
Isolation and purification of OMVs from culture medium	15
Staining and confocal laser scanning microscopy	16
Flow cytometry	18
Transmission Electron Microscopy (TEM)	18
Results and discussion	19
Detection of <i>S. vesiculosa</i> M7 ^T and <i>B. abortus</i> 2308 by confocal microscopy	19
Staining of <i>S. vesiculosa</i> M7 ^T with different membrane dyes	19
Staining of <i>S. vesiculosa</i> M7 ^T OMVs with Nile red, SYTO [®] RNaselect [™] , DiIC 18-DS and DAPI	20
Staining of <i>S. vesiculosa</i> M7 ^T OMVs with Nile red and SYTO [®] RNaselect [™]	20
Staining of <i>S. vesiculosa</i> M7 ^T OMVs with DiIC-18-DS and SYTO [®] RNaselect [™]	24
Staining of <i>B. abortus</i> 2308 OMVs with Nile Red and SYTO [®] RNaselect [™]	26
Staining of <i>B. abortus</i> 2308 OMVs with DiIC-18-DS and SYTO [®] RNaselect [™]	26
Detection of <i>S. vesiculosa</i> M7 ^T and <i>B. abortus</i> 2308 OMVs by flow cytometry	28
Visualization of <i>S. vesiculosa</i> M7 ^T OMVs by electron microscopy	30
Conclusions	32
References	33

Abstract

Outer membrane vesicles (OMVs) were recently described as a new type of secretion system. Moreover, it has been observed that some bacteria can secrete two different types of OMVs, one formed only by outer membrane and periplasmic components, and a second type that includes also inner membrane and cytoplasmic components. This opens the field to the presence in these vesicles of cytoplasmic components such as sRNAs. Among the multiple functions of these sRNAs, one of them is the interference with host functions, as it has been described for the first time for an *Escherichia coli* sRNA acting on *Caenorhabditis elegans*. *Brucella* species produce such OMVs, and our hypothesis is that they probably contain both types of vesicles. If so, they could contain sRNAs with the potential of interfering with the host. To test this we have isolated and visualized *Brucella abortus* 2308 OMVs and their content by different techniques such as confocal microscopy, flow cytometry and electron microscopy, using *Shewanella vesiculosa* M7^T to standardize all protocols. Our results indicate that the three methods are useful for different purposes, and thus complementaries, although they need to be improved to fully demonstrate the presence or not of RNA in some of the OMVs.

Keywords: OMVs, sRNA, *Shewanella vesiculosa* M7^T, *Brucella abortus* 2308, confocal microscopy, electron microscopy, flow cytometry

Resumen

Las vesículas de membrana externa (VMEs) han sido descritas recientemente como un nuevo sistema de secreción. Además, se ha observado que algunas bacterias pueden secretar dos tipos diferentes de VMEs, uno formado sólo por membrana externa y componentes periplásmicos, y el otro tipo además de estos componentes incluye también membrana interna y componentes citoplasmáticos. Esto permite pensar que estas últimas vesículas pueden contener componentes citoplasmáticos como sRNAs. Estas moléculas tienen múltiples funciones incluyendo la interferencia con las funciones del hospedador, como ha sido descrito por primera vez para sRNAs de *Escherichia coli* que son capaces de actuar en *Caenorhabditis elegans*. Las especies de *Brucella* producen VMEs, por lo que pensamos que estas bacterias probablemente contienen los dos tipos de vesículas y de ser así, podrían contener sRNAs que tendrían capacidad de interactuar con el hospedador. Para probar esto aislamos y visualizamos VMEs de *Brucella abortus* 2308 y su contenido mediante diferentes técnicas, tales como microscopia confocal, citometría de flujo y microscopia electrónica, usando *Shewanella vesiculosa* M7^T para estandarizar todos los protocolos. Nuestros resultados indican que los tres métodos son adecuados para diferentes propósitos, y por lo tanto complementarios, aunque necesitan ser mejorados para demostrar por completo la presencia o no de RNA en algunas de las VMEs.

Palabras clave: VMEs, sRNA, *Shewanella vesiculosa* M7^T, *Brucella abortus* 2308, microscopia confocal, citometría de flujo, microscopia electrónica.

Introduction

The secretion of different compounds is crucial for bacteria, because thus they can interact with the external environment, detect changes in it, and respond facing them. Bacteria secrete products by different secretory systems. There are 7 commonly accepted and different secretion systems in bacteria. The type I, type III, type IV and type VI transfer the secreted material directly into the extracellular milieu or into another cell, while the type II and type V secretion systems are two-step processes in which proteins are transported first through the inner membrane (IM) and then through the outer membrane (OM) (Abdallah *et al.*, 2007, Kuehn and Kesty, 2005). Finally, the type VII secretion system is a specific secretion system of mycobacteria that transports extracellular proteins across their hydrophobic and highly impermeable cell wall (Abdallah *et al.*, 2007). Aside from these secretion systems the existence of outer membrane vesicles (OMVs) has been reported in gram negative bacteria. These OMVs were first observed in the 1960s, by electron microscopic studies of bacterial structures (Kim *et al.*, 2015). However, it was thought that they had a residual role. Nevertheless, in recent years, the number of studies about OMVs has increased significantly demonstrating that OMVs play an important role in survival and function of gram negative bacteria, since containing different biologically active proteins and they are able to carry out diverse biological processes (Kulp and Kuehn, 2010). Moreover, these vesicles can be incorporated within the cells by the endocytic pathway, and their content may act in the host. For this reason, the study of the composition of these vesicles is of great importance.

1. Composition of outer membrane vesicles

OMVs are spheroid particles with a size approximately between 20-250 nm of diameter (Pérez-Cruz *et al.*, 2013). OMVs contain bacterial lipids, outer membrane proteins, periplasmic content and other insoluble components that are released to the environment to carry out different functions (signaling molecules, toxins, etc ...) (Deatherage and Cookson, 2012). Moreover, although these OMVs are originated from the bacterial outer membrane, their composition reflects a specific enrichment and depletion of different outer membrane proteins and lipids (Park *et al.*, 2011), and therefore, they are not vesicles formed by random fragments of outer membrane, but, as it will be explained later, they reflect a specific formation mechanism.

In addition to these components, OMVs can contain other typically cytoplasmic components such as DNA. In 1989, Garon *et al* observed that vesicles of *Borrellia burgdorferi* contained DNA that was protected from degradation, since it was detected after treatment of purified vesicles with DNAsas. Later, the presence of vesicles and lineal chromosomal and plasmid (lineal and circular) DNA was detected in other bacteria (table 1) (Dorward and Garon, 1990). Over time, DNA and

other cytoplasmic compounds, such as ATP, have been found in many other pathogenic and non-pathogenic gram negative bacteria, such as *Shewanella vesiculosa* M7^T, *Neisseria gonorrhoeae* DMS 15130, *Pseudomonas aeruginosa* PAO1 and *Acinetobacter baumannii* AB41 (Pérez-Cruz *et al.*, 2013; Pérez-Cruz *et al.*, 2015). However, little is known about the mechanism by which these components are incorporated into OMVs. Several models have been proposed to explain the incorporation of such components into the vesicles. The first model proposes that extracellular DNA present in the medium due to cell lysis can be internalized in the vesicles by a mechanism similar to bacterial transformation (Renelli *et al.*, 2004). The second model proposes that DNA somehow passes through the periplasm in a protected form and is included in a conventional OMV (Pérez-Cruz *et al.*, 2015). Finally, the third model proposes that bacteria can produce OMVs with cytoplasmic content, such as DNA, as was demonstrated to *S. vesiculosa* M7^T and some pathogenic bacteria (Pérez-Cruz *et al.*, 2013; Pérez-Cruz *et al.*, 2015). In other words, it has recently been shown that gram negative bacteria are able to produce two types of vesicles at the same time (Pérez-Cruz *et al.*, 2013; Pérez-Cruz *et al.*, 2015). The first one is derived exclusively from the outer membrane, is formed by a single membrane, and does not contain cytoplasmic components; while the second type is derived from both outer and inner membrane, is then limited by a double membrane, and contains cytoplasmic components (figure 1). Therefore, the last type of OMVs corresponds with vesicles that could contain nucleic acids and other cytoplasmic compounds, such as ATP.

Table 1. Export of Vesicle-associated DNA by bacteria. Adapted from Dorward and Garon, 1990.

Species and strain	Types of DNAs
<i>Agrobacterium tumefaciens</i> 15955	Circular plasmid
<i>Bordetella pertussis</i> 3779	Lineal chromosomal
<i>Borrelia burgdorferi</i> Sh-2-82	Lineal plasmid, circular plasmid, lineal chromosomal
<i>Escherichia coli</i> 11775	Lineal chromosomal
<i>Haemophilus influenzae</i> clinical	Circular plasmid
<i>Haemophilus parainfluenzae</i> 33392	Circular plasmid
<i>Moraxella osloensis</i> 199976	Circular plasmid, lineal chromosomal
<i>Neisseria gonorrhoeae</i> 31426	Circular plasmid
<i>Pseudomonas aeruginosa</i> 10145	Lineal chromosomal
<i>Salmonella typhimurium</i> 13311	Lineal chromosomal
<i>Serratia marcescens</i> 13880	Lineal chromosomal
<i>Shigella dysenteriae</i> 13313	Circular plasmid, lineal chromosomal
<i>Shigella flexneri</i> 29903	Circular plasmid, lineal chromosomal
<i>Staphylococcus aureus</i> 12600	Not determined
<i>Streptococcus sanguis</i> 10556	Not determined
<i>Yersinia pestis</i> EV76	Circular plasmid, lineal chromosomal

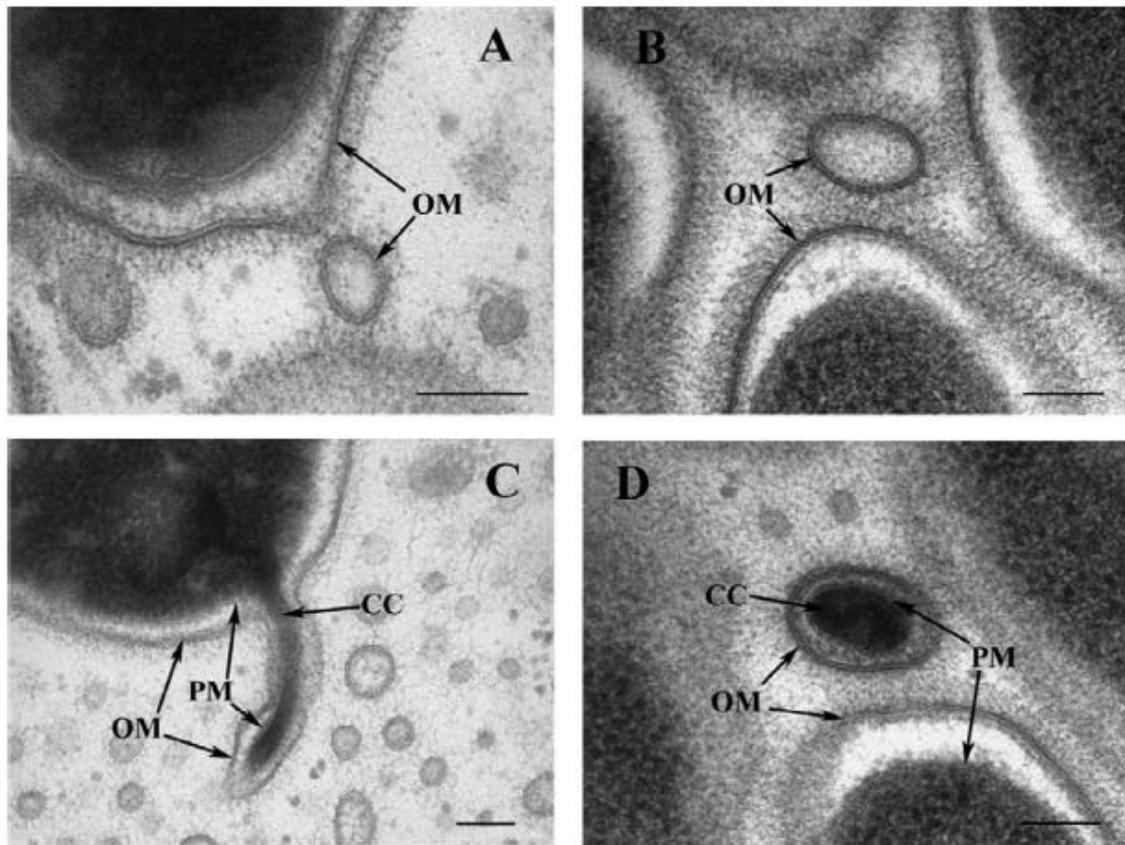


Figure 1. TEM micrographs of ultrathin sections from *S. vesiculosa* M7^T prepared by HPF-FS. (A and B) A view of OMVs extruded from cells. Only one bilayer is observed around the vesicles, with the same structure as the outer membrane (OM) of the cell (arrows). (C) OMVs being released from cells and dragging the plasma membrane (PM) and a portion of the cytoplasmic content (CC) in addition to the OM. (D) The same type of vesicle observed in panel C but once outside the cell. Bars, 100 nm (A, C) and 200 nm (B, D). Adapted from Pérez *et al.*, 2013.

2. Formation of OMVs

The actual mechanism of OMVs formation is currently unknown. However, there are three different mechanisms proposed (figure 2) (Mashburn-Warren *et al.*, 2006; Kim *et al.*, 2015; Kuehn and Kesty, 2005):

- Model 1. OMVs are formed when the outer membrane (OM) expands faster than the peptidoglycan layer. This produces localized detachment sites between the peptidoglycan and the OM layers due to a deficiency of lipoprotein linkages. If this asymmetric growth continues, areas of detachment will bulge and OMVs will be released from the outer membrane.
- Model 2. OMVs are formed when products released by the replacement of the peptidoglycan layer are not efficiently internalized into the bacterial cytoplasm to be

degraded and reused. This causes its accumulation in the periplasmic space generating a local bulge in the OM. Finally, the mechanic movement of OM produces the release of these products in the OMVs.

- Model 3. The chemical composition of LPS is an important mediator for the formation of OMVs. This model is based in the charge-to-charge repulsion resulting from the negative charge of B-band LPS, that generate instability of the membrane and a bulge which initiates the formation of OMVs. Moreover, PQS (Pseudomonas Quinolone Signal) mediates OMVs formation. These molecules sequester divalent ions (Mg^{+2}) that destabilize the outer membrane and then, promote the vesiculation.

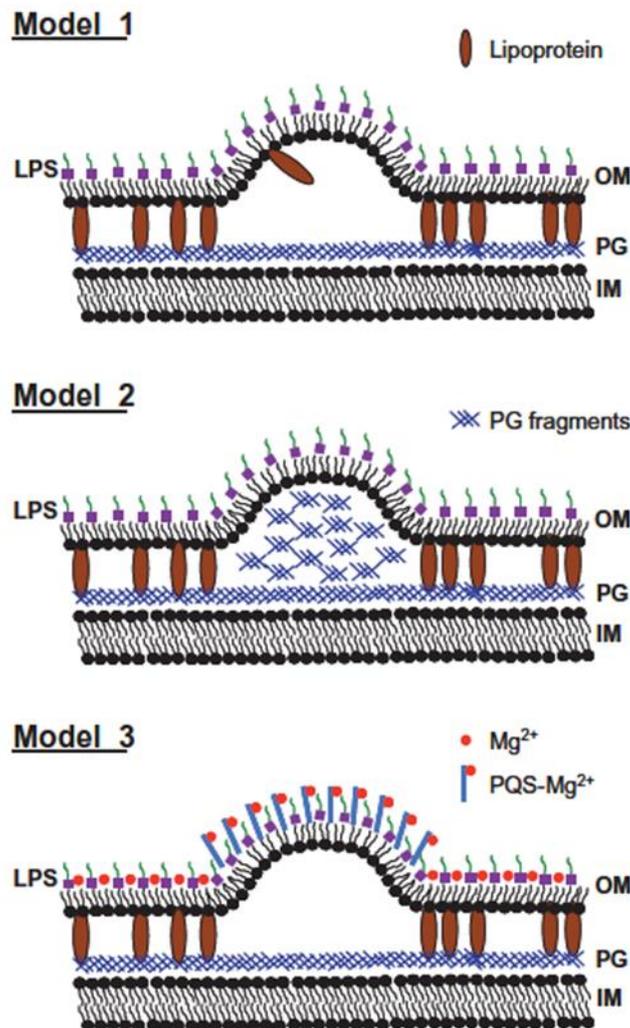


Figure 2. Proposed models for biogenesis of Gram-negative bacterial OMVs. Model 1: OMVs are liberated from regions on the cell surface where there are fewer amount of lipoproteins due to the faster expansion of the outer membrane. Model 2: Increase of blebbing of the outer membrane due to increase turgor pressure caused by accumulation of peptidoglycan fragments in the periplasm. Model 3: PQS sequesters the positive charge of Mg^{+2} , which results in enhanced anionic repulsion between LPS molecules and membrane blebbing. PQS, Pseudomonas quinolone signal. Mashburn-Warren *et al.*, 2006

The biogenesis of these OMVs is not a random process, but a process highly regulated. It was thought because the composition of vesicles is similar but not identical to outer membrane of bacteria, as it would be to expect if they were formed randomly. For example, Park *et al.* in 2011 showed that *Edwardsiella tarda* OMVs had a different composition than outer membrane (figure 3). The OMVs production is regulated by growth conditions and genetic control. As for growth conditions, it was shown that high growth temperatures can augment the number of OMVs produced (Kulp and Kuehn, 2010). This could be due to the activation of stress response pathways by generation of denatured proteins. Moreover, it is possible that this may also be due to more fluid membranes, augmentation of rate of cell division, and therefore high rates of cell wall growth and turnover. In fact, it is known that in exponential phase there is an increased production of OMVs. In addition, some bacteria produce more amount of OMVs when they grow with little nutrients, as OMVs contain enzymes that might degrade nutrients at a distance and convert them into nutrients that can be used by the bacteria (Kulp and Kuehn, 2010). Finally, external conditions that generate envelope stress also cause an increase of OMVs production in some bacteria, such as *E. coli* (Kulp and Kuehn, 2010; Deatherage and Cookson, 2012). As for genetic control, it was shown that some mutations favour the vesiculation. For example, mutations of components of

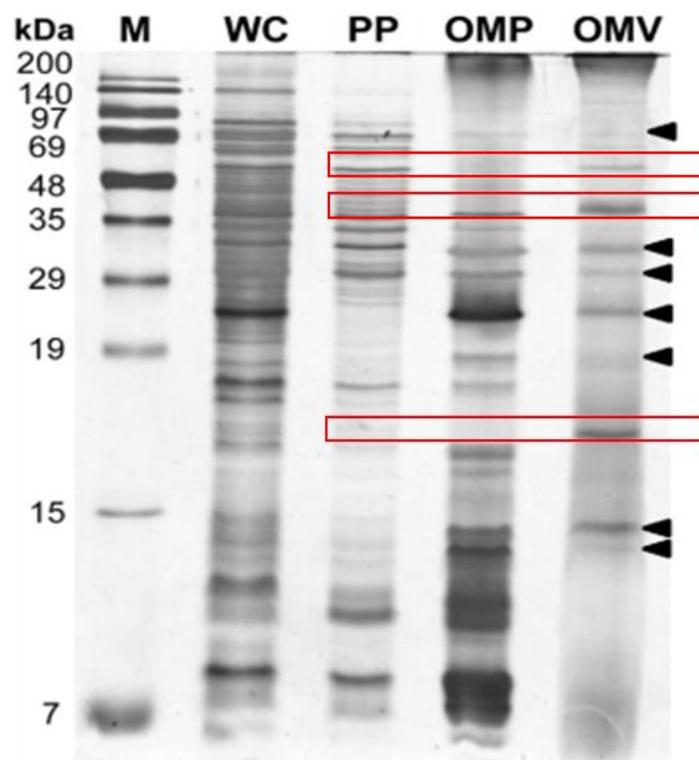


Figura 3. SDS-PAGE protein profiles of WCL, PPs, OMPs and OMVs. Arrowheads indicate OMVs polypeptides with molecular weights the same as those of OMPs while red box indicate OMVs polypeptides with different molecular weights to those of OMPs and PP. M: protein marker lane; WCL: whole cell lysate, PP: periplasmic proteins; OMPs: outer membrane proteins; OMVs: outer membrane vesicles. Adapted from Park *et al.*, 2011.

the Tol/Pal system cause an increase of OMVs production (Kulp and Kuehn, 2010). Moreover, mutations in some genes encoding cell-envelope-localized proteins, such as yphA and nlpA also favour the vesiculation; as happens with several genes involved in the SigmaE stress response pathway (Kulp and Kuehn, 2010). Moreover of these mutations exist others that also favour the vesiculation, so as we said previously, the vesiculation is a highly regulated process, therefore it is easy think that this process plays an important role in many bacterial functions and ultimately in the bacterial survival.

3. Functions of OMV

OMVs contain components that they are able to carry out many biological functions. In this way, OMVs are involved in the pathogenesis, biofilms formation, communication between species, nutrients acquisition and transfer of genetic material (Kulp and Kuehn, 2010).

3.1. Pathogenesis

Diverse Gram-negative pathogens released OMVs to promote their action in the host, since OMVs contained many virulence factors (table 2). For example, the adhesins are very important for pathogenic bacteria, especially for colonization of host tissues, because they mediate the coaggregation (Kulp and Kuehn, 2010). Therefore, the presence of multivalent membrane adhesion complexes in the OMVs can facilitate this process. Moreover, the formation of OMVs can also protect toxins of degradation with environment proteases, since their localization in membrane or lumen protect of their degradation. Finally, OMVs formation can facilitate the action of toxins a high concentrations and their action in a specific place.

3.2. Communication between species

Bacteria could use their OMVs to interact with other bacteria and thereby modulating the microbial environments and bacteria themselves (Kim *et al.*, 2015). That is to say, OMVs release by bacteria contain bioactive molecules which allowing positive or negative bacterial communication with others organisms (Deatherage and Cookson, 2012).

3.3. Biofilm formation

OMVs provide the majority of LPS to biofilm formation (Kulp and Kuehn, 2010). Moreover OMVs are associated with the entire biofilm matrix. It was showed that the addition of OMVs to a *Helicobacter pylori* culture stimulated biofilm production (Kulp and Kuehn, 2010). Therefore, OMVs allow biofilm formation, that is to say, their presence in biofilms are not solely the result of their entrapment in the matrix. Moreover, it is possible that OMVs stimulate biofilm formation due to surface DNA (Kulp and Kuehn, 2010).

Table 2. Different virulence factors in bacteria. Amano *et al.*, 2010

Species	Virulence factors
<i>Pseudomonas aeruginosa</i>	Proelastase, phospholipase C, hemolysin, murein hydrolases, alkaline phosphatase, Cif 2-Heptyl-3-hydroxy-4-quinolone (<i>Pseudomonas</i> quinolone signal; PQS)
<i>Helicobacter pylori</i>	Vacuolating cytotoxin (VacA), urease, <i>Helicobacter</i> cysteine-rich proteins (Hcp)
<i>Porphyromonas gingivalis</i>	Proteases (Arg-gingipain, Lys-gingipain), fimbriae
<i>Treponema denticola</i>	Proteases, protease and adhesion (dentilisin)
<i>Aggregatibacter actinomycetemcomitans</i>	Leukotoxin, peptidoglycan-associated lipoprotein
Enterotoxigenic <i>Escherichia coli</i> (ETEC)	Heat-labile enterotoxin (LT)
Shiga toxin-producing <i>E. coli</i> (STEC)	Shiga toxin
Enterohemorrhagic <i>E. coli</i> (EHEC)	Cytolysin A (ClyA), Shiga toxin
<i>Actinobacillus pleuropneumoniae</i>	Proteases, Apx toxins
<i>Borrelia burgdorferi</i>	Outer surface proteins (OspA, OspD)
<i>Bacteroides fragilis</i>	Haemagglutinin
<i>Salmonella typhi</i>	ClyA, outer membrane protein (OmpC)
<i>Shigella flexneri</i>	Invasion plasmid antigens (IpaB, IpaC, IpaD)
<i>Shigella dysenteriae</i>	Shiga toxin
<i>Xenorhabdus nematophilus</i>	Chitinase, bacteriocin, adhesin, pore-forming toxin
<i>Campylobacter jejuni</i>	Cytotoxic distending toxin (CDT)

3.4. Nutrient acquisition

As mentioned previously, OMVs contain degradative enzymes and receptor that may contribute to nutrient acquisition and therefore, to bacterial survival. For example, in OMVs of *P. aeruginosa* there are an active aminopeptidase (Kulp and Kuehn, 2010). This enzyme is able to release into the environment amino acids that may be critical to bacterial growth. Moreover, their OMVs contain PQS capable of binds iron, which is essential for bacterial viability and often limiting in biological environments (Kulp and Kuehn, 2010). Then, the nutrients are accessible in order that the bacteria can catch them.

3.5. Transfer of genetic material

The presence of nucleic acids within bacterial OMVs is a new horizontal gene transfer mechanism between different strains of gram-negative bacteria and even across species (Kulp and Kuehn, 2010). This mechanism allows the prokaryotic evolution, but also can allow the resistance or virulence gene transfer between bacteria. Moreover, this mechanism can allow the communication between different kingdoms, as it was shown for *E. coli* and *Caenorhabditis elegans* (Liu *et al.*, 2012). In this case, noncoding RNA of *E. coli* regulate gene expression and physiology conditions of *C. elegans*.

However, all these OMVs functions are not carried out of an isolated way, but many of them are carried out of a simultaneous way, because the same type of molecule can perform several of these functions together such as RNA.

4. Possible roles of RNA contained within OMVs

A high percentage of the bacterial genome is transcribed as non-coding RNA molecules, such as small intergenic RNAs (sRNAs) (Ortega *et al.*, 2014). These non-coding RNAs play an important role in bacteria, because regulate post-transcriptionally multiple processes. For example, they can control uptake and assimilation of nutrients, cell-to-cell communication, envelope homeostasis, biofilm formation and stress responses (Ortega *et al.*, 2014). Moreover, non-coding RNA may play an important role in host-microbe interaction (Knip *et al.*, 2014). In fact, RNA have been found to be mobile within organisms, of the same species or of different species (Knip *et al.*, 2014); even between different kingdoms, for instance between animals and bacteria (Liu *et al.*, 2012). So, RNA can be other molecule of communication between kingdoms. However, the mechanism by which these RNAs pass from one organism to another without being degraded by environment RNase is unknown. One of the proposed mechanisms for this exchange involved the formation of membrane vesicles, which it has been shown at least in the case of *Trypanosoma cruzi* parasite (Garcia-silva *et al.*, 2014). This is thought because, as it was said previously, it has been found that there are OMVs which contain cytoplasmic content, such as DNA. So, it is also possible that there may be bacterial RNA. Moreover, this RNA within vesicles may be protected of environment RNases degradation, allowing its arrival at the host in which exert its function. Until now, the presence of diverse types of RNA only had been demonstrated in the membrane vesicles derived from eukaryotic microorganisms (Barteneva *et al.*, 2013). Nevertheless, still it is unknown if the presence of RNA in eukaryotic membrane vesicles is translatable an gram negative bacteria, such as *S. vesiculosa* M7^T in which it is known that produce OMVs with cytoplasmic content , and more specifically a pathogens gram negative bacteria, such as *Brucella sp.* where this RNA may act as new virulence factor.

***S. vesiculosa* M7^T**

S. vesiculosa M7^T is a cold-adapted Antarctic bacteria that produce huge amounts of OMVs. So, it is an excellent model for studying the vesiculation process. Moreover, as mentioned before *S. vesiculosa* M7^T produces different types of OMVs, including vesicles with a single bilayer and another more complex type with a double bilayer and containing cytoplasmic components.

Brucella sp.

The members of the genus *Brucella* are causative agents of brucellosis, a zoonotic with 500,000 new cases per year (Atluri *et al.*, 2011), although the real number of cases could be much higher. Brucellosis is a chronic infectious bacterial disease that affects several species of domestic and wild animals, as well as humans (Galinska and Zagorski, 2013). This disease is a worldwide spread zoonotic transmitted from domestic animals to humans, through consumption of

unpasteurized dairy products or contact with infected animals (Atluri *et al.*, 2011). Moreover, it is known that three members of the genus *Brucella*, *Brucella mellitensis*, *Brucella abortus* and *Brucella suis* are major causes of economic loss and human suffering as well as potential bioterrorism threats (Lamontagne *et al.*, 2006). Therefore, the study of this genus is very important.

Brucella, in association with animals or humans, is a strictly intracellular live bacteria, so the production of infections depends of the ability of *Brucella* to survive to the harsh environmental conditions that find along its replicative cycle in different cell types, such as ROI's, acid pH and nutritional deprivation (Roop *et al.*, 2004). In consequence, the host adaptation and the intracellular survival mechanisms are an essential virulence factor to *Brucella*, but not restricted to this genus. In fact, many pathogens in their interaction with the host are able to, on the one hand to detect changes in the environment and respond to these changes altering the expression of their genes, and on the other hand to modify somehow the behavior of the host, altering the host capacity to respond to infection. In the vast majority of cases reported in the literature, this host modification is accomplished by the secretion of virulence factor of diverse nature, for example proteins, lipids or carbohydrates. Therefore, to understand how host modification is produced is necessary to know the different secretion systems in bacteria, as well as the molecules involved in this modification.

Hypothesis and objectives

Brucella species produce OMVs. Our hypothesis is that there are probably two populations of OMVs, the ones derived exclusively from the outer membrane and the ones derived from the inner and outer membranes. If so, this second set of OMVs could contain sRNAs with the potential of interfering with the host, a function known to be performed by *Brucella*.

Therefore, the objectives proposed for this study are:

- Standardized protocols to isolate and visualize OMVs by confocal and electron microscopy, and flow cytometry, using *S. vesiculosa* M7^T.
- Isolate and visualize *B. abortus* 2308 OMVs and their content by confocal microscopy and flow cytometry.

Materials and methods

Bacterial strains and growth conditions

In this study, we used *S. vesiculosa* M7^T, a cold-adapted Antarctic bacterium, which grows at temperatures ranging from -4 °C to 34 °C (Pérez-Cruz *et al.*, 2013). *S. vesiculosa* M7^T was provided by E. Mercade research group. To do the different experiments, this bacteria was grown on Trypticase soy agar (TSA) (Conda) for 5 days at room temperature, and on Trypticase soy broth (TSB) (Conda) for 2 days at 15 °C. Moreover, we used *B. abortus* 2308, a pathogenic bacterium, which was grown at 37°C with 5% CO₂ on TSB (Conda) until early exponential phase. For liquid cultures, an orbital shaker at 100 rpm was used.

In addition, in some cases TSB was ultracentrifuged at 40,000 X g and filtrated through 0.22 µm-pore-size-filter before bacterial inoculation to removed fluorescent particles. Then, this medium was used to grow *S. vesiculosa* M7^T or *B. abortus* 2308 in the same conditions that in culture medium without untracentrifuge.

Isolation and purification of OMVs from culture medium

OMVs were isolated from liquid cultures of *S. vesiculosa* M7^T and *B. abortus* 2308 as previously described (Pérez-Cruz *et al.*, 2013), with some modifications (figure 4). Briefly, 300 ml of TSB was inoculated with the necessary amount of a previously grown culture of bacteria to obtain an OD of 0.01. Once grown until exponential phase, the cells were pelleted by centrifugation at 10,000 X g for 10 min at 4 °C in an Avanti J-301 centrifuge (Beckman coulter) with JA-10 rotor (Beckman), and the supernatant was filtered through 0.22- µm-pore-size filters

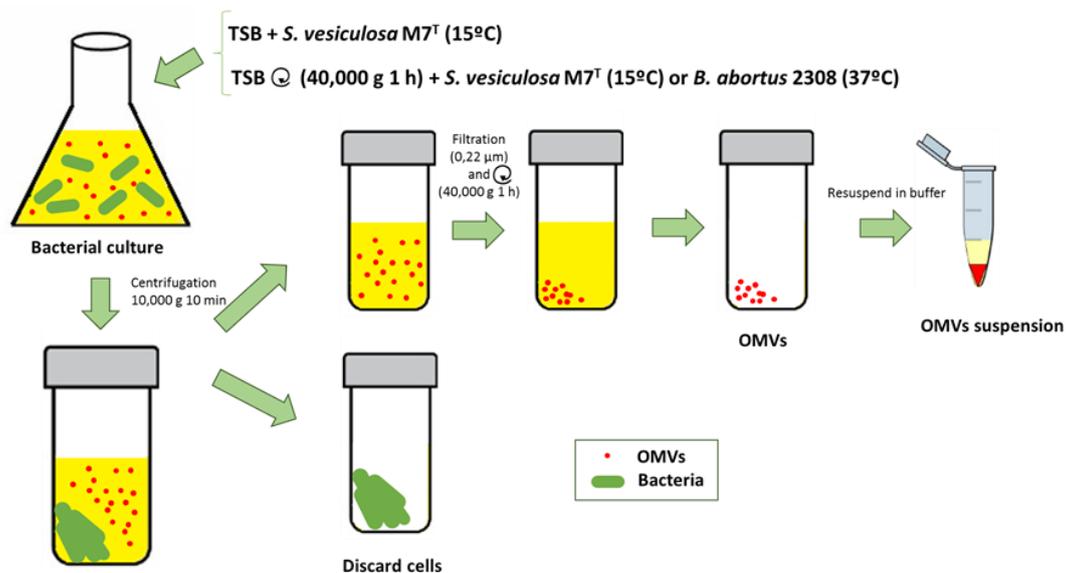


Figure 4. Schedule of OMVs isolation. The most representative process steps of OMVs isolation are show

(Millipore) to remove remaining bacterial cells. As a control, filtered supernatants were checked for bacterial contamination by plating 0.5 mL of supernatant on a TSA plate followed by 5 days incubation at room temperature for *S. vesiculosa* M7^T and culturing 1 mL of supernatant on a TSB medium followed by 2 days incubation at 37° for *B. abortus* 2308. The filtered supernatants were then centrifuged at 40, 000 X g for 1 h at 4 °C in an Avanti J-301 centrifuge (Beckman coulter) with JA-30.50 rotor (Beckman). Pelleted vesicles were resuspended in 8 mL of 50 mM HEPES (pH 6.8) (Sigma) and centrifuged at 40,000 X g for 1 h at 4°C in an Optima Max XP centrifuge (Beckman coulter) with TLA-55 rotor (Beckman). Then, vesicles were resuspended in 1 mL of 50 mM HEPES (pH 6.8). Finally, OMVs were stored at -20 °C until use.

Staining and confocal laser scanning microscopy

OMVs samples were incubated with 0.5 µg/mL of Nile Red (Sigma) or 1 µmol/ L of red fluorescent Lipophilic Tracer DiIC-18-DS (Life Technologies) and 1 µmol/L of SYTO ® RNASelect™ Green Fluorescent Cell Stain (Life Technologies). Moreover, 1 ng/ml of Dapi (AppliChem) was used to same experiments. All samples were incubated during 45 min at 37 °C in orbital shaker. Bacteria sample were incubated with 1 µmol/ L of red fluorescent Lipophilic Tracers DiI, DiD oil, DiIC-18-DS, SP-DiIC18 and 5’5-pH2-DiIC 18 (Life Technologies) during 45 min at 37 °C in orbital shaker (figure 5). After incubation samples were washed twice in PBS (1x) followed by centrifugation at a speed of 13, 600 rpm 3 min in the case of bacteria and by

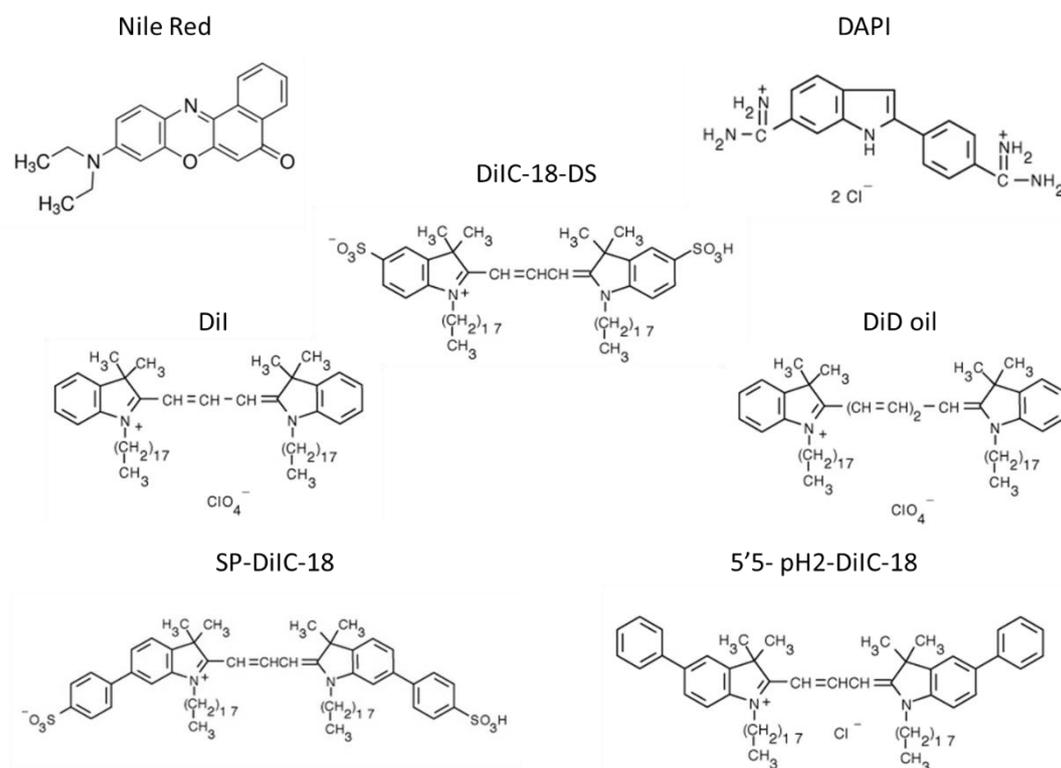


Figure 5. Chemical structure of different stain used. It was shown the chemical structure of Nile red, DAPI, DiIC-18-DS, DiI, DiD oil, SP-DiIC-18 and 5’5-pH2-DiIC-18.

filtration through 30,000 Dalton filters and centrifugation at 4,000 rpm 10 min in the case of OMVs. Once stained, 2 μL of OMVs and bacteria were placed onto a glass slide with 5 μL of mounting medium, Mowiol (Sigma).

Confocal images (1024 x 1024 pixels) were acquire sequentially on a SP-5 laser-scan microscope (Leica microsystems) with 63x 1.4 NA objective, a 2 Airy units pinhole, x7 electron zoom and 400 Hz speed using LAS AF acquisition software. Samples were excited sequentially with 405 nm, 488 nm, 543 nm and 633 nm laser lines and emission capture between 580-650 nm (Nile red), 509-540 nm (Syto RNaselect), 414-473 nm (DAPI), 555-600 nm (DiIC-18-DS), 553-625 nm (DiI), 643-725 nm (DiD oil), 553-625 nm (SP-DiIC 18) and 604-650 nm (5'5 pH2-DiIC 18) (figure 6). Images are presented after digital adjustment of brightness and contrast to maximize signal.

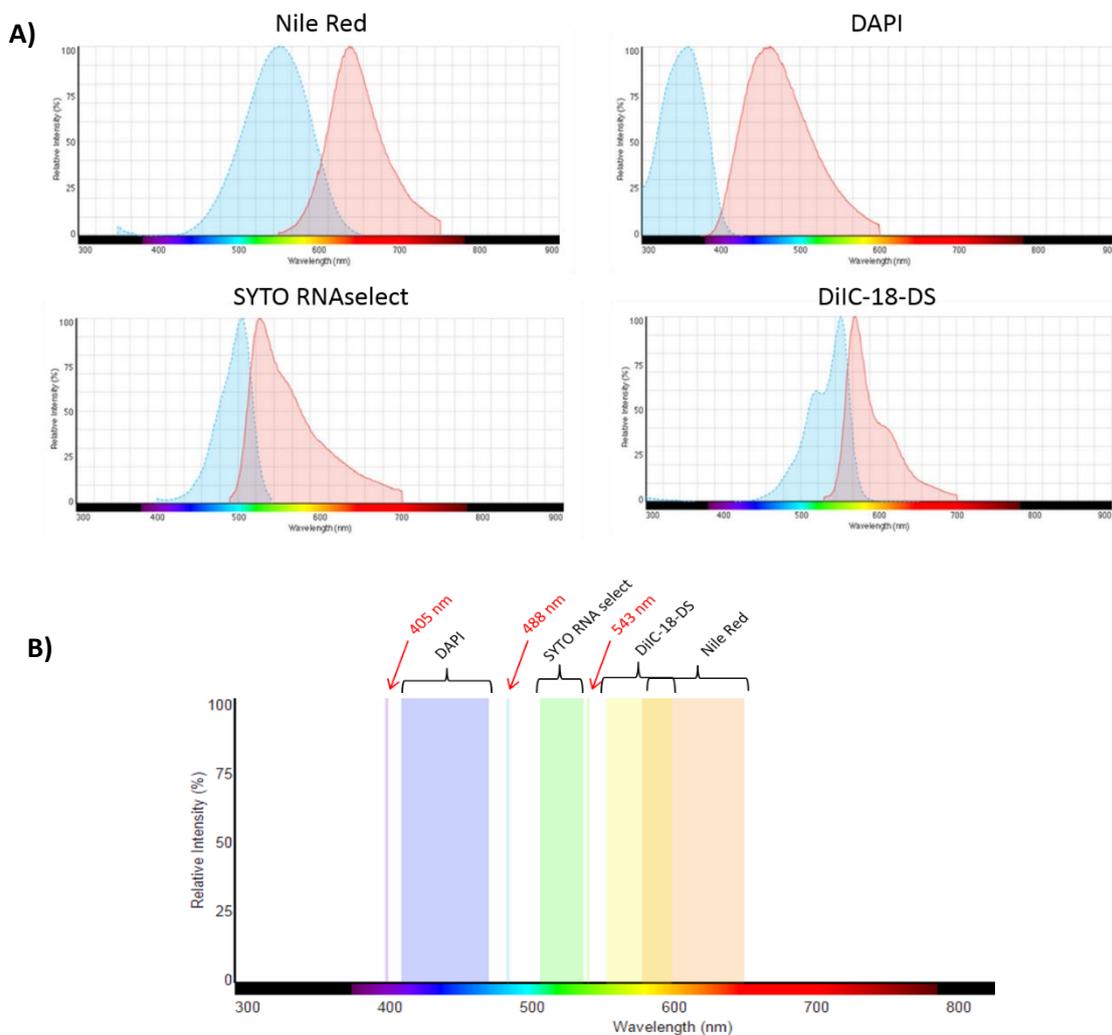


Figure 6. Fluorescence spectra of dyes used to stain OMVs. (A) Spectra of Nile red, DAPI, SYTO RNaselect and DiIC-18-DS. (B) Laser and emission spectra of dyes used to study OMVs by confocal microscopy.

Flow cytometry

S. vesiculosa M7^T and *B. abortus* 2308 OMVs were incubated with 0.5 µg/mL of Nile Red (Sigma). Samples were incubated during 45 min at 37 °C in orbital shaker. Then, samples were visualized by MACSQuant® VYB flow cytometer using MACSQuantify™ software. This flow cytometer allows detection of particles until 50 nm, so it is possible detect the majority of OMVs. Samples were excited with 405 nm, 488 nm and 561 nm lasers and emission capture at 450/50 nm (DAPI), 525/50 nm (SYTO RNaselect) and 615/20 nm (Nile red). Finally, images were analyzed using MACSQuantify™ software.

Transmission Electron Microscopy (TEM)

Isolated OMVs and bacteria were centrifuged at 40,000 X g for 1 h at 4 °C in an Optima Max XP centrifuge (Beckman coulter) with TLA-55 rotor (Beckman). Then, pelleted samples were fixed with 1 mL of 3% glutaraldehyde (25% glutaraldehyde; 0.4M phosphate buffer; 0.5% Cl₂Ca; 4% O₄Os) in 0.12 M phosphate buffer (pH 7.2-7.4) (NaH₂PO₄H₂O; K₂HPO₄) during 10 min at room temperature. After, samples were washed two times with 0.12 M phosphate buffer (pH 7.2-7.4) and each time, they were centrifuged at 14,000 rpm for 30 min. Then, samples were post-fixed with osmium tetroxide (glucose; 0.4 M phosphate buffer (pH 7.2-7.4); 0.5% Cl₂Ca; 4% O₄Os) during 3 h at room temperature in mild agitation and protected from light. After, pellets were washed 15 min two times at 4°C with washing solution (mother solution; 0.2 N NaOH) pH 4.5-4.6. Then, pellets were contrasted with contrast solution of uranyl acetate (mother solution pH 4.5-4.6; 0.2 N NaOH; uranyl acetate) during 90 min at 4°C, and washed two times during 15 min at 4 °C with washing solution pH 4.5-4.6. After, samples were dehydrated following the next series: 1 min in 15% acetone, 10 min in 30% acetone; 10 min in 50 % acetone; 10 min in 70 % acetone; 15 min in 80% acetone; 2 x 10 min in 95% acetone; 2 x 10 min in 100 % anhydrous acetone. Finally, pellets were included in araldite (Durcupan AMC Fluka). For that, anhydrous acetone were replaced by a mixture with 75 % anhydrous acetone and 25% B mixture [epoxiresin (chemical 4411); hardener (chemika 44612); plasticizer (chemical 44614); accelerator (chemika 44613)] during 2 h. Then, it was replaced by a mixture with 25 % anhydrous acetone and 75% B mixture. Finally, pellets were left in a pure B mixture overnight. The next morning B mixture was replaced and put to polymerize at 60°C during 3 days. Then, samples were cut to do semithin and ultrathin sections and placed on nickel grids, and examined at 80 kV with a JEOL-JEM-1011 transmission electron microscope. Images were captured digitally by an ORISUS SC 1000 CCD (Gatan).

Results and discussion

The research group that I incorporate for the development of this work is focused on the study of the role that possible sRNAs in *Brucella* OMVs play during eukaryotic cell infection. Therefore, the aim that we proposed was to study if *B. abortus* 2308 OMVs contain some type of RNA inside by confocal microscopy and flow cytometry, and also visualize OMVs of *B. abortus* 2308 by electron microscopy.

To carry out the objective proposed, previously all protocols were standardized with *S. vesiculosa* M7^T, a gram negative bacteria no pathogenic in which had already detected the presence of vesicles with outer and inner membranes and hence with cytoplasmic compounds, such as DNA (Pérez-Cruz *et al.*, 2013).

Detection of *S. vesiculosa* M7^T and *B. abortus* 2308 by confocal microscopy

Staining of *S. vesiculosa* M7^T with different membrane dyes

To study OMVs, initially we stained *S. vesiculosa* M7^T with different membrane dyes: Nile red, DiI, DiD oil, DiIC-18-DS, SP-DiIC-18 and 5'5 pH2-DiIC-18-DS. In this manner, we wanted to see that dyes were more efficient to stain bacterial membrane, and therefore they will stain better the membrane of OMVs. Thus, once stained the bacteria we observed by confocal microscopy that only Nile red and DiIC-18-DS were able to stain efficiently *S. vesiculosa* M7^T membrane (figure 7A). This is known because when we observe the images obtained by bright field microscopy (figure 7B), we can observe the same amount of bacteria in the samples stained with Nile red and DiIC-18-DS dyes that when we detect the fluorescence by confocal microscopy.

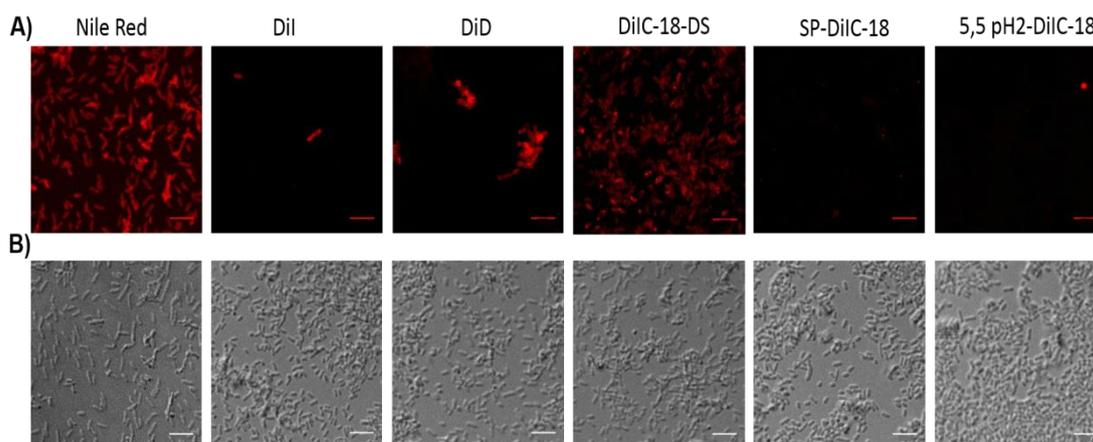


Figure 7. Staining of *S. vesiculosa* M7^T with different membrane dyes. (A) Confocal microscopy analysis from *S. vesiculosa* M7^T stained with different lipid tracer dyes: DiI, DiD, DiIC-18-DS, SP-DiIC-18 and 5'5 pH 2-DiIC-18-DS. (B) Bright field images of *S. vesiculosa* M7^T stained with different membrane dyes. The scale bar is equivalent to 5 μ m in the images.

However, we can observe more bacteria in the samples stained with DiI, SP-DiIC-18 and 5'5 pH2-DiIC-18-DS dyes by bright field microscopy that when we detect the fluorescence of these samples (figure 7). In this way, for further experiments we will use only Nile red or DiIC-18-DS to stain OMVs because they can stain efficiently *S. vesiculosa* M7^T membrane.

Staining of *S. vesiculosa* M7^T OMVs with Nile red, SYTO® RNaselect™, DiIC 18-DS and DAPI

Once selected the membrane dyes that we would use to stain OMVs, and having selected SYTO RNaselect to stain RNA and DAPI for DNA, we perform controls to see whether these dyes were detected in some emission channel that were not theirs. To test this, *S. vesiculosa* M7^T OMVs were stained with one dye: Nile red, DiIC-18-DS, SYTO RNaselect or DAPI. Thus, as we can see in figure 8, samples of OMVs stained with Nile red, DiIC-18-DS and SYTO RNaselect, in addition to emit fluorescence in itself emission spectra, and also we can detect fluorescence in DAPI channel in all cases. Moreover, we can observe that OMVs stained with DAPI emit fluorescence in DAPI channel, but also in Nile red and SYTO RNaselect emission channel (figure 8D). Based on these data, we decided that we will not use DAPI to stain nucleic acids, because, we could not trust of the detected signal. Thus, hereafter OMVs only will be stained with Nile red or DiIC-18-DS to stain the membrane and with SYTO RNaselect to stain RNA.

Staining of *S. vesiculosa* M7^T OMVs with Nile red and SYTO® RNaselect™

We wanted to examine whether OMVs from *S. vesiculosa* M7^T contains RNA, by applying a fluorescent labelling method. Using this method, lipids of OMVs were labelled with the dye Nile red and RNAs with SYTO RNaselect dye.

Thereby, if we only observe the images of *S. vesiculosa* M7^T OMVs stained with Nile red and SYTO RNaselect, it is possible think that these OMVs contain nucleic acid, and more specifically RNA (figure 9A). Since, we detected fluorescence in Nile red and SYTO RNaselect spectrum, and some of this fluorescence colocalized. However, when culture medium control was performed, it was detected similar fluorescent signal to the obtained in images of OMVs samples (figure 9B). This indicates that any of the materials used for microscopy have autofluorescent particles or they are able to be stained with these dyes employed. It is a problem, because all particles of less than 200 nm, including OMVs, will be seen as dots. Thus, it is difficult to distinguish whether we are observing OMVs or other particles present in the medium.

Thus, to detect from where were the fluorescent particles, we realized different controls. Mounting medium was not responsible for the fluorescent particles detected in the control; because when we observed only mounting medium we did not detect fluorescence (figure 10A). Moreover, neither fluorescence was detected when we visualized only Nile red with mounting medium (figure 10B). Thereby, we thought that fluorescent particles detected in the control were in the culture medium.

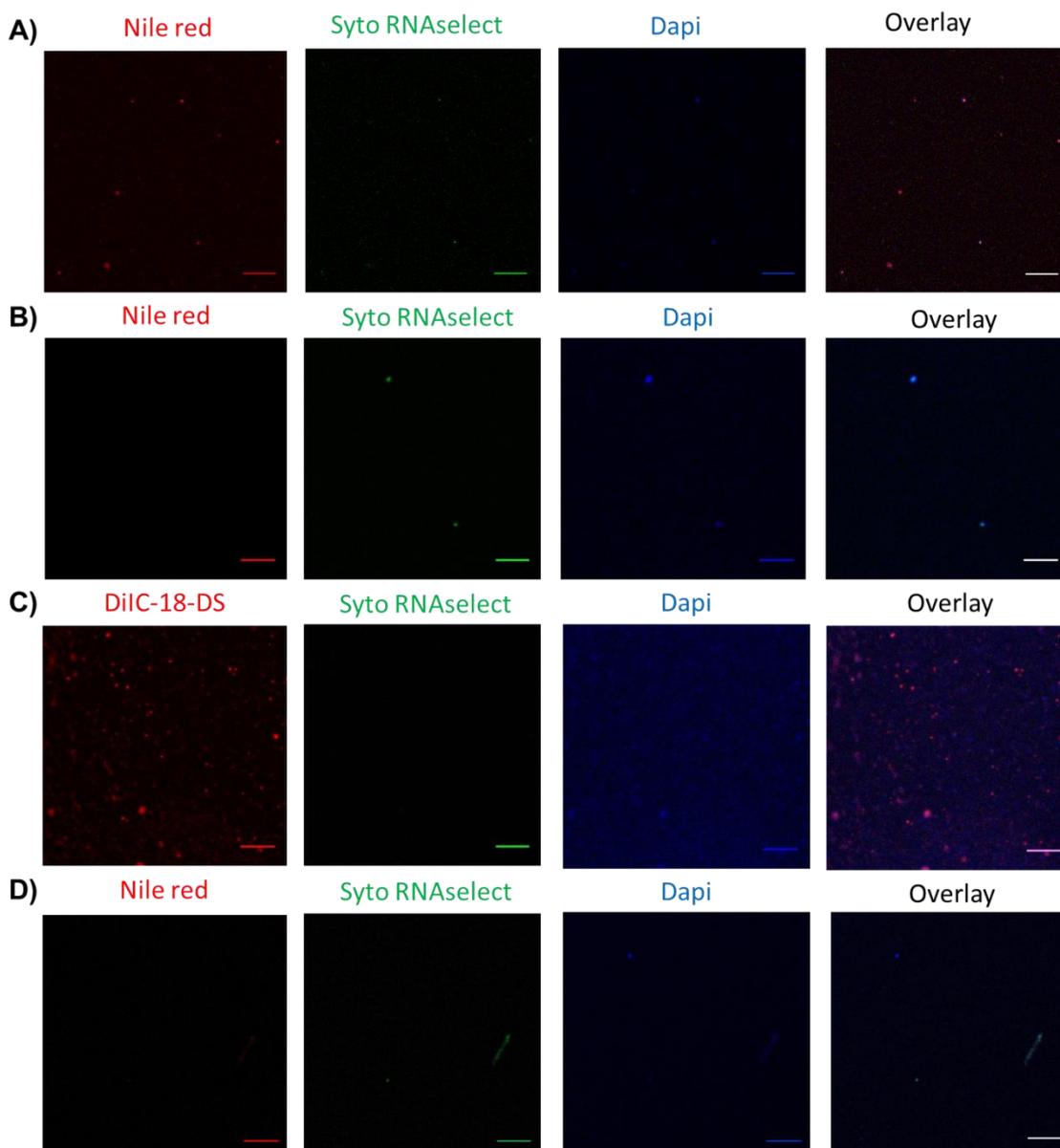


Figure 8. Control of dyes used in confocal microscopy analysis. (A) Control of Nile red emission in others spectrums. OMVs were stained only with Nile red. (B) Control of SYTO® RNaselect™ emission in others spectrums. OMVs were stained only with SYTO® RNaselect™. (C) Control of DiIC-18-DS emission in others spectrums. OMVs were stained only with DiIC-18 DS. (D) Control of DAPI emission in others spectrums. OMVs were stained only with DAPI. The scale bar is equivalent to 5 μm in the images.

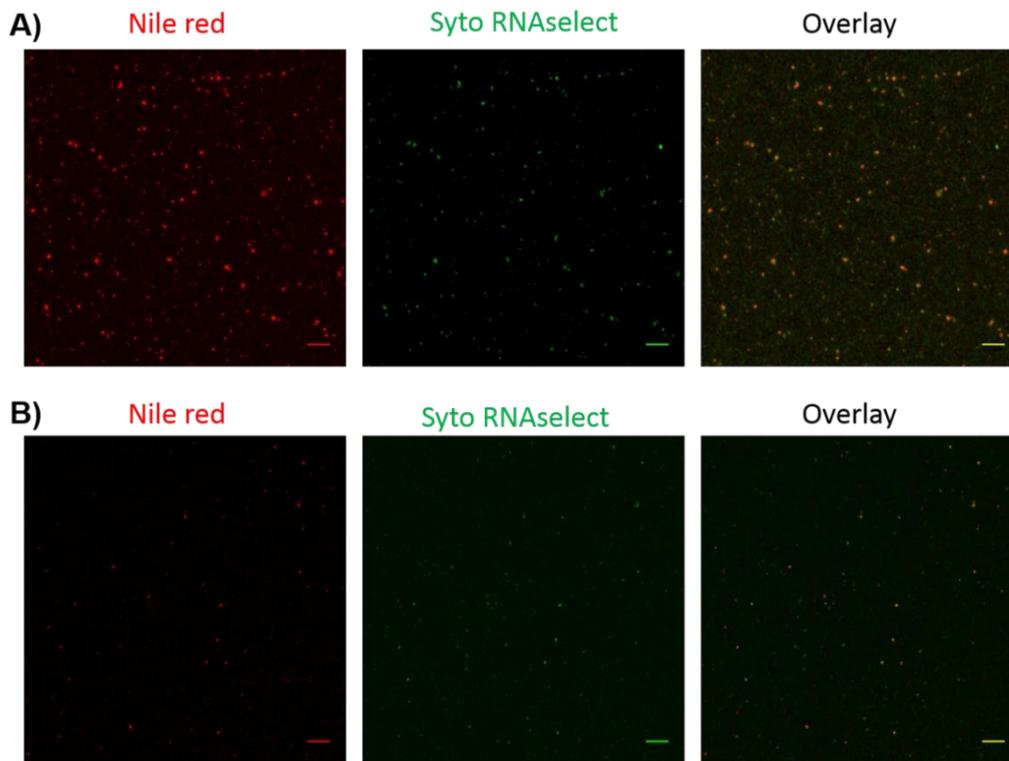


Figure 9. Confocal microscopy analysis of OMVs from *S. vesiculosa* M7^T. (A) OMVs obtained in normal medium stained with membrane dye, Nile red (red) and RNA-specific dye, SYTO RNaselect (green). (B) Medium control without OMVs stained with membrane dye, Nile red (red) and RNA-specific dye, SYTO RNaselect (green). The scale bar is equivalent to 5 μm in the images.

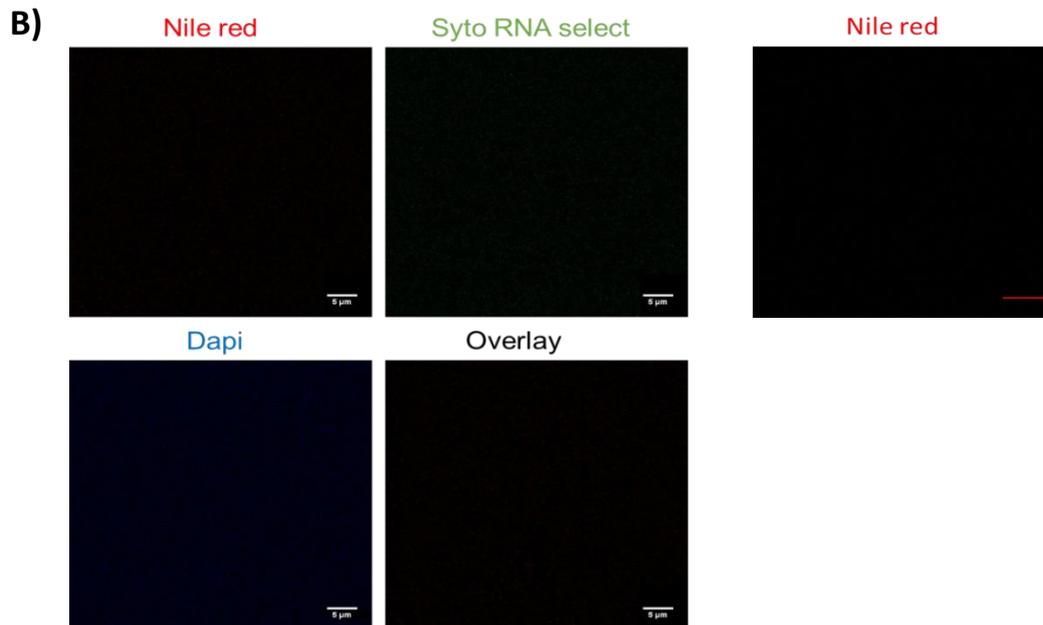


Figure 10. Confocal microscopy analysis. (A) Mounting medium fluorescence detected in Nile red, SYTO RNaselect and DAPI emission channels. (B) Confocal microscopy analysis of Nile red control. The scale bar is equivalent to 5 μm in the images.

Therefore, to try to remove fluorescent particles, culture medium was ultracentrifuged before inoculation of the bacteria (clarified medium). Thus, we obtained that when isolated OMVs were stained with Nile red and SYTO RNaselect, less amount of fluorescent dots were detected suggesting that in this case we would only be seeing the vesicles and the rest of fluorescent particles would have been removed of culture medium (figure 11A). Moreover, only some of these vesicles would contain nucleic acid, including RNA. However, when the control of clarified culture medium was performed, we observed that had still fluorescent particles in the culture medium and these could be detected in Nile red and SYTO RNaselect emission channels (figure 11B). Although in clarified culture medium control, as we can see in figure 11B, the amount of fluorescent particles detected in Nile red spectrum is lower than the amount of fluorescent particles detected when we use culture medium without clarify (figure 11B) suggesting that the utilization of clarified culture medium is better to visualized only OMVs present in the sample, as well as to detect which contain nucleic acid, specially RNA. However, it remains difficult to ensure that we are seeing vesicles.

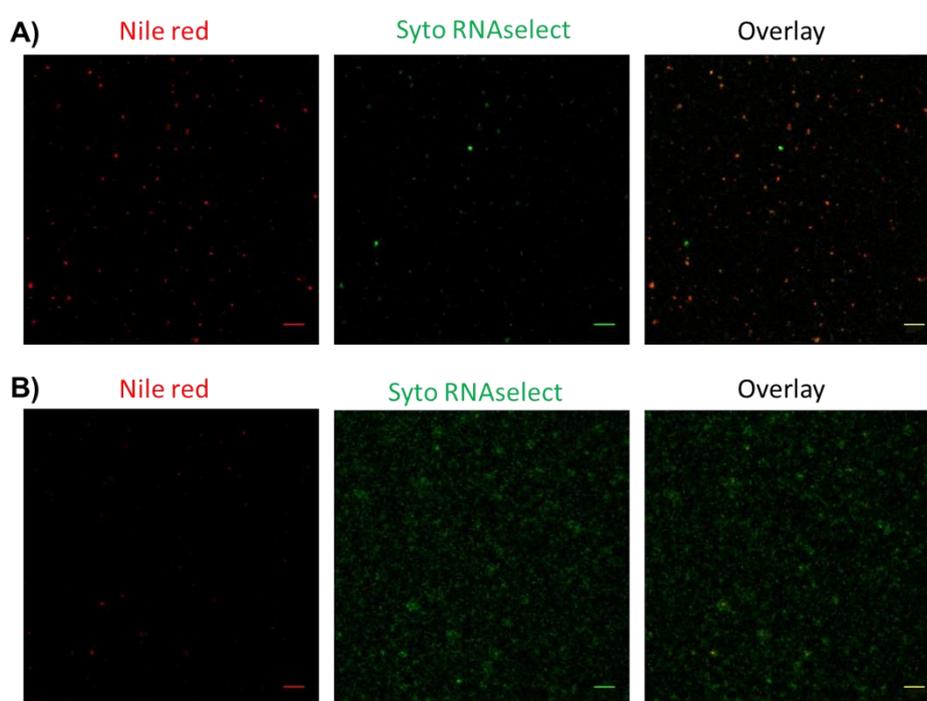


Figure 11. Confocal microscopy analysis of OMVs from *S. vesiculosa* M7^T (A) OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red (red) and RNA-specific dye, SYTO RNaselect (green). (B) Ultracentrifuged medium control without OMVs stained with membrane dye, Nile red (red) and RNA-specific dye, SYTO RNaselect (green). The scale bar is equivalent to 5 μ m in the images

Thus, we tried to remove all fluorescent particles of culture medium by filtration through 0.05 μ m pore-size-filter. However, it was not practicable, although fluorescent particles were not detected in the control of this culture medium (data not shown), because bacteria could not grow

in this medium. This is likely because the filtration removed any component present in the culture medium necessary for growth of the bacteria.

Staining of *S. vesiculosa* M7^T OMVs with DiIC-18-DS and SYTO® RNaselect™

On the other hand, DiIC-18-DS was used to repeat the same experiment that we did with Nile red. DiIC-18-DS is a specific membrane dye that only emits fluorescence when it is attached to a lipid membrane. Thus, we wanted to try remove all background detected in the culture medium. Moreover, for this assay we also used SYTO RNaselect to stain RNA in OMVs.

Thus, when we stain isolated OMVs with DiIC-18-DS and SYTO RNaselect, we only detected fluorescence in the DiIC-18-DS spectrum, so we thought that the sample contained OMVs without RNA (figure 12A). However, when control of culture medium was performed fluorescent particles were also detected in the DiIC-18-DS spectrum (figure 12B), although in lesser abundance than in OMVs samples. Moreover, in the control fluorescent particles were also detected in the SYTO RNaselect spectrum, although these particles did not colocalize with the particles stained with DiIC-18-DS (figure 12B).

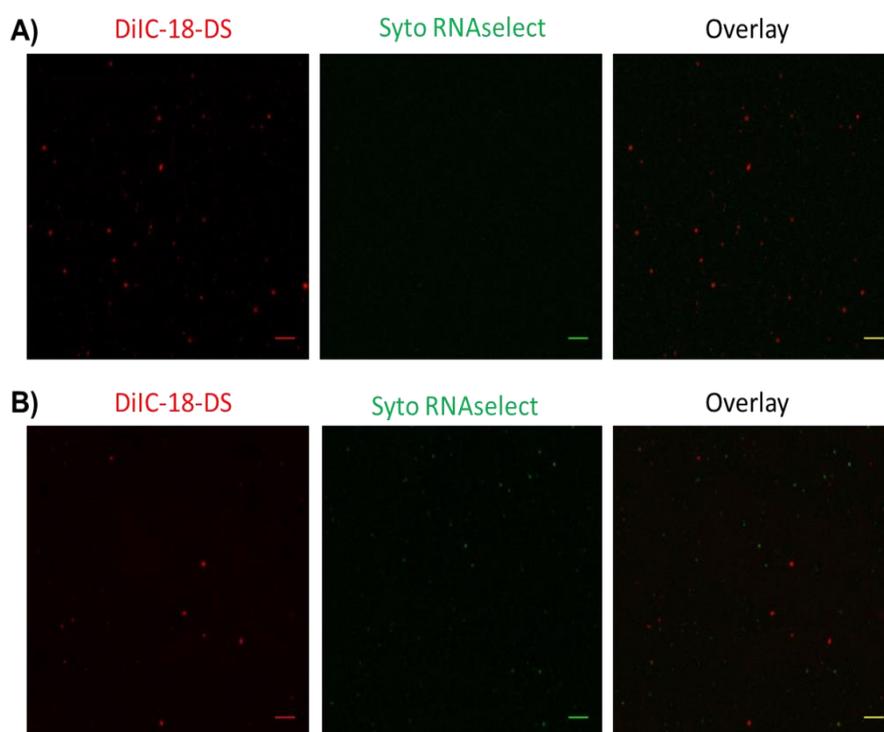


Figure 12. Confocal microscopy analysis of OMVs from *S. vesiculosa* M7^T. (A) OMVs obtained in normal medium stained with lipid tracer dye, DiIC-18-DS (red) and RNA-specific dye, SYTO RNaselect (green). (B) Medium control without OMVs stained with lipid tracer dye, DiIC-18-DS (red) and RNA-specific dye, SYTO RNaselect (green). The scale bar is equivalent to 5 μ m in the images.

For this reason, the experiment was repeated using OMVs obtained in clarified culture medium. Thereby, when these OMVs were stained with DiIC-18-DS and SYTO RNaselect, we detected particles stained with both dyes (figure 13A). However, fluorescent particles did not colocalize. In this way, if these particles were OMVs, they would not contain RNA. However, it is difficult to determine whether we are actually seeing OMVs or not, because as in previous controls, when we did the control of clarified culture medium stained with DiIC-18-DS fluorescent particles were detected. Moreover, in this case, fluorescence was detected in a similar amount to OMVs samples (figure 13B). Also, we detected fluorescence in SYTO RNaselect spectrum and some of these particles colocalized. The large amount of fluorescence detected in the spectrum of DiIC-DS could be due to this dye precipitated. Therefore, a control was performed using only DiIC-DS without culture medium or vesicles where we can see that really this dye form precipitates because we can detect intense fluorescence in DiIC-18-DS emission channel and due to this intense fluorescence we can detect too in SYTO RNA select emission channel (figure 14).

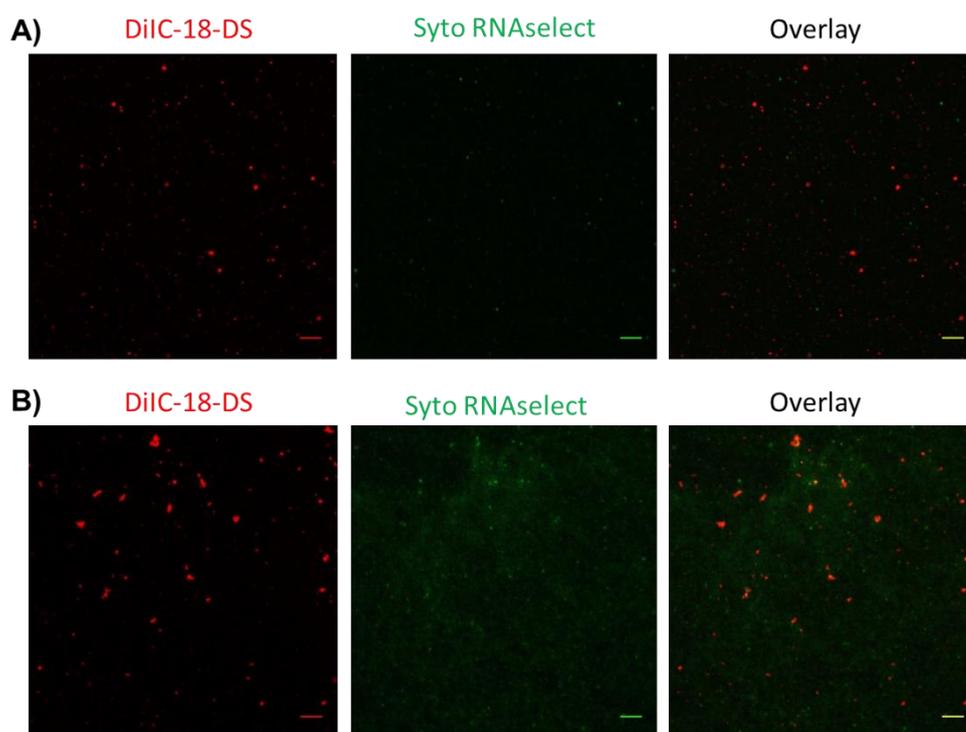


Figure 13. Confocal microscopy analysis of OMVs from *S. vesiculosa* M7^T. (A) OMVs obtained in ultracentrifuged medium stained with lipid tracer dye, DiIC-18-DS (red) and RNA-specific dye, SYTO RNaselect (green). (B) Ultracentrifuged medium control without OMVs stained with lipid tracer dye, DiIC-18-DS (red) and RNA-specific dye, SYTO RNaselect (green). The scale bar is equivalent to 5 μ m in the images.

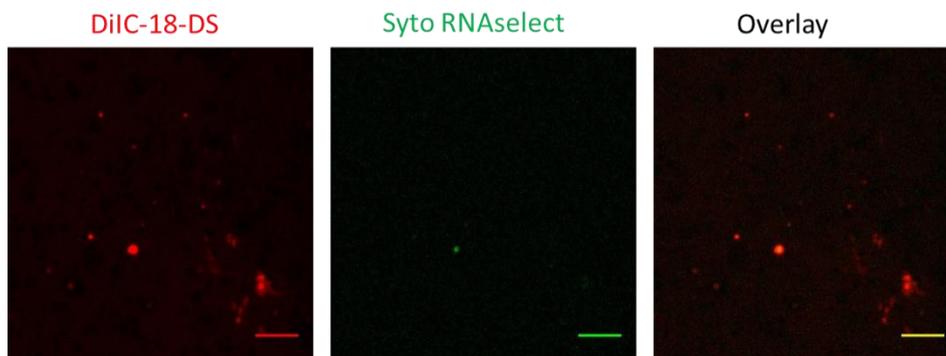


Figure 14. Confocal microscopy analysis of only DiIC-18-DS. Fluorescence was detected in DiIC-18-DS and SYTO RNaselect emission channel. The scale bar is equivalent to 5 μ m in the images.

Staining of *B. abortus* 2308 OMVs with Nile Red and SYTO® RNaselect™

To despite of differences found with this method, we tried to see OMVs of *B. abortus* 2308, our bacteria of interest, by confocal microscopy. In this case, in order to remove all fluorescent particles present in the culture medium, we used directly OMVs isolated from clarified culture medium.

Thus, we observed that when samples of isolated *B. abortus* 2308 OMVs were stained with Nile red and SYTO RNaselect, fluorescence was detected both Nile red and SYTO RNaselect spectrum, and all signals colocalized (figure 15A), so they could be OMVs with RNA. This was confirmed by the control, because in this case fluorescence was not detected in the control when we stained with Nile red (figure 15B), on the contrary that before. However, it is unlikely that they are OMVs with RNA, because as others authors have shown bacteria produce less than 1% of OMVs with cytoplasmic components (Pérez-Cruz *et al.*, 2013, Pérez-Cruz *et al.*, 2015). Thus, according to these data, it is impossible that all OMVs observed contain RNA, so more assays are necessary to show if our data are true or false.

Staining of *B. abortus* 2308 OMVs with DiIC-18-DS and SYTO® RNaselect™

Similar data were obtained when *B. abortus* 2308 OMVs were stained with DiIC-18-DS. Thus, fluorescence was detected when we stained with DiIC-18-DS and SYTO RNaselect, but in this case not all signals colocalized (figure 16A), that it is more similar to what we expected. Moreover, in this case, we did not detected fluorescence in the control of centrifuged culture medium when we stained with DiIC-18-DS (figure 16B). Thus, this suggests that the fluorescence detected correspond with OMVs and that a percentage of them may contain RNA.

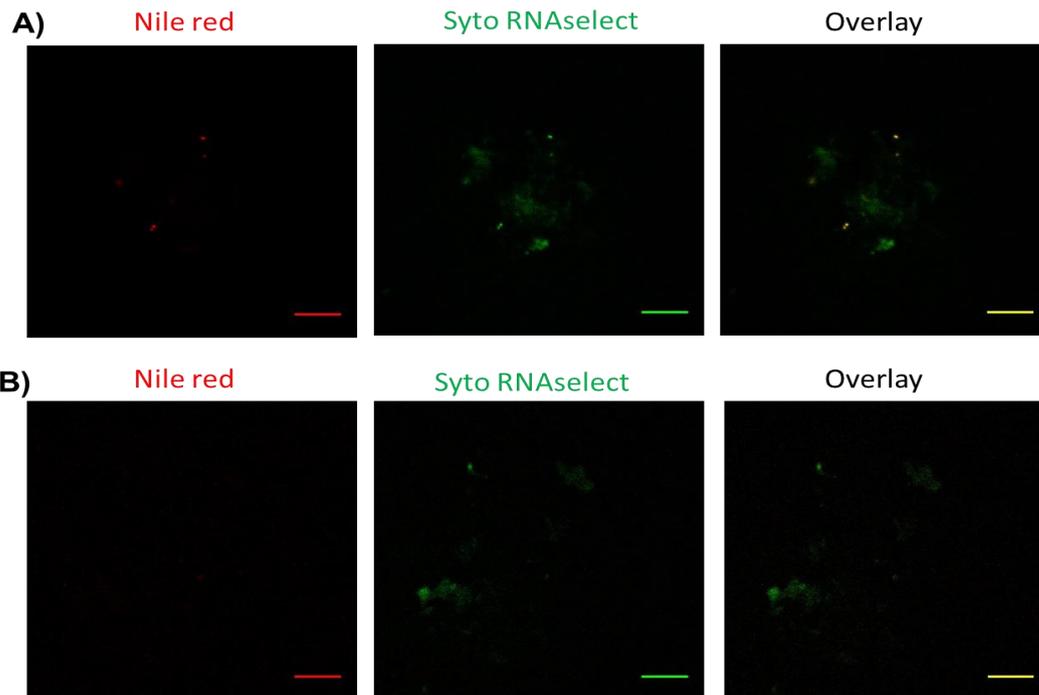


Figure 15. Confocal microscopy analysis of OMVs from *B. abortus* 2308. (A) OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red (red) and RNA-specific dye, SYTO RNaselect (green). (B) Ultracentrifuged medium control without OMVs stained with membrane dye, Nile red (red) and RNA-specific dye, SYTO RNaselect (green). The scale bar is equivalent to 5 μm in the images.

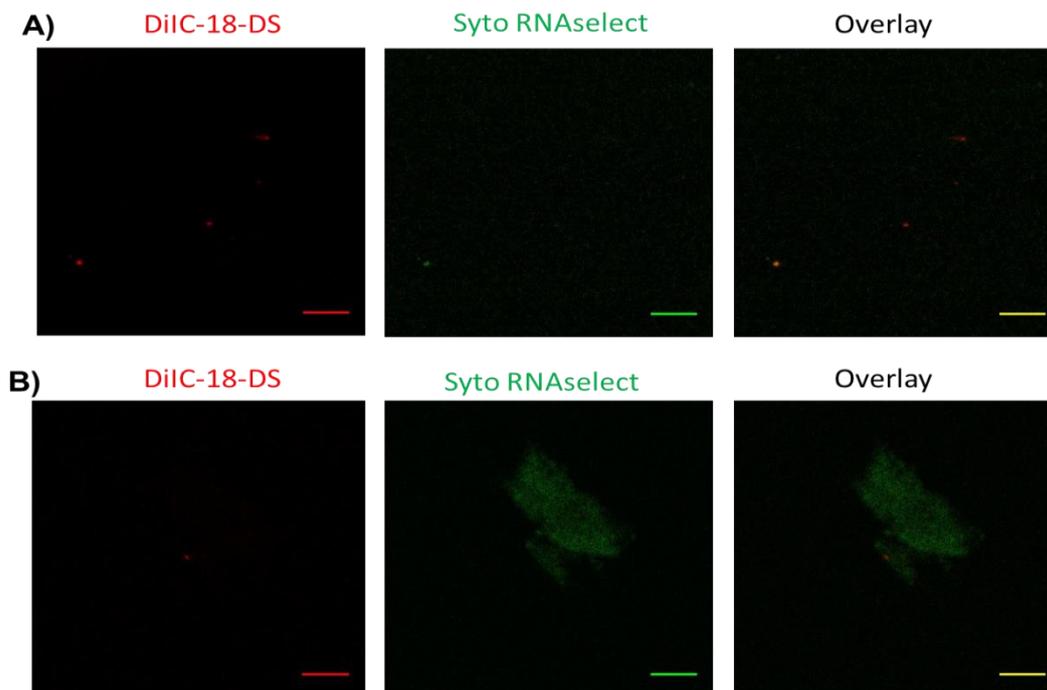


Figure 16. Confocal microscopy analysis of OMVs from *B. abortus* 2308. (A) OMVs obtained in ultracentrifuged medium stained with lipid tracer dye, DiIC-18-DS (red) and RNA-specific dye, SYTO RNaselect (green). (B) Ultracentrifuged medium control without OMVs stained with lipid tracer dye, DiIC-18-DS (red) and RNA-specific dye, SYTO RNaselect (green). The scale bar is equivalent to 5 μm in the images.

However, all these data are very preliminary and more studies are necessary to determine whether OMVs contain or not RNA. Moreover, it is necessary improve on the detection of OMVs by confocal microscopy, because until we are not able to eliminate partially or totally the fluorescence of the culture medium, we cannot be sure whether we are seeing OMVs or not. In fact, we will do density gradients with Optiprep™ to purify OMVs and thus, try to remove the background. Moreover, it is also necessary confirm these data by other assays, such as electron microscopy and RNA-seq.

Detection of *S. vesiculosa* M7^T and *B. abortus* 2308 OMVs by flow cytometry

Until now, we had only been trying detect OMVs and their content by confocal microscopy. However, it was decided to test whether other technique such as flow cytometry was a better method to detect these particles and their content. To accomplish this, we used a flow cytometer capable to detect particles until 50 nm as allowed detect the majority of OMVs, because as it was mentioned before OMVs have a size approximately between 20-250 nm of diameter (Pérez-Cruz *et al.*, 2013). Moreover, another incentive was that this technique should allow distinguish between OMVs and fluorescent particles detected in the control of clarified medium by confocal microscopy assays, since this technique allow discriminate particles by size and complexity.

Thus, we can observe that we cannot distinguish any population through size and complexity in *S. vesiculosa* M7^T OMVs without stain (figure 17A). However, in samples of *S. vesiculosa* M7^T OMVs stained with Nile red we can observe two populations, P1 and P2, and these populations do not appear in the control of clarified medium stained with Nile red (figure 17B and C). Moreover, if we only study these two populations we can see that P1 is a fluorescent population for Nile red channel, but it is not fluorescent for SYTO RNaselect and DAPI channels (figure 18A). While P2 is a fluorescent population for Nile red channel but also it is partially fluorescent

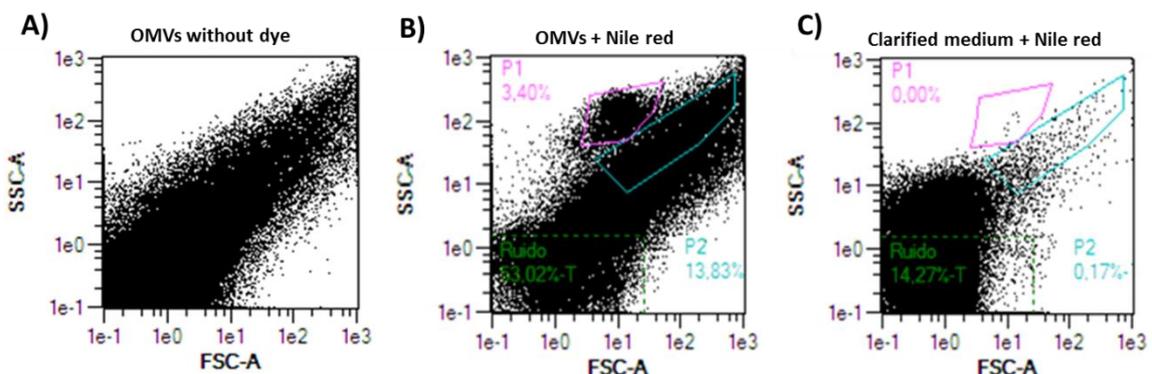


Figure 17. Flow cytometry analysis of OMVs from *S. vesiculosa* M7^T. (A) OMVs obtained in ultracentrifuged medium without stain. (B) OMVs obtained in centrifuged medium stained with membrane dye, Nile red. (C) Ultracentrifuged medium control without OMVs stained with membrane dye, Nile red.

for SYTO RNaselect and DAPI channels (figure 18B). Therefore, with these preliminary data it is possible to think that P1 population corresponds with OMVs, while P2 population corresponds with fluorescent particles present in the culture medium or with dye aggregates.

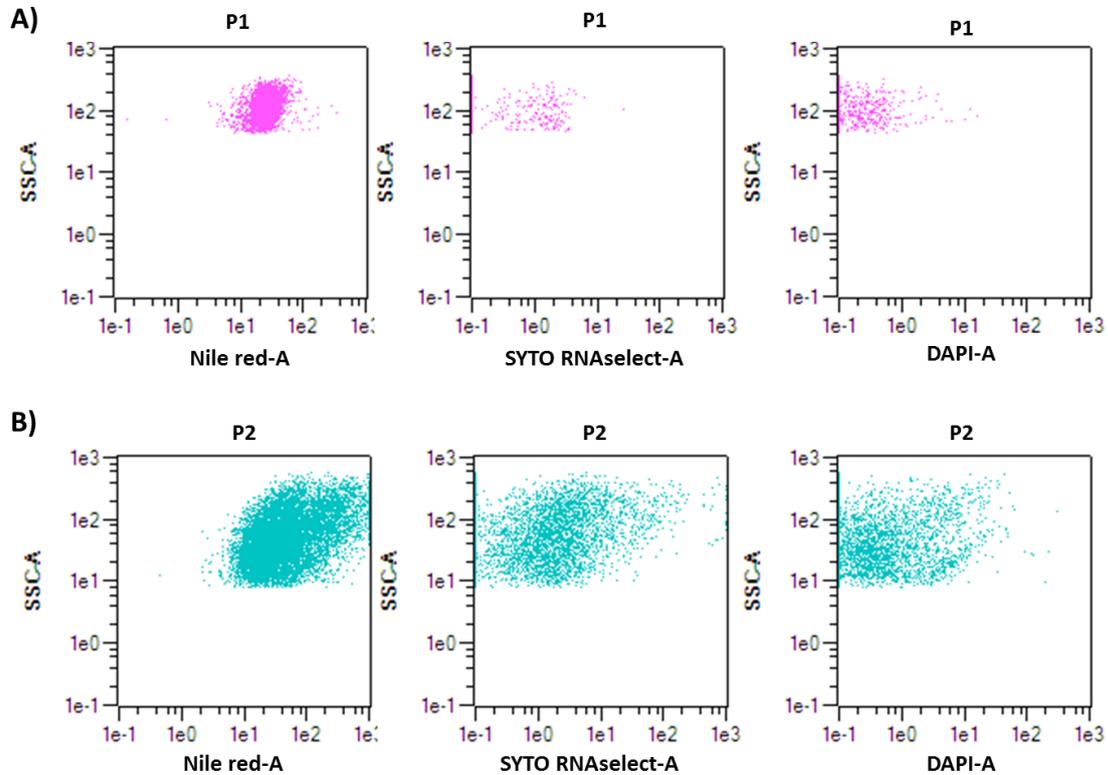


Figure 18. Flow cytometry analysis of OMVs from *S. vesiculosa* M7^T. (A) Fluorescence analysis of population 1 (P1) of OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red. (B) Fluorescence analysis of population 2 (P2) of OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red.

Similar result it was obtained to *B. abortus* 2308 OMVs. However, in this case the population that it can correspond with OMVs, P1, was detected in other position, that is to say, it would be a population with less complex OMVs, possibly because it is also a population with smaller particles, since it appears shifted to the left in the X axis (figure 19). This is logical since *B. abortus* 2308 OMVs have a size between 30-178 nm (Pollak *et al.*, 2012) while *S. vesiculosa* M7^T OMVs have a size between 25-200 nm (Pérez-Cruz *et al.*, 2013).

However, more studies are necessary to reach to confirm these data. Moreover, these experiments should be repeated using SYTO RNaselect and DAPI to detect whether these OMVs contain nucleic acids, especially RNA.

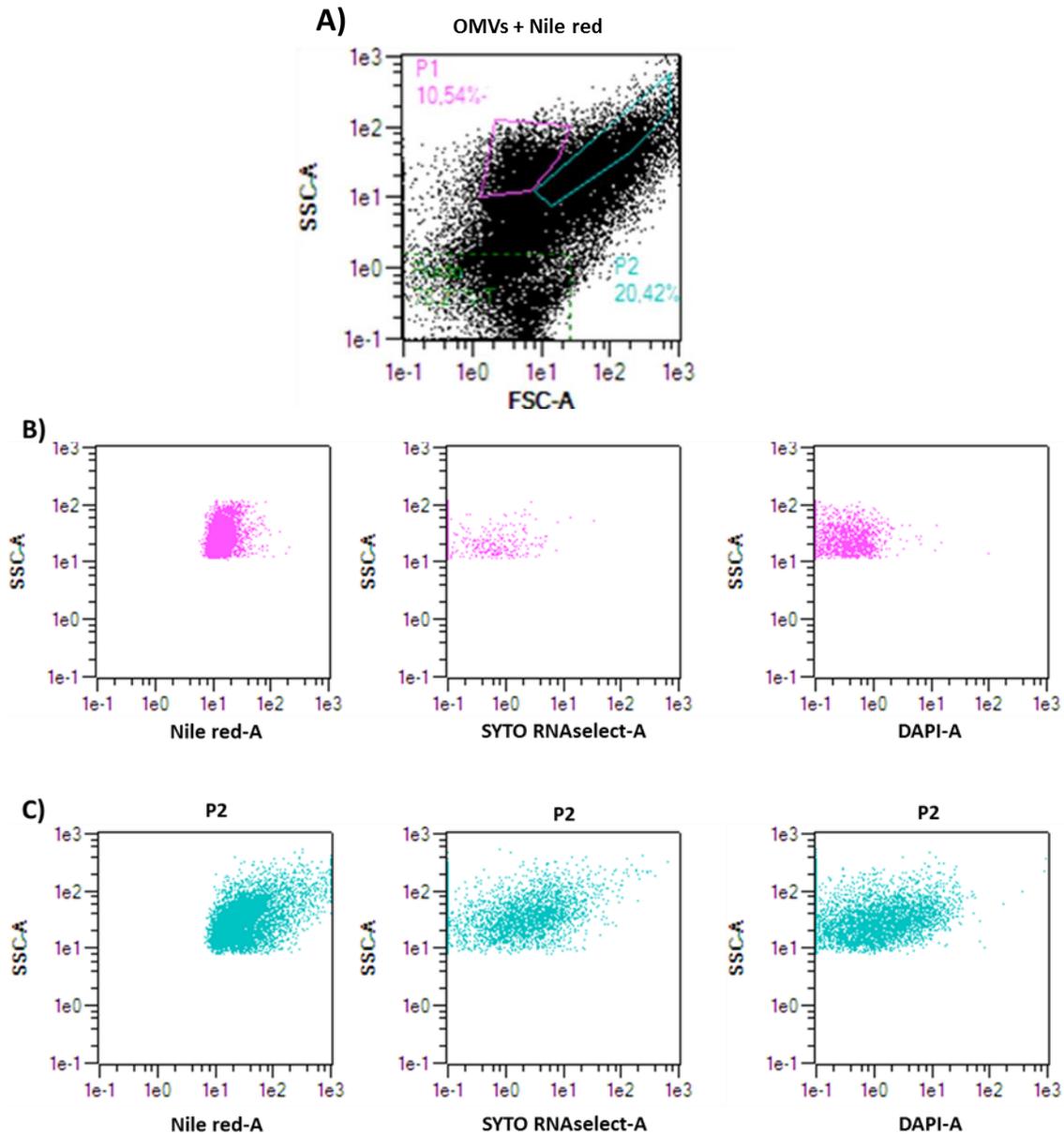


Figure 19. Flow cytometry analysis of OMVs from *B. abortus* 2308. (A) OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red. (B) Fluorescence analysis of population 1 (P1) of OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red. (C) Fluorescence analysis of population 2 (P2) of OMVs obtained in ultracentrifuged medium stained with membrane dye, Nile red.

Visualization of *S. vesiculosa* M7^T OMVs by electron microscopy

In addition to study if OMVs contain RNA or not, we wanted visualize if *B. abortus* 2308 is able to secrete different types of membrane vesicles as it had been previously demonstrated in others bacteria such as *S. vesiculosa* M7^T (Pérez-Cruz *et al.*, 2013, Pérez-Cruz *et al.*, 2015). Thus, we employed *S. vesiculosa* M7^T to standardize the protocol of TEM, in order to work as little as possible with a pathogen such as *B. abortus* 2308.

In this manner, TEM observations of ultrathin sections of OMVs and whole cells of *S. vesiculosa* M7^T revealed that this bacteria really produces two types of OMVs (figure 20A). One type of OMVs with a single bilayer, exhibiting similar structure as the outer membrane (OM) of the cell (figure 20B). The second type of OMVs that we observed was OMVs with a double bilayer. Moreover, we clearly observed a OMVs with a double bilayer precisely at the moment of formation (figure 20C) which confirmed that the external membrane derived from the cell OM, and the inner membrane corresponded to the cell plasma membrane as previously Pérez-Cruz *et al.*, 2013 had shown for this bacteria.

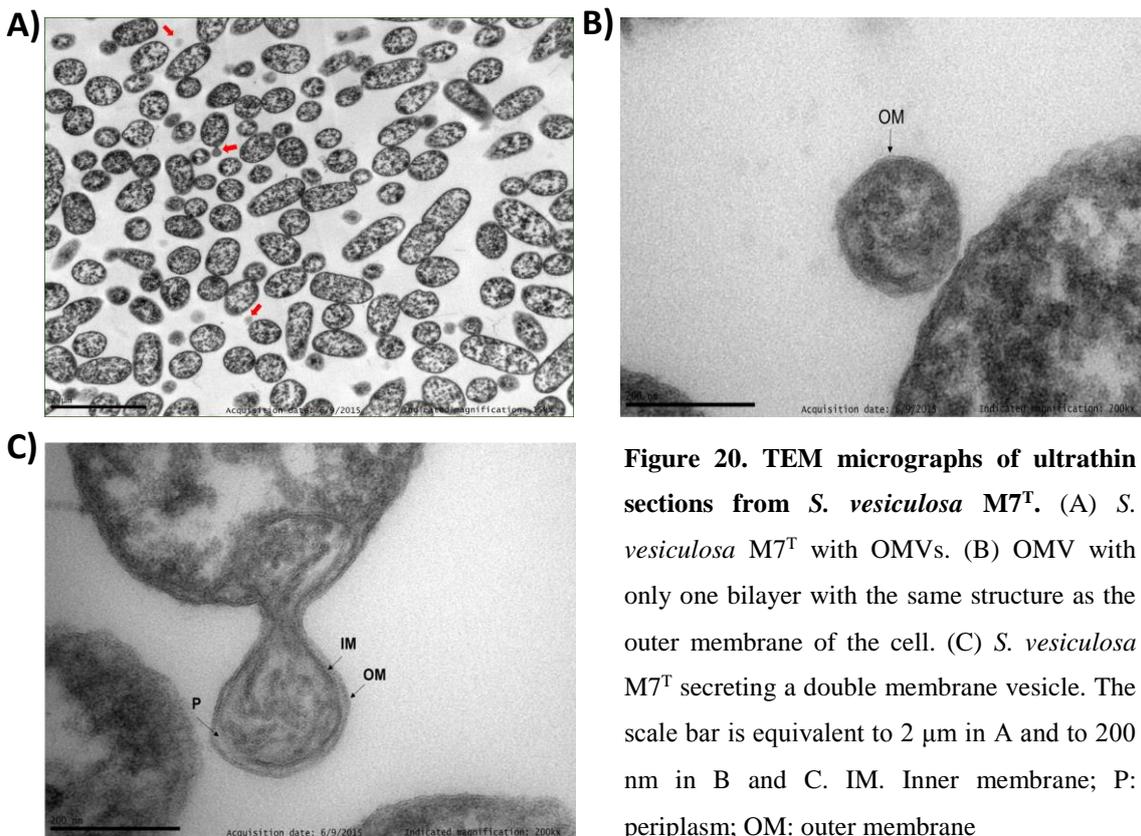


Figure 20. TEM micrographs of ultrathin sections from *S. vesiculosa* M7^T. (A) *S. vesiculosa* M7^T with OMVs. (B) OMV with only one bilayer with the same structure as the outer membrane of the cell. (C) *S. vesiculosa* M7^T secreting a double membrane vesicle. The scale bar is equivalent to 2 μ m in A and to 200 nm in B and C. IM. Inner membrane; P: periplasm; OM: outer membrane

Once standardized the protocol with *S. vesiculosa* M7^T the same experiment should be realized with *B. abortus* 2308 to determinate whether this bacteria can generate both types of vesicles, and therefore possess cytoplasmic components within them, such as sRNAs.

Finally, although this work is very preliminary may be of great interest because if we get show the presence of sRNAs within OMVs, we could discover a new system of bacterial interference with host functions, and thus OMVs are able to convert in a new virulence factor in *B. abortus* 2308 and in others pathogenic bacteria because this will be expandable to others pathogenic bacteria.

Conclusions

- Nile red and DiIC-18-DS are membrane dyes capable of efficiently staining the *S. vesiculosa* M7^T membrane, while DiI, DiD oil, SP-DiIC-18 and 5'5 pH2-DiIC-18-DS do not stain all the cells, and as such, are not useful for this study.
- DAPI is not a suitable dye for detecting DNA in vesicles, as OMVs stained with Nile red, DiIC-18-DS and Syto® RNaselect™ also emit in its emission spectrum.
- TSB is not a good culture medium to see OMVs by confocal microscopy because it has many fluorescent particles of similar size to the vesicles. Ultracentrifuged TSB is a better alternative because it has less fluorescent particles.
- Confocal microscopy is a simple and rapid method to detect OMVs, as well as the components that they contain, including RNA, but it needs to be improved.
- Confocal microscopy assays suggests that both *S. vesiculosa* M7^T as *B. abortus* 2308 OMVs could contain RNA, but this needs to be confirmed by other assays, such as electron microscopy or RNA-seq.
- Flow cytometry assays using the MACSQuant® VYB flow cytometer allow detection of OMVs, so it may be a good method to study its contents, although more controls are necessary, as the vesicles are very close to the background noise.
- OMVs with one or two bilayer have been detected in *S. vesiculosa* M7^T by electron microscopy. Using carrier particles of defined sizes will help in the characterization of the purified vesicles.
- The work performed with *S. vesiculosa* M7^T has proved to be useful to streamline the work with pathogens like *B. abortus* 2308.

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Introduction

Outer membrane vesicles (OMVs) were recently described as a new type secretion system. These OMVs are small spheroid particles with size approximately between 20-250 nm of diameter (Perez-Cruz *et al.*, 2013). OMVs contain many different components that are released to the environment to carry out different functions (Deatherage and Cookson, 2012; Dorward and Garon, 1990). Although there has been a historical controversy about their content, it has recently been observed that some bacteria can secrete two different types of OMVs, one formed only by outer membrane and periplasmic components, and a second type that includes also inner membrane and cytoplasmic components (Perez-Cruz *et al.*, 2013; Perez-Cruz *et al.*, 2015). This opens the field to the presence in these vesicles of others cytoplasmic components such as sRNAs. Among the multiple functions of these sRNAs, one is the interference with host functions, as it has been described for the first time for an *Escherichia coli* sRNA acting on *Caenorhabditis elegans* (Knip *et al.*, 2014).

Hypothesis

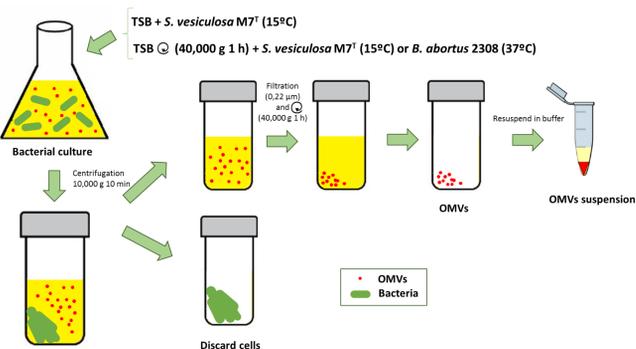
Brucella probably contain both types of vesicles. If so, they could contain sRNAs with the potential of interfering with the host.

Objective

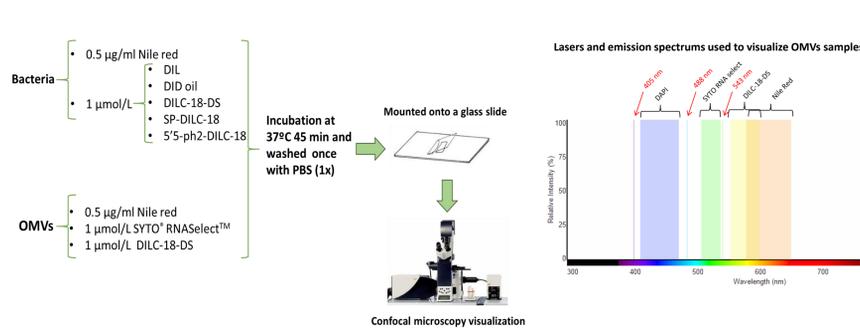
Isolate and visualize *Brucella abortus* 2308 OMVs and their content by different techniques such as confocal and electron microscopy, using *Shewanella vesiculosa* M7^T to standardize all protocols.

Materials and methods

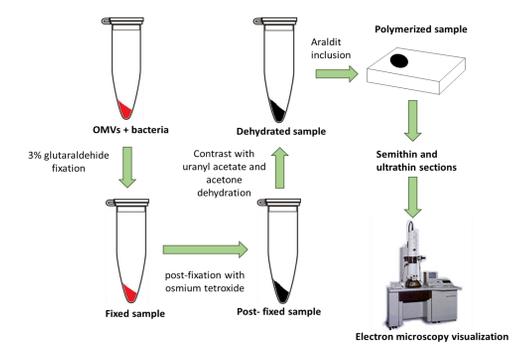
OMVs isolation



Confocal microscopy analysis



Electron microscopy analysis



Results

S. vesiculosa M7^T with different membrane dyes

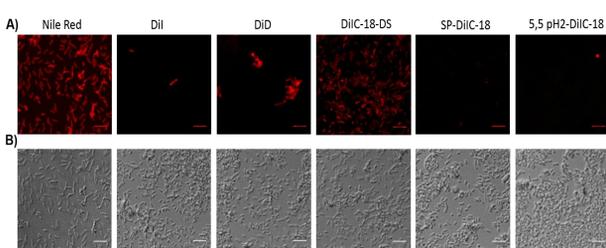


Figure 1. Staining of *S. vesiculosa* M7^T with different membrane dyes. (A) Confocal microscopy analysis from *S. vesiculosa* M7^T stained with different lipid tracer dyes: Nile red, DiI, DiD oil, DiIc-18-DS, Sp-DiIc-18 and 5'5 pH2-DiIc-18. (B) Bright field images of *S. vesiculosa* M7^T stained with different membrane dyes. The scale bar is equivalent to 5 µm in the images.

S. vesiculosa M7^T OMVs with Nile red, Syto[®] RNAselect™ and DiIc-18-DS

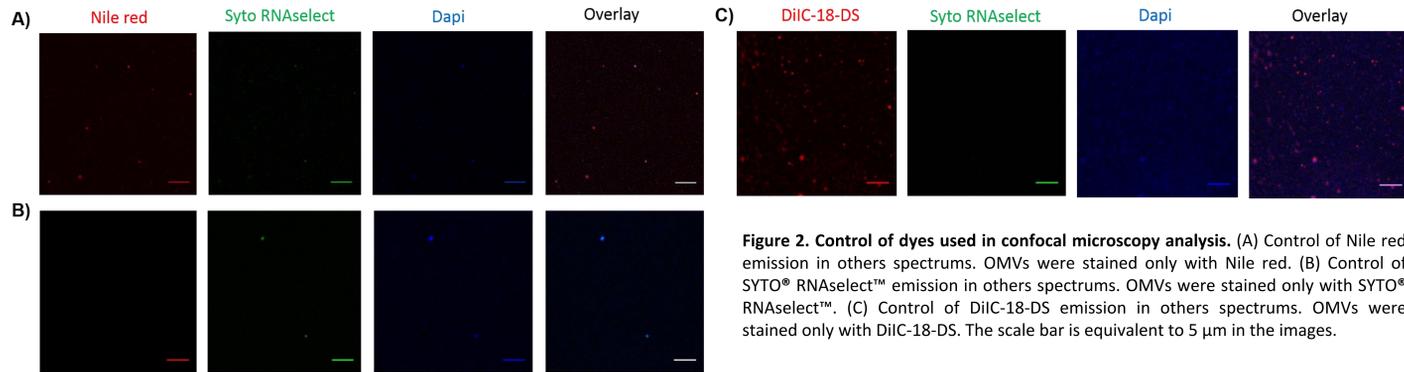


Figure 2. Control of dyes used in confocal microscopy analysis. (A) Control of Nile red emission in others spectrums. OMVs were stained only with Nile red. (B) Control of SYTO[®] RNAselect™ emission in others spectrums. OMVs were stained only with SYTO[®] RNAselect™. (C) Control of DiIc-18-DS emission in others spectrums. OMVs were stained only with DiIc-18-DS. The scale bar is equivalent to 5 µm in the images.

S. vesiculosa M7^T OMVs with Nile red and Syto[®] RNAselect™

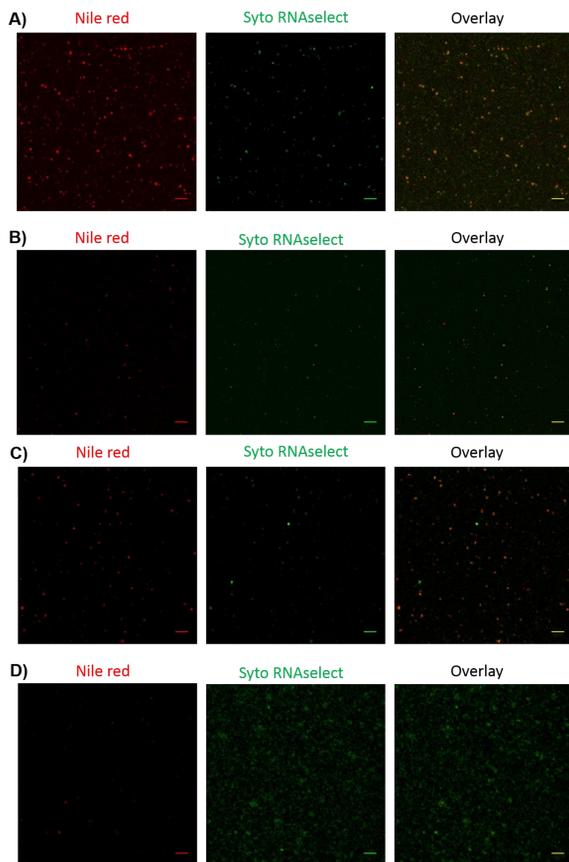


Figure 3. Confocal microscopy analysis of OMVs from *S. vesiculosa* M7^T stained with membrane dye, Nile red (red) and RNA-specific dye, Syto[®] RNAselect™ (green). (A) Staining of OMVs obtained in normal medium. (B) Medium control without OMVs. (C) Staining of OMVs obtained in centrifuged medium. (D) Centrifuged medium control without OMVs. The scale bar is equivalent to 5 µm in the images.

S. vesiculosa M7^T OMVs with DiIc-18-DS and Syto[®] RNAselect™

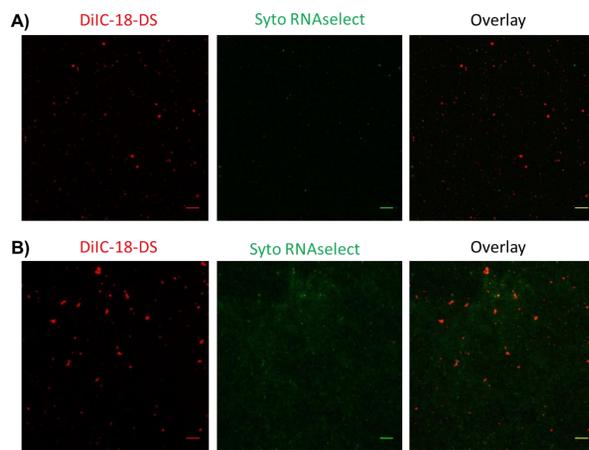


Figure 4. Confocal microscopy analysis of OMVs from *S. vesiculosa* M7^T stained with lipid tracer dye, DiIc-18-DS (red) and RNA-specific dye, Syto[®] RNAselect™ (green). (A) Staining of OMVs obtained in centrifuged medium. (B) Centrifuged medium control without OMVs. The scale bar is equivalent to 5 µm in the images.

B. abortus 2308 OMVs with DiIc-18-DS and Syto[®] RNAselect™

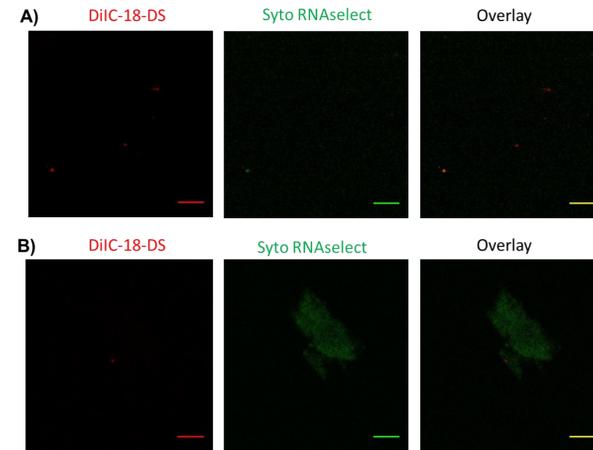


Figure 5. Confocal microscopy analysis of OMVs from *B. abortus* 2308 stained with lipid tracer dye, DiIc-18-DS (red) and RNA-specific dye, Syto[®] RNAselect™ (green). (A) Staining of OMVs obtained in centrifuged medium. (B) Centrifuged medium control without OMVs. The scale bar is equivalent to 5 µm in the images.

Visualization of *S. vesiculosa* M7^T OMVs by electron microscopy

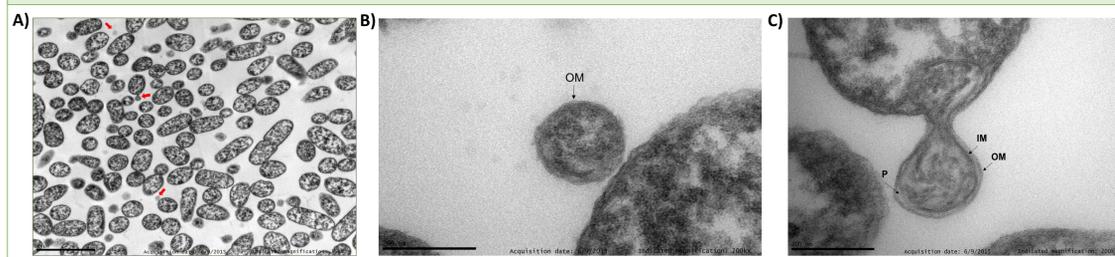


Figure 6. TEM micrographs of ultrathin sections from *S. vesiculosa* M7^T. (A) *S. vesiculosa* M7^T with OMVs. (B) OMV with only one bilayer with the same structure as the outer membrane of the cell. (C) *S. vesiculosa* M7^T secreting a double membrane vesicle. The scale bar is equivalent to 2 µm in A and to 200 nm in B and C. IM: Inner membrane; P: periplasm; OM: outer membrane

Conclusions and further research

- Nile red and DiIc-18-DS are membrane dyes capable of efficiently staining the *S. vesiculosa* M7^T membrane, while DiI, DiD oil, SP-DiIc-18 and 5'5 pH2-DiIc-18-DS are not suitable to stain *S. vesiculosa* M7^T membrane.
- DAPI is not a suitable dye for this experiment because OMVs stained with Nile red, DiIc-18-DS and Syto[®] RNAselect™ can be detected in its emission spectrum.
- TSB is not a good culture medium to see OMVs by confocal microscopy because it has many fluorescent particles. However, centrifuged TSB is a better alternative because it has less fluorescent particles.
- Confocal microscopy is a simple and rapid method to detect OMVs, as well as the components that they contain, including RNA, but it needs to be improved.
- Confocal microscopy assays suggests that both *S. vesiculosa* M7^T as *B. abortus* 2308 OMVs could contain RNA, but it will be confirmed by other assays, such as electron microscopy or RNA-seq. Moreover, flow cytometry will be used to count the total number of OMVs and the number of them with RNA to obtain the percentage of OMVs with RNA that produces *B. abortus* 2308.
- OMVs with one or two bilayer have been detected in *S. vesiculosa* M7^T by electron microscopy, and we will try to detect and quantify both OMVs types in *B. abortus* 2308.
- Density gradients with Optiprep™ will be used to purify OMVs and thus, try to remove the background in confocal microscopy.
- The work performed with *S. vesiculosa* M7^T has proved to be useful to streamline the work with pathogens like *B. abortus* 2308.

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