Chapter 3
Biogenesis and Function of Extracellular Vesicles in Gram-Positive Bacteria, Mycobacteria, and Fungi

Ainhoa Palacios, Carolina Coelho, Maria Maryam, Jose L. Luque-García, Arturo Casadevall, and Rafael Prados-Rosales

Abstract The production of membrane vesicles (MVs) has been documented in all domains of life. Justification for the historical lack of interest in the study of vesicle biogenesis in Gram-positive bacteria, mycobacteria, and fungi is based on the difficulty in explaining how MV can traverse the thick cell wall. For this reason, the scientific landscape has been dominated by studies examining vesicle biogenesis in microorganisms that lack cell walls or Gram-negative bacteria, since they possess an outer membrane layer. Evidence of MV production by cell-walled microorganisms is now available from different experimental approaches including, isolation of MVs from culture supernatant, compositional analysis, visualization of vesiculation events, and genetic studies. Strikingly, more recent studies have shown that beside

A. Palacios
CIC bioGUNE, Technological Park Bizkaia, Derio, Spain

C. Coelho
Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Medical Research Council Centre for Medical Mycology, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK

Department of Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, UK

M. Maryam · A. Casadevall
Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

J. L. Luque-García
Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, Madrid, Spain

R. Prados-Rosales (✉)
CIC bioGUNE, Technological Park Bizkaia, Derio, Spain

Faculty of Medicine, Department of Preventive Medicine and Public Health, and Microbiology, Autonoma University of Madrid, Madrid, Spain
e-mail: rafael.prados@uam.es

M. Kaparakis-Liaskos, T. A. Kufer (eds.), *Bacterial Membrane Vesicles*,
https://doi.org/10.1007/978-3-030-36331-4_3

the fundamental differences in the architecture of the cell envelope of Gram-positive and Gram-negative bacteria, a similar mechanism of cell wall remodeling may govern the release of MVs. Here, we describe the current understanding of vesicle biogenesis in cell-walled microorganisms, discussing novel mechanisms of vesicle production, methods to study MVs, cargo, and functions of MVs as well as medical applications of naturally produced MVs.

3.1 Introduction

The notion that Gram-negative bacteria produce outer membrane vesicles (OMVs) has been an accepted phenomenon for some time. However, the realization that cell-walled organisms such as fungi, mycobacteria, and Gram-positive bacteria also make MVs has only taken root in the past decade. The major hurdle in considering MV production by cell-walled microbes was the belief that the cell wall was a rigid structure that prevented vesicular transit. In 2007, the fungus *Cryptococcus neoformans* was shown to produce MVs and to use them for export of macromolecular compounds including virulence factors (Rodrigues et al. 2007, 2008b). Subsequently, several other fungal species including *Candida albicans* (Vargas et al. 2015), *Saccharomyces cerevisiae* (Oliveira et al. 2010), *Histoplasma capsulatum* (Albuquerque et al. 2008; Nimrichter et al. 2016), *Sporothrix brasiliensis* (Ikeda et al. 2018), *Paracoccidioides brasiliensis* (Vallejo et al. 2011), *Alternaria infectoria* (Silva et al. 2014), and *Malassezia sympodialis* (Johansson et al. 2018) were shown to be producers of MVs (Table 3.1). The experience with fungal MVs prompted a search for comparable structures in Gram-positive bacteria. The report of the proteomic composition of isolated *Staphylococcus aureus* MVs provided an early example in cell-walled bacteria (Lee et al. 2009). Subsequent studies with the bacterium *Bacillus anthracis* showed that it packaged its toxins into vesicles (Rivera et al. 2010). An initial hint that anthrax toxin components were packaged into vesicles came from immunogold electron microscopy, indicating that these were secreted at discrete sites in the cell wall (Rivera et al. 2010). The production of MVs has now been identified to occur by numerous Gram-positive bacteria (Liu et al. 2018) and mycobacteria (Prados-Rosales et al. 2011) (Table 3.1). The demonstration that vesicle biogenesis is altered in some mutants of cell-walled organisms (Brown et al. 2015) suggest that, as in Gram-negative bacteria, MV production by Gram-positive organisms might be a genetically regulated process. Recent reports have demonstrated a similar mechanism for MV release from *Bacillus subtilis* (Toyofuku et al. 2017) and OMV release from *Pseudomonas aeruginosa* (Turnbull et al. 2016), indicating that beside the fundamental differences in cell envelope architecture, common mechanisms may govern this process in prokaryotes. The clinical relevance of MVs produced by pathogenic species of cell-walled organisms is apparent from several studies. Both beneficial and detrimental effects have been associated with MVs. Moreover, several groups have provided evidence that isolated MVs may represent a good platform for vaccine development in animal

Table 3.1 List of cell-walled microorganisms where MVs have been demonstrated

Species	References
Gram-positive bacteria	
Bacillus anthracis	Rivera et al. (2010)
Bacillus subtilis	Brown et al. (2014)
Bifidobacterium longum	Kim et al. (2016a)
Clostridium perfringens	Jiang et al. (2014)
Enterococcus faecalis	Kim et al. (2016b)
Lactobacillus casei	Dominguez Rubio et al. (2017)
Lactobacillus plantarum	Li et al. (2017)
Lactobacillus reuteri	Grande et al. (2017)
Lactobacillus rhamnosus	Behzadi et al. (2017)
Listeria monocytogenes	Lee et al. (2013b)
Propionibacterium acnes	Jeon et al. (2017)
Streptococcus agalactiae (group B streptococcus)	Surve et al. (2016)
Streptomyces coelicolor	Schrempf et al. (2011)
Streptomyces lividans	Lee et al. (2009)
Staphylococcus aureus	Lee et al. (2009)
Streptococcus mutans	Liao et al. (2014)
Streptococcus pneumoniae	Olaya-Abril et al. (2014)
Streptococcus pyogenes (group A streptococcus)	Resch et al. (2016)
Streptococcus suis	Haas and Grenier (2015)
Mycobacteria	
Mycobacterium avium	Prados-Rosales et al. (2011)
Mycobacterium bovis BCG	Prados-Rosales et al. (2011)
Mycobacterium kansasii	Prados-Rosales et al. (2011)
Mycobacterium phlei	Prados-Rosales et al. (2011)
Mycobacterium smegmatis	Prados-Rosales et al. (2011)
Mycobacterium tuberculosis	Prados-Rosales et al. (2011)
Mycobacterium ulcerans	Marsollier et al. (2007)
Fungi	
Candida albicans	Albuquerque et al. (2008)
Cryptococcus gatii	Bielska et al. (2018)
Candida parapsilosis	Albuquerque et al. (2008)
Cryptococcus neoformans	Rodrigues et al. (2007)
Histoplasma capsulatum	Albuquerque et al. (2008)
Malassezia sympodialis	Gehrmann et al. (2011)
Paracoccidioides brasiliensis	Vallejo et al. (2011)
Saccharomyces cerevisiae	Albuquerque et al. (2008)
Sporothrix schenckii	Albuquerque et al. (2008)

models (see Chap. 10). Given the relevance of MVs in clinical settings, the feasibility of MVs as a diagnostic element is starting to be appreciated.

3.2 Evidence for MVs in Cell-Walled Organisms

Although the existence of MV transport systems in cell-walled microbes is now generally accepted, acceptance of this notion took a while to acquire traction. The problem with MVs gaining general acceptance was the result of criticisms that ranged from physical concerns to intellectual arguments and these took almost a decade to be resolved. The three major criticisms will be considered separately.

1. *Cell walls are rigid structures that prevent the passage of such large structures as MVs*. The notion that cell walls precluded passage of MVs due to their rigidity and the absence of large pores is known as the physical criticism. This criticism persisted despite the fact that cell walls were known to be flexible structures that could be easily rearranged during budding and, in the case of the fungi, hyphal formation. This criticism was addressed by demonstrating vesicles in the cell wall during what appeared to be a transfer process. For *C. neoformans* there was electron microscopic evidence of vesicles transiting the cell wall (Rodrigues et al. 2007). For *S. aureus*, scanning electron microscopy showed vesicle-like structures with comparable dimensions to MVs protruding from the cell wall surface (Lee et al. 2009). Subsequent work using freeze–fracture electron microscopy revealed vesicles entering or creating pores in the fungal cell wall (Wolf et al. 2014). Recently, liposomes containing amphotericin b were shown to transit the cell wall of *C. neoformans* and *C. albicans* from the outside to the inside, when added exogenously (Walker et al. 2018), making a compelling case for the notion that the living cell wall is a pliable and deformable structure and selectively porous, that is, not a barrier to vesicular transit.
2. *MVs form from self-assembly of lipids released from cells*. The criticism that MVs were artifacts from the self-assembly of lipid molecules released from live or dead cells was difficult to counter because lipids are notoriously able to form micelles. Furthermore, there was data that lysis of *B. subtilis* cells by phages could produce voluminous amounts of membrane lipids that could self-assemble into vesicles. For example, the expression of endolysins by phage in *Bacillus subtilis* led to explosive lysis of the bacterial cells that was associated with vesicle formation (Toyofuku et al. 2017, 2019). The lipid self-assembly criticism was answered by a series of experiments that made a compelling case against this explanation. Several bacteria MVs were shown to have lipid and protein composition different than whole bacterial cells, which argued against their emergence as a consequence of cellular lysis (Coelho et al. 2019; Olaya-Abril et al. 2014). In an experiment designed to rule out the self-assembly explanation *C. neoformans* polysaccharide was added to *B. subtilis* cultures and then localized by immunogold labeling (Brown et al. 2014). If MVs were the result of lipid self-

assembly one would have expected to see the fungal polysaccharide inside the vesicles, but this was not the case providing strong evidence against the lipid self-assembly origin explanation (Brown et al. 2014). In fungi, the finding that MVs were used in two-way communication between fungal cells indicated a new specific function for these structures that made a strong case against an artifactual nature (Bielska et al. 2018; Rodrigues and Casadevall 2018). Finally, the visualization of MVs emerging from *Listeria monocytogenes* inside epithelial cells through live cell imaging provided compelling visual evidence for their physiological existence (Coelho et al. 2019).

3. *The absence of null mutants for MV production* in vesicle-producing organisms. This criticism followed from the viewpoint of genetic reductionism, which posited that if MVs were real then there must be a complex machinery that could allow the isolation of null mutants for MV production. Remarkably, those who espoused such views never doubted the existence of cell membranes despite the fact that no mutant could exist without a cell membrane. The absence of null mutants for MV production could reflect the fact that vesicle formation is an integral part of cell membrane remodeling, and as such, would be produced by any microbe. Nevertheless, this criticism has now been muted by the discovery of genes that regulate MV production in fungi (Oliveira et al. 2010), mycobacteria (Rath et al. 2013), and Gram-positive bacteria (Resch et al. 2016).

Today, fungi are accepted to be producers of MVs and these have been described in *C. neoformans, C. albicans* (Vargas et al. 2015), *S. cerevisiae* (Oliveira et al. 2010), *H. capsulatum* (Nimrichter et al. 2016), *Sporothrix brasiliensis* (Ikeda et al. 2018), *P. brasiliensis* (Vallejo et al. 2011), and *A. infectoria* (Silva et al. 2014), as well as the skin commensal *M. sympodialis* (Johansson et al. 2018). For recent reviews on this topic see also de Toledo Martins et al. (2019); Joffe et al. (2016); Rodrigues et al. (2015).

The first observation that MVs could be produced by a Gram-positive bacterium was reported in 1990 but this was not investigated further (Dorward and Garon 1990). It was not until 2009 when a proteomic study of *S. aureus* MVs set the stage for modern research into the biogenesis of MVs by Gram-positive bacteria (Lee et al. 2009). That finding was followed by studies in *B. anthracis*, *L. monocytogenes*, several species of *Streptococcus* (Haas and Grenier 2015; Olaya-Abril et al. 2014; Surve et al. 2016), and *Lactobacillus* (Li et al. 2017), to name a few.

The first evidence that a bacterial strain from the *Mycobacterium* genus releases MVs was reported in 2007 in *M. ulcerans* (Marsollier et al. 2007). MVs were isolated from the extracellular matrix of *M. ulcerans* and were enriched in mycolactone, the main virulence factor of this pathogen. Subsequent studies validated this study and supported the notion that MV biogenesis is a conserved phenomenon in the *Mycobacterium* genus. Indeed, a 2011 report demonstrated MV production in the two most medically important strains of this genus, *Mycobacterium tuberculosis* (Mtb) and Bacillus Calmette-Guérin (BCG) (Prados-Rosales et al. 2011). Analysis of MV biogenesis was extended to other slow growers and fast growers from the same genus and all of them showed the capacity to release MVs

(Prados-Rosales et al. 2011). Evidence of MV production was not only demonstrated by the possibility of MV isolation from culture supernatant but also by the visualization of MV release events using electron microscopy techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM) during an ongoing infection (Prados-Rosales et al. 2011). Further characterization of Mtb and BCG MVs showed a similar size distribution than that of Gram-negative OMVs (Prados-Rosales et al. 2011).

3.3 Methods for the Study of MVs in Organisms with a Thick Cell Wall

Most of the studies on MVs in Gram-positive bacteria, fungi, and mycobacteria involve an initial purification from laboratory culture supernatants followed by the concentration and characterization of these structures. Few studies have attempted MV isolation from host-related compartments. Mass spectrometry has been used to define the MV-associated protein and lipid cargo, and electron microscopy to visualize isolated MVs or vesiculation events from living cells. The methodology to study MVs should be designed carefully attending to the final use of isolated material. If the study involves the interaction of isolated MVs with host cells, the isolation and purification steps should go as far as eliminating most of the unwanted contaminants. This would also apply for cargo identification by mass spectrometry.

3.3.1 Methods of Isolation

As previously mentioned, the isolation process is one of the most challenging approaches during the study of MVs (Mateescu et al. 2017). Since there is not an ideal technique to isolate MVs, most groups combine several approaches with the aim of reducing contaminants, which are typically co-isolated with the MVs pool, and to minimize sample loss. The protocol that has been mostly used to isolate MVs derived from Gram-positive bacteria, mycobacteria, and fungi involve clarification of the supernatant using low-speed centrifugation followed by filtration to remove microorganisms and the collection of an MV-rich faction after subsequent ultracentrifugation (Brown et al. 2015; Prados-Rosales et al. 2014a). Although many studies have performed MVs characterization from the pelleted MV preparation after ultracentrifugation, additional separation steps are desired to increase the purity of the MV pool and avoid the carryover of unwanted cellular material. For instance, an additional step in the form of sucrose cushion density gradient was applied during isolation of MVs from *Streptococcus* or *Streptomyces* (Gurung et al. 2011; Schrempf et al. 2011). Similarly, density gradient ultracentrifugation was used to further purify ultracentrifuge-isolated MVs from Mtb (Prados-Rosales et al. 2011), *S. aureus* (Lee

et al. 2009), *S. pneumoniae* (Olaya-Abril et al. 2014), and *C. perfringens* (Jiang et al. 2014) cultures. More recently, size-exclusion chromatography was combined with clarification and ultracentrifugation of culture supernatants to isolate MVs from *C. albicans* biofilms (Zarnowski et al. 2018). The major disadvantage in applying additional purification steps during MV isolation is the decrease in vesicle yield (Chutkan et al. 2013).

MVs have also been isolated from samples different from axenic cultures. In a study reporting a pulmonary allergic reaction to *S. aureus* MVs in mice, vesicles from this organism were isolated from house dust by removing bacteria from dust previously dissolved PBS and subsequent ultracentrifugation (Kim et al. 2012). MVs from *S. aureus* have also been isolated from skin lavage fluids obtained from patients with atopic dermatitis (Hong et al. 2011) and lung tissue from *S. aureus*-infected mice (Gurung et al. 2011) using a similar approach.

One of the major limitations of methods used to isolate MVs, in general, is the difficulty in separating MV subpopulations. The development of new approaches with the sufficient resolution capacity to achieve the separation of such populations will increase the understanding of the intrinsic heterogeneity of MVs.

3.3.2 Cargo Identification

The definition of MV composition is critical to understanding their role in different biological processes. MVs contain a diverse variety of cargo including lipids, proteins, nucleic acids, and metabolites. In the case of Gram-positive bacteria, mycobacteria, and fungi derived-MVs, most studies have been focused on the definition of the protein composition of isolated MVs by biological mass spectrometry; or by biochemical assays such as antibody-based assays, which include ELISA or Immunoblot (Lee et al. 2009; Prados-Rosales et al. 2011). Fewer studies report the lipid profile of MVs from cell-walled microorganisms and to a lesser extent the presence of nucleic acids or metabolites. Compared to Gram-negative bacterial OMVs, quantitative data regarding the relative incorporation of cellular material, including lipids and proteins into MVs are not available for cell-walled microorganisms. This information is necessary to better understand the process of Gram-positive MV biogenesis. Moreover, one aspect usually unattended to fully establish the unique association of a specific cellular component to MVs is the lack of additional experiments including the composition of the cell membrane. Elucidation of MV composition will shed light into the enrichment phenomenon of specific MV components.

3.3.2.1 Identification of MV-Associated Proteins

The protein content of MVs derived from cell-walled microorganisms can be assessed by antibody-based assays or by high-throughput mass spectrometry

approaches. Usually, the selection of the antibody to detect the MV-associated protein is preceded by a mass spectrometry approach where the catalogue of MV proteins has been defined. In the latter, a cleanup step in the form of acetone precipitation is usually performed followed by in-solution trypsin digestion and peptide purification by solid phase extraction (ZipTip) before analysis by mass spectrometry (Prados-Rosales et al. 2014a). As mentioned above, to fully establish the enrichment of a specific protein within the MV it is critical to compare the abundance of the protein hit in the MV relative to the one in the cellular membrane. Most of the approaches used to analyze MV protein composition from cell-walled microorganisms are based on electrospray ionization (ESI)-based nano liquid chromatography coupled to mass spectrometry (nanoLC-MS/MS) (Kim et al. 2015). To increase the number of identified proteins, some studies incorporate a one-dimensional gel electrophoresis separation step before in-gel trypsin digestion (Lee et al. 2009). However, one of the potential issues associated to this step is the low recovery of large and/or highly hydrophobic peptides from the gel. This shortcoming is especially relevant for the study of the protein content of MVs given that these are likely to have a significant content of membrane proteins.

One of the hallmarks of the protein catalogue of MVs from pathogenic cell-walled microorganisms is the fact that virulence-associated proteins are overrepresented. This is true for Mtb (Lee et al. 2015; Prados-Rosales et al. 2011), *S. aureus* (Gurung et al. 2011; Lee et al. 2009), *L. monocytogenes* (Lee et al. 2013b), *B. anthracis* (Rivera et al. 2010), *Streptomyces coelicolor* (Schrempf et al. 2011), *S. pneumoniae* (Olaya-Abril et al. 2014), *C. neoformans* (Rodrigues et al. 2008a), *H. capsulatum* (Albuquerque et al. 2008), *P. brasilensis* (Vallejo et al. 2011), and *C. perfringens* (Jiang et al. 2014). In the particular case of Mtb, an enrichment of lipoproteins in isolated MVs was reported. These classical toll-like receptor 2 (TLR2) ligands that are involved in the interference of the antigen presentation process during their interaction with host cells (Fulton et al. 2004), were not detected in isolated MV from the environmental mycobacterial strain *M. smegmatis* (Prados-Rosales et al. 2011).

3.3.2.2 Identification of MV-Associated Lipids

A few studies have attempted the lipid characterization of isolated MV from cell-walled microorganisms. Lipidomic analysis of isolated MVs from the two Gram-positive bacteria *B. anthracis* and *S. pneumoniae* shows an enrichment in short-chain saturated fatty acids relative to the corresponding cell membranes (Olaya-Abril et al. 2014; Rivera et al. 2010), suggesting that membrane fluidity might be an important factor for MV release. In a different study with Mtb, MV lipid analysis was performed by thin-layer chromatography (TLC) and showed enrichment in polar lipids including phosphatidylinositol mannosides (PIMs) and cardiolipins, suggesting that the origin of mycobacterial MV might be the cellular membrane (Prados-Rosales et al. 2011). Lipidomic analysis of isolated *C. neoformans* MVs showed the presence of the glycosphingolipid (GlcCer) and sterol derivatives

(Rodrigues et al. 2007). Considering that these lipids are enriched in membrane microdomains (Muniz and Riezman 2000), their presence in isolated MVs would suggest that MV release events occur at these discrete regions of the membrane. Similar to *C. neoformans*, lipidomic analysis of isolated MVs from *H. capsulatum* showed the increased abundance of two species of hydroxylated fatty acids, containing 16 or 18 carbons (Albuquerque et al. 2008). As discussed by the authors, the similarity between the lipid composition of mammalian exosomes and fungal MVs in these particular lipids, would suggest a close mechanism of biogenesis. Lipidomic studies of MVs from cell-walled microorganisms are critical to understanding vesicle biogenesis. Consequently, more studies incorporating state-of-the-art methodology are needed to shed light to this conserved biological phenomenon.

3.3.2.3 Identification of MV-Associated Nucleic Acids

Several important recommendations have been proposed by the international society for extracellular vesicles when attempting the analysis of nucleic acids, especially RNA, in extracellular vesicles to exclude the possibility that they are contaminants (Mateescu et al. 2017). Similarly, the study of the presence of DNA in isolated MVs should include enzymatic digestion of isolated material to determine the degree of DNA association with MVs. MVs from *C. neoformans*, *C. albicans*, *P. brasiliensis, M. sympodialis* (Rayner et al. 2017), and *S. cerevisiae* contain different types of RNA, including mature tRNAs, mRNAs, and noncoding RNAs (Peres da Silva et al. 2015). The potential role that RNA delivered by MVs might have in the target cells is still a matter of investigation.

3.3.3 Quantification, Labeling, and Visualization of MVs

Determination of the number and size of MVs is critical to understand how these structures are produced. This information would be essential to assess the feasibility of MVs as novel vaccines. Quantitative measurements of MVs from Gram-positive bacteria, mycobacteria, and fungi have been mostly performed by the determination of either vesicle mass, protein content, or the relative amount of lipids. Alternatively, determination of the amount of MVs has been performed by radiolabeling of growing cells with lipid precursors and by measuring radioactive counts in cells, vesicle pellet, and supernatant after ultracentrifugation (Brown et al. 2014). However, none of these methods provides absolute quantification of MVs. Labeling of MVs has also been used as a method for quantification or visualization of MVs. The first attempts at labeling MVs were performed with lipophilic dyes such as DiI, DiO, and PKH26 (Morales-Kastresana and Jones 2017; Morales-Kastresana et al. 2017). However, this approach has proven problematic given that these lipophilic dyes caused unacceptable alterations in size and morphology, presumably due to aggregation events (Morales-Kastresana et al. 2017). The authors of these works have

cautioned against the use of DiI or PKH26 and we join them in urging the discontinuation of these protocols. We have used a fatty-acid-BODIPY conjugate that is added to the culture medium and incorporated into bacterial cells (Coelho et al. 2019), which allowed us to isolate MVs that maintained the same size and density, as observed by DLS characteristics and density gradient centrifugation. This technique has the advantage that it can be tailored to each individual organism, i.e., it is easy to change the lipid species that are tagged, particularly when combined with the knowledge of lipid composition of the MVs under study. Another widely used tool is to detect MVs by immunodetection to one of the surface-associated proteins (Kowal et al. 2016). This technique is only limited by the quality of the antibody used and has allowed affinity purification of MVs subpopulations in mammalian cells (Kowal et al. 2016). Additional tools are needed to perform in vivo tracking and highly sensitive tracking, to allow for more specific labeling and separation. A very successful alternative was genetic coupling of fluorophores (or luciferase) to proteins secreted in MVs (Lai et al. 2015, 2014).

The diversity of microbes entails that for each microbe, MV-specific markers (or at least MV-enriched markers) need to be selected and validated. The task of selecting an appropriate, robust MV marker is further complicated since MVs may be significantly different depending on media or culture conditions used, and therefore one would have to find the one marker that is conserved throughout a wide variety of experimental conditions. However, the rewards to be reaped would be immense: in addition to live-tracking of MVs these tools could allow quick and selective purification, via columns or sorting, and could allow for detection of very low amounts of these MVs.

Technologies have been developed to perform the automatic analysis and quantification of individual MVs, including the tunable resistive pulse sensing (tRPS), nanoparticle tracking analysis (NTA), high-resolution flow cytometry (hFC), or dynamic light scattering (DLS) (Maas et al. 2015). While all of these methods acquire information related to particle size, only hFC has the potential of providing an absolute number of MVs. Advantages and limitations of these technologies have been discussed previously (Szatanek et al. 2017). In the context of Gram-positive bacteria, NTA has been successfully applied to the study of *Sporothrix brasiliensis* MVs (Ikeda et al. 2018) and tRPS was used to determine vesicle size in Mtb (Athman et al. 2015). Also very extended in the bacterial and fungal MVs field is the use of either DLS or transmission electron microscopy (TEM) to assess the size distribution of the vesicle population. Electron microscopy is another complementary approach to characterize MVs populations. TEM was used to visualize MVs and to study size and morphology of these structures in many Gram-positive, mycobacteria, and fungal species (Brown et al. 2015). TEM provides valuable information about the heterogeneity of the MV population. Limitations associated with this technique are the required number of steps for sample processing that usually introduces many morphological artifacts. Scanning electron microscopy (SEM) has also been utilized to visualize vesicle release events in bacterial and fungal cells (Prados-Rosales et al. 2011; Silva et al. 2014). Although this information is valuable to ascertain the capacity of a specific strain to release MVs, the possibility

that these structures are confounded with other cell surface-associated molecules requires strict validation of the obtained results. Demonstration of MV production in many species of Gram-positive bacteria usually combines several microscopy approaches such as TEM, SEM, FC, or atomic force microscopy (AFM) (Liu et al. 2018). The recently developed technique of cryoelectron microscopy (cryo-EM) reduces considerably the steps in sample processing and allows the determination of sample morphology in a close-to-native state (Glaeser and Hall 2011). A recent study combined NTA, tRPS, and cryo-EM to determine the size distribution of isolated *S. aureus* MVs. Interestingly, each method provided a different size distribution and showed the monodispersed nature of the MV population, and highlighted the necessity of combining complementary approaches to characterize MVs in cell-walled microorganisms (Tartaglia et al. 2018).

3.4 Mechanisms of MV Biogenesis in Cell-Walled Microorganisms

The limited research into MVs from Gram-positive bacteria, mycobacteria, and fungi was influenced by the belief of the potential interference from the thick cell wall for MV transport to outside the cell. This, in turn, makes it more challenging to provide an explanation for the mechanism(s) of vesicle biogenesis in cell-walled microorganisms. It seems intuitive that a cell wall remodeling process that would allow vesicular transit must govern MV biogenesis in these microorganisms (Fig. 3.1). Hypotheses for MV production in cell-walled microorganisms are mostly based on proteomics data from isolated vesicles, assuming that MV-associated proteins are involved in such remodeling processes. The proposal that cell wall-modifying enzymes can alter cell wall thickness and facilitate MV release provides an example for this notion. Isolated MVs from *S. aureus* (Lee et al. 2009) or *M. tuberculosis* (Prados-Rosales et al. 2011) were found to contain peptidoglycan degrading enzymes. Similarly, glycosyl hydrolases with substrate specificity for cell wall polymers have been identified in proteomic screens of EVs from several fungal species (Albuquerque et al. 2008; Oliveira et al. 2010; Rodrigues et al. 2007) (Fig. 3.1a, b). Alternatively, it was proposed that MVs can exert a change in turgor pressure after budding from the cell membrane forcing their pass through the cell wall (Rodrigues et al. 2008a; Vallejo et al. 2012a) (Fig. 3.1b). Derived from ultrastructural studies is the hypothesis that MVs can transit through pores originated within the cell wall (Rodrigues et al. 2008a; Vallejo et al. 2012a). Of note, as previously demonstrated the pore size in *S. cerevisiae* varies from 50 to 300 nm, depending on the growing conditions (de Souza Pereira and Geibel 1999). Modification of cell wall permeability has also been observed during melanization by *C. neoformans*. In this case, melanization reduces the cell wall pore size and induces the accumulation of MVs in the cell wall (Jacobson and Ikeda 2005) (Fig. 3.1b).

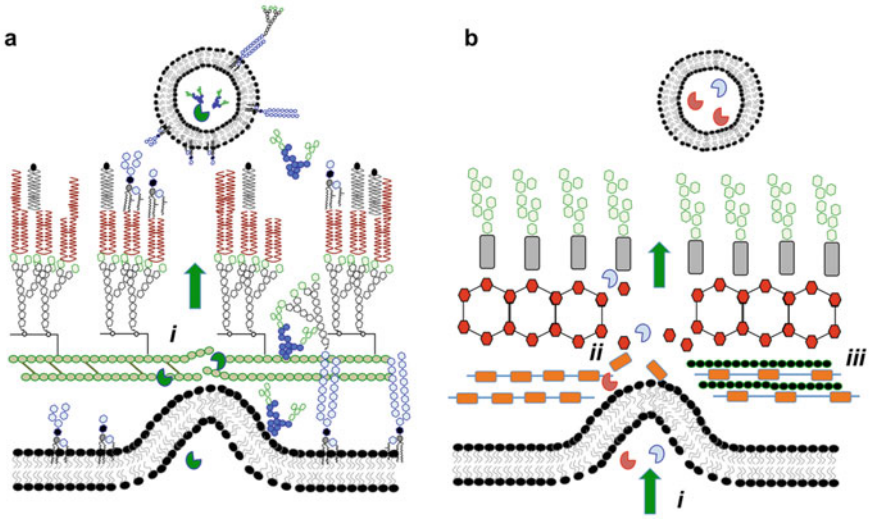

Fig. 3.1 MV formation in cell-walled microorganisms. Hypothesis on how MVs get through thick cell walls. (**a**) Scheme of the mycobacterial cell envelope. As documented in proteomics studies for either *M. tuberculosis* or Gram-positive bacteria such as *S. aureus*, the presence of cell wall-modifying enzymes (**i**) could loosen the wall skeleton facilitating the release of MVs (green arrow). (**b**) Scheme of the fungal cell wall. Release of MVs (green arrow) by fungal cells may be regulated by the porosity of the cell wall, which can be modified by turgor pressure (**i**), cell wall modifying enzymes (**ii**) or melanization (**iii**)

3.4.1 Fungi

The complexity involved in generating vesicles and loading them with such varied cargo implies the existence of complex machinery. The current view is that MVs in fungi are assembled in multivesicular bodies that then release these structures outside of the cell membrane to transit through the cell wall into the extracellular space (Casadevall et al. 2009). Consistent with this notion, numerous genes and signaling pathways have been implicated in MV production. Analysis of MV production in *S. cerevisiae* strains deficient in *Sec4p* and *Snf7*, which are involved in Golgi-derived exocytosis and multivesicular body (MVB) formation, respectively, showed that these produced vesicles with altered composition (Oliveira et al. 2010). Golgi reassembly and stacking protein (GRASP) was implicated in vesicle release and the packaging on RNA in *C. neoformans* vesicles (Kmetzsch et al. 2011; Peres da Silva et al. 2018). In *C. albicans*, the *Sap2* gene and TOR pathway have been implicated in extracellular vesicle production (Gil-Bona et al. 2015).

3.4.2 Gram-Positive Bacteria and Mycobacteria

As in Gram-negative bacteria, no gene mutation abolishing MV has been found for either Gram-positive bacteria or mycobacteria. The absence of null mutants has led to suggestions that vesicle biogenesis is either an essential process or that physical changes may give rise to vesicles irrespective as an integral property of membranes. Nevertheless, few reports have established the connection between defects in vesicle biogenesis and genetics in cell-walled microorganisms relative to Gram-negative bacteria, raising the possibility that null mutants may still be found in the future. Deletions in *L. monocytogenes sigB* (RNA polymerase sigma factor σ^B) (Lee et al. 2013b) or *M. tuberculosis* vesicle biogenesis and immune response regulator (*virR*) (Rath et al. 2013) and transmembrane component of the phosphate-specific transport (Pst) *pstA1* (White et al. 2018) were shown to alter MV production. In the particular case of *L. monocytogenes*, quantification of MVs in the wild type and *sigB* mutant strains was determined by protein concentration, raising the question whether such differences could be attributable to the potential differential capacity of both strains to load proteins on vesicles. Similarly, there is evidence that a two-component system, CovRS (control of virulence regulator-sensor) regulates the production of MVs in *Streptococcus pyogenes* (group A streptococcus (GAS)) (Resch et al. 2016). It was observed that those GAS strains harboring a CovRS genetically disrupted manifested increased MV production compared to those with an intact system. Nevertheless, the exact mechanism by which MV biogenesis is controlled is still unknown. Recently, two independent reports on the Gram-positive bacteria *B. subtilis* and *S. aureus*, have shed some light into mechanisms for MV biogenesis. Mytomicin C-induced genotoxic stress in *B. subtilis* stimulated the expression of endolysins encoded by prophages, which altered cell wall permeability and facilitated MV production (Toyofuku et al. 2017). Of note, a similar mechanism was demonstrated in the Gram-negative bacterium *P. aeruginosa* (Turnbull et al. 2016). However, while in *B. subtilis* MV are released through pores originated in the peptidoglycan, *P. aeruginosa* cells explode and MVs are formed from membrane fragments (Turnbull et al. 2016). In *S. aureus*, the release of MV enriched in lipoproteins was dependent on surfactant-like molecules such a phenol-soluble modulins (PSMs). Apparently, PSMs facilitates MV release by increasing cell membrane fluidity (Schlatterer et al. 2018). In the same bacteria, antibiotics induced MV production by a phage-dependent or phage-independent fashion, depending on the mode of action of the antibiotic. Thus, oxidative stress-inducing antibiotics can induce *S. aureus* MV production through endolysin-triggered cell death (phage-dependent) and β-lactams do it by increasing the permeability of the peptidoglycan layer in phage-independent way (Andreoni et al. 2019). The finding that iron limitation regulates MV release in *M. tuberculosis* (Prados-Rosales et al. 2014c) supports the notion that MV biogenesis is important for the survival strategy of this pathogenic bacterium. In that study, it was shown that in vitro growth of *M. tuberculosis* under iron starvation triggered MV production and those vesicles were enriched in Fe-loaded siderophores. Importantly, growth restoration was

achieved when siderophore-deficient *M. tuberculosis* mutants were supplemented with low iron MVs, indicating that they could serve as iron donors. These findings entertain the notion that those MVs can potentially contribute to the bacterial persistence by benefiting the local community.

3.5 MV Cargo

3.5.1 Fungi

Fungal MVs cargo includes proteins, lipids, nucleic acids, and carbohydrates. Compositional analysis of MVs from different fungal species revealed proteins from different functional categories such as metabolism of proteins or carbohydrates, response to stress, transport, or signaling (de Toledo Martins et al. 2019). Common to other pathogenic organisms is the fact that MVs from pathogenic fungal species are loaded with proteins associated with virulence. For instance, laccase, urease, and phosphatase activities have been demonstrated in isolated MVs from *C. neoformans* (Rodrigues et al. 2008a). Similarly, phosphatase activity was detected in MVs from *P. brasiliensis* (Vallejo et al. 2012a). Other proteins related to fungal virulence and also found in MVs are those involved in controlling the REDOX balance of the fungal cell, such as catalase B or superoxide dismutase (Albuquerque et al. 2008; Vallejo et al. 2012b). Several hypotheses of MV function have been proposed based on protein composition of fungal MVs. MVs may participate in the fungal cell wall remodeling process due to the fact that cell wall glycosyl hydrolases such as endochitinase and glucanase were identified in isolated MVs (Albuquerque et al. 2008); MVs may participate in cell wall biogenesis as they deliver glycoconjugates to the outermost part of the cell wall (Rodrigues et al. 2007; Vallejo et al. 2011).

Although there are few studies of RNA analysis in fungal MVs, a comparative study of RNA content of fungal MVs from *S. cerevisiae*, *C. neformans*, and *C. albicans* revealed the intraluminal association of RNA with MVs as demonstrated by enzymatic degradation studies, demonstrating the role of vesicles as RNA transporters (Peres da Silva et al. 2015). For a recent review on this topic see also de Toledo Martins et al. (2019).

3.5.2 Gram-Positive Bacteria

Gram-positive MVs harbor a variety of components including proteins, toxins, nucleic acids, lipids and polysaccharides, small metabolites, and antibiotics. These data offer a first insight into their possible functions, as well as mechanisms required for MV biogenesis. While there is more available information in protein and nucleic acid composition, due to the wider availability of technology, information on other types of MV components is starting to emerge.

The production of MV in Gram-positive bacteria was first studied in the case of *S. aureus* that led to the identification of 90 MV-associated proteins using a proteomic approach. The proteins found in MVs were comprised mostly of cytoplasmic proteins in addition to extracellular and membrane-associated proteins (Lee et al. 2009). That study was followed by proteomic analysis of various other Gram-positive bacteria MVs including *B. subtilis* (Brown et al. 2014), *B. anthracis* (Rivera et al. 2010), *L. monocytogenes* (Coelho et al. 2019), *S. coelicolor* (Schrempf et al. 2011), C. *perfringens* (Jiang et al. 2014), and *S. pneumoniae* (Olaya-Abril et al. 2014).

Virulence factors, including toxins, constitute a significant portion of the protein cargo. MVs isolated from *B. anthracis* consisted of biologically active toxins including edema factor, lethal factor, protective antigen, and anthrolysin (Rivera et al. 2010). MVs containing α-hemolysin and various pore-forming toxins have been identified in several other Gram-positive bacteria including *S. aureus*, *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* (Jeon et al. 2016; Olaya-Abril et al. 2014; Resch et al. 2016; Surve et al. 2016; Thay et al. 2013). The virulence factors Internalin B (InlB) and Listeriolysin O (LLO) were reported in MVs isolated from *L. monocytogenes* (Coelho et al. 2019; Lee et al. 2013b).

MVs can carry components important for bacterial survival, including a role in nutrient scavenging. MVs of *B. subtilis* contain the virulence factor SunI that provide immunity against the bacterial antibiotic sublancin (Dubois et al. 2009). Proteomic analysis of MVs revealed the presence of proteins involved in regulating the levels of drug resistance in Gram-positive bacteria. Specifically, MVs isolated from *S. aureus* ATCC 14458 were loaded with beta-lactamase protein BlaZ, that can bind to beta-lactam antibiotics and protect the neighboring bacteria against ampicillin drug (Lee et al. 2013a). Another study showed the presence of MsrR, a membrane-associated protein, in the MVs of *S. aureus* (Tartaglia et al. 2018). MsrR is known to be involved in providing resistance against methicillin and teicoplanin and also affects the synthesis of various virulence factors like alpha-toxin and protein A (Rossi et al. 2003). *S. coelicolor* and *S. aureus* produce MVs containing iron-binding proteins that help in storage of iron and survival in iron restricted environment (Schrempf et al. 2011; Surve et al. 2016), in agreement with what has been found in other classes of bacteria, as discussed above.

Schrempf et al., studied blue-pigmented exudate droplets, approximately 1 mm in size, obtained on the sporulated lawns of *S. coelicolor* M110. Inside these droplets, they found antibiotics such as actinorhodin. Electron microscopic view showed that these droplets were filled with vesicular structures. The proteomic analysis of the exudates revealed the presence of several other proteins important in survival and defense mechanisms (Schrempf et al. 2011).

MV cargo from commensal bacteria can also mediate beneficial effects on the mammalian hosts. MVs isolated from *Bifidobacterium longum*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus rhamnosus* contain proteins responsible for the probiotic effects of the bacterium (Behzadi et al. 2017; Dominguez Rubio et al. 2017; Kim et al. 2016a; Li et al. 2017).

The presence of nucleic acids is also a constant for Gram-positive bacteria MVs. PCR analysis demonstrated the genes for 16S ribosomal RNA, α-toxin, and perfringolysin O in MVs from *Clostridium perfringens* (Jiang et al. 2014). Similarly, *Streptococcus mutans* produced MVs containing extracellular DNA during biofilm formation. The secretion of extracellular DNA during biofilm formation plays an important role in bacterial adhesion and the stabilization and maturation of the biofilm structure (Liao et al. 2014).

Another critical component of MVs is their lipids and some studies have started to explore the lipid composition of Gram-positive MVs. The lipid membrane composition of Gram-positive MVs is not entirely dissimilar from that of the bacterial cell membrane, but lipidomic analysis of *B. anthracis* (Rivera et al. 2010) and *S. pneumoniae* (Olaya-Abril et al. 2014) MVs revealed some significant differences as well. In comparison to the bacterial cell membrane, the isolated MVs were highly enriched in short-chain saturated fatty acids (such as myristic, palmitic, lauric, and pentadecanoic acids). In contrast, in *L. monocytogenes* (Coelho et al. 2019) MVs were enriched in unsaturated fatty acids compared to bacterial cells. To date only one study characterized metabolites in Gram-positive MVs. In *L. monocytogenes* some small metabolites such as ornithine, pyruvic acid, and sugars such as xylitol and D-mannose were found in MVs extract (Coelho et al. 2019).

3.5.3 Mycobacteria

Compositional proteomic analysis of *M. tuberculosis* MVs identified 42 MV-associated proteins, where the putative TLR2 ligands such as the lipoproteins 19 kDa, LppX, and LprG, a well-known group of TLR2 ligands that interfere with the antigen presentation process in dendritic cells and macrophages, were the most abundant proteins (Prados-Rosales et al. 2011). This enrichment phenomenon was also observed in BCG MVs but not in *M. smegmatis* MVs. Results from this report were validated by a second study, which implemented a label-free mass spectrometry quantification of Mtb MVs, identifying 287 proteins. Among the most prevalent proteins of MVs were SodB, EsxN, LppX, PstS1, LpqH, KatG, GlnA1, Apa, FbpA, FadA3, Mtc28, AcpM, Fba, and Prs. Notably, only Lppx, PstS1, and LpqH accounted for more than 6% of the total vesicular proteins (Lee et al. 2015). In another study, it was revealed that Mtb enhances the release of MVs upon iron starvation and that such low iron MVs are loaded with siderophores (Prados-Rosales et al. 2014c).

3.6 Cell-Walled Organisms-Derived MV Functions

3.6.1 Fungi

In the sections below we briefly summarize some functions and topics involving fungal MVs. For recent reviews in this topic see also de Toledo Martins et al. (2019); Joffe et al. (2016); and Rodrigues et al. (2015).

MVs function as vehicles for non-classical secretion of many different components including proteins, small molecules, lipids, and nucleic acids. Packaging multiple components into MVs has the obvious advantage that these are delivered as a concentrated bolus and thus avoid the dilution that would occur had they been released at the cell membrane and diffused away. Extracellular vesicles from *C. neoformans*, *S. cerevisiae*, and *P. brasiliensis* have each been shown to package small RNAs (Peres da Silva et al. 2015). The function of these RNAs is not known but their presence in MVs raises the possibility that they are used in communication and/or modulation of target cells. Proteomic analysis of MV protein content from *C. neoformans*, *H. capsulatum*, and *P. brasiliensis* has shown great variability in composition with the cargo including structural proteins and enzymes (Rodrigues et al. 2014; Vallejo et al. 2012b). Many of the proteins found in MV lack signal peptides associated with conventional secretion and vesicles represent a mechanism for unconventional secretion (Rodrigues et al. 2014).

For *C. neoformans* MVs transport components associated with virulence such as polysaccharides and laccase (Rodrigues et al. 2008a, 2007) that can modulate macrophage functions (Oliveira et al. 2010). Lipid staining was used to document punctate structures in the *C. neoformans* capsule that could represent MVs (Nicola et al. 2009). MVs from the highly pathogenic *Cryptococcus gattii* responsible for the Vancouver outbreak are taken up by macrophages where they impair cellular functions and facilitate the growth of less pathogenic strains (Bielska et al. 2018). *C. albicans*-derived vesicles activate NF-κB in murine macrophages and this effect is highly dependent on MV lipid composition (Wolf et al. 2015). Host defenses against MVs include Galectin-3, which is lytic to *C. neoformans* MVs (Almeida et al. 2017). *C. neoformans* MVs have also been shown to be disrupted by other host proteins such as albumin (Wolf et al. 2012). Antibodies to epitopes in the fungal cell wall of *H. capsulatum* interfere with vesicle release (Matos Baltazar et al. 2016), suggesting that adaptive immune responses during infection can modulate fungal physiology to reduce the secretion of vesicle-associated virulence factors.

3.6.2 Gram-Positive Bacteria

Some of the Gram-positive bacterial MVs proteins are related to virulence and are involved in drug resistance, host cell invasion, immune system evasion, cytotoxicity, and pathogenicity. Proteomic analysis showed that *S. aureus* produces MVs

containing superantigens that can result in nonspecific activation of host T-cells and thus MV may help the bacterium to evade host immune system (Lee et al. 2009). Other virulence factors involved in immune evasion included IgG-binding protein SbI, lactamase, coagulase, lipase, and N-acetylmuramoyl-1-alanine amidase (Lee et al. 2009). MVs isolated from Group B *Streptococcus* contained hyaluronate lyase and possessed collagenase activity that can result in the breakdown of extracellular structures (Surve et al. 2016). Some other virulence factors contributing to the spread and cellular invasion of bacteria included cysteine protease (Staphopain A) and serine protease (exfoliative toxins) identified in *S. aureus* MVs (Jeon et al. 2016; Lee et al. 2009).

MV-associated toxins play an important role in pathogenesis by forming pores in the host membrane and leading to cytotoxicity and apoptosis, consistent with the virulence functions of Gram-positive MVs. Importantly, toxins carried in MVs may explain secretion of toxins that lack any export or secretion signal sequences, an example of which are pneumolysin-containing MVs produced by *S. pneumoniae* (Hirst et al. 2008; Olaya-Abril et al. 2014). To further support an MV-associated secretion, activity of these toxins is usually dependent upon its association with vesicles. For instance, in the case of *S. aureus* the MV-associated α-hemolysin proved to be more cytotoxic and induced necrosis in comparable amounts of soluble α-hemolysin (Hong et al. 2014); or the mycolactone toxin present in the *M. ulcerans* MVs showed a more potent toxic effect on non-phagocytic cells relative to pure toxin (Marsollier et al. 2007).

MVs serve not only as attack weapons but also in defensive roles. MVs can bind to antibiotics (Lee et al. 2013a; Rossi et al. 2003) or help in collecting micronutrients from the extracellular environment, such as iron (Jeon et al. 2016; Schrempf et al. 2011).

While there have been several studies on the response triggered by bacterial MVs in host cells, including the toxic and immunomodulatory effects of MVs, one area that is still relatively unexplored is the role of MVs in intercellular communication among bacterial communities. MVs have been shown to be critical for the formation of biofilms, through export of DNA and proteins, but certainly other intercellular communications take advantage of MVs. For example, *B. subtilis* produces MVs for striking different compositions in different stages of growth (Kim et al. 2016b). Are these serving as communication devices, for example, in quorum sensing, as is proposed for the Gram-negative OMVs? If in mixed communities, MVs have been shown as defenses from antibiotics produced by other bacterial species, it seems logical that MVs serve as interspecies communication devices in symbiotic relationships to help both species thrive.

3.6.3 Mycobacteria

Production of MVs by Mtb was shown to occur during an ongoing infection (Prados-Rosales et al. 2011), suggesting that released MVs and their corresponding cargo

have the capacity of interfering with the surrounding environment. Considering that these MVs are enriched in lipoproteins which, via TLR2, are able to reduce the capacity of dendritic cells and macrophages to present antigen, it is reasonable to hypothesize that Mtb might use these structures to modify the nearest environment to its benefit. Supporting this notion was the observation of an increase in bacterial burden in lungs and spleen, as well as lung inflammation, in Mtb-infected mice, which were treated with intratracheal administration of Mtb MVs (Prados-Rosales et al. 2011). Mtb MVs have gained interest in the context of antigen transfer during the prime immune response to Mtb infection. In an attempt to provide an explanation to the observed robust immune response to Mtb beside the impaired capacity of infected DC for antigen presentation, the hypothesis of antigen export to bystander uninfected DC was proposed. A major premise for antigen export from infected phagocytes is that Mtb-related antigens associate with host membranes and are incorporated into the host endocytic machinery resulting in eukaryotic vesicles harboring bacterial antigens. Two recent reports indicate that the landscape of antigen export by Mtb-infected phagocytes is more complex than expected (Srivastava and Ernst 2014; Srivastava et al. 2016). Strikingly, they showed that antigen transfer was not dependent on eukaryotic exosomes or apoptotic bodies derived from macrophages. Another recent report exploring the origin of MVs in Mtb-infected macrophages showed that exosomes and bacterial-derived extracellular vesicles might represent two independent populations of vesicles, suggesting a scenario where antigen transfer could be mediated by bacterial derived MVs (Athman et al. 2015).

3.7 MVs in Medicine

3.7.1 Clinical Implications of MVs

Examples of both detrimental and beneficial effects of MVs from cell-walled microorganisms have been provided. The production of MVs by many pathogenic species of Gram-positive bacteria, mycobacteria, and fungi suggests a connection between microbial pathogenesis and vesicles. Studies where isolated MVs were administered locally before challenge with the corresponding pathogenic species translated into exacerbation of the disease. In *C. neoformans*, this approach resulted in an enhanced efficiency in crossing the blood–brain barrier by the fungus (Huang et al. 2012). In *M. tuberculosis*-infected mice a Koch's phenomenon was observed in mice previously infused with Mtb MVs (Prados-Rosales et al. 2011). In fact, some of these findings could help to explain the occurrence of such detrimental effects at distant locations from the infection site. Similarly, it was reported that there was an association between preterm pregnancy termination and exposure of mice to *S. agalatiae* (GBS) MVs. In this particular case, documentation of MVs traveling through the uterine reproductive tract was reported (Surve et al. 2016). Conversely, MVs produced by nonpathogenic variants of cell-walled microorganisms have been

shown to provide a benefit or at least no negative effect for the host. The best examples of this type of effect are MVs from probiotic microorganisms such as those from the *Bifiobacterium* and *Lactobacillus* genus. Importantly, such probiotic effects were observed with isolated MVs but not with whole cells, highlighting again the potential of these structures in reaching distant sites of the host more efficiently than a whole bacterial cell (Liu et al. 2018).

3.7.2 MVs as Vaccine

The observation that the administration of MVs prior to infection with pathogenic cell-walled microorganisms promote disease, contrasts with scenarios where naturally produced MVs have been shown to serve as potential vaccines. The potential advantages of using MVs as vaccines are *(i)* they are easy to obtain; *(ii)* they have intrinsic adjuvant properties; and *(iii)* they lack the ability to replicate like live bacteria. Protective effects of isolated MVs have been shown for *M. tuberculosis* (Prados-Rosales et al. 2014b), *B. anthracis* (Rivera et al. 2010), *S. pneumoniae* (Olaya-Abril et al. 2014), *C. perfringes* (Jiang et al. 2014), *S. aureus* (Choi et al. 2015) and *C. albicans* (Vargas et al. 2015). Among beneficial effects derived from vaccination with MV are the extended life of the infected host, reduced inflammation, or lower bacterial or fungal burden in organs. One of the major issues associated with the use of naturally produced MVs from bacteria is their potential toxicity. Although this problem is greater with Gram-negative bacteria because of the presence of LPS in OMVs, toxicity issues may also appear in Gram-positive MVs. A recent study generated genetically engineered *S. aureus* strains to produce MVs with limited toxicity (Wang et al. 2018) and propose them as a vaccine platform.

3.7.3 MVs in Biofilm Production

It is believed that environmental microbial communities are primarily organized in biofilms, which creates one of the most recalcitrant issues in clinical settings and represent a leading source of antibiotic-resistant infections. The biofilm matrix constituted by exopolysaccharides, proteins, and DNA, among other molecules, protects the bacterial and or fungal communities from external insults. MVs have been implicated in biofilm formation, presumably due to their role in transporting the cargo used in biofilm formation. Association between MVs and the biofilm formation was studied in *M. ulcerans* (Marsollier et al. 2007), *B. subtilis* (Brown et al. 2014), *S. mutants* (Liao et al. 2014), and *C. neoformans* (Robertson et al. 2012). MVs were visualized in association with the outermost layer of the extracellular matrix of *B. subtilis* and *M. ulcerans* biofilms. For *C. albicans,* MVs produced in biofilm conditions carry components used in matrix construction and differ from those produced by planktonic cells (Zarnowski et al. 2018). Such biofilm-promoting

vesicles reduce the antifungal susceptibility of *C. albicans* cells and thus represent an example of community sharing of resources (Zarnowski et al. 2018). The addition of subinhibitory concentrations of EDTA to *C. neoformans* interfered with both MV release and biofilm formation (Robertson et al. 2012).

3.7.4 MVs in Diagnosis

The literature on the use of MVs from cell-walled microorganisms as a platform for the diagnosis of infectious diseases is scarce. In a previous report, the human serologic response to isolated *M. tuberculosis* MVs was evaluated. A signature for TB was identified and consisted of three proteins that were specifically recognized by both smear-positive and smear-negative TB patients but not in healthy controls with or without latent tuberculosis infection (Ziegenbalg et al. 2013). These studies supported the use of Mtb MVs as a source of biomarkers. More recently, the finding that microbiota-derived MVs could be detected in urine or blood opens the door to the use of these components as a surrogate of the health status of the bacterial communities of our gut (Kang et al. 2013; Lee et al. 2017; Park et al. 2017).

3.8 Unsolved Problems and Concluding Remarks

The discovery of MVs in cell-walled microorganisms such as fungi, Gram-positive bacteria, and mycobacteria has opened a whole new set of questions and avenues of investigation that were not considered in microbiology until very recently. Some of the new problems are common to both fungi and bacteria, while others are specific to each class of microorganisms.

3.8.1 Cell Wall Transport

Perhaps the most intriguing question raised by the discovery of MVs in cell-walled microorganisms is: how do these structures get through the cell wall? This question is intriguing because it challenges long held impressions of cell walls and their functions. In fact, the discovery of MVs in this class of microorganisms was probably delayed by the fact that their existence did not fit within the paradigm that cell walls were rigid and permeable only to small molecules. This view of cell walls meant that there was no need to consider extracellular vesicles and when such structures were occasionally noted in electron microscopy images these were dismissed as artifacts from the association of lipids released from dead and dying cells. In microbiology, fields are organized based on the species of the microbe such that communities working on different microbes constitute different fields

(Casadevall and Fang 2015). Hence, different fields have demonstrated different levels of acceptance of MVs. At this present time, the fungal and mycobacterial fields have wholeheartedly accepted the existence of MVs while the same notion has found different acceptance in various Gram-positive bacterial fields. Nevertheless, the increasing accumulation of papers from independent groups reporting MVs in different Gram-positive bacteria means that it will hopefully be only a matter of time before there is universal acceptance of this phenomenon.

3.8.2 Mechanism of Vesicle Production and Sorting Contents

The discovery of MV provides a transport system for great myriad of biomolecules that is not dependent on classical secretion mechanisms. MVs have been shown to carry proteins, polysaccharides, lipids, and even small molecules such as siderophores. Vesicle production poses different questions in fungi, Gram-positive bacteria, and mycobacteria. In fungi, the current view is that MVs are produced in vesicular bodies that then release these structures into the space between the cell membrane and cell wall for the crossing of the latter (Casadevall et al. 2009). For Gram-positive and mycobacteria, these vesicles are presumably produced at cell membranes for their transit across the cell wall. At this time there is little information regarding how OMVs and MVs are made and loaded with their cargo. Given that MVs have numerous roles in the extracellular space from biofilm formation, to communication, and promoting microbial virulence, one can imagine that there must exist a mechanism that sorts the cargo into vesicles depending on their intended role.

3.8.3 Role of Vesicles in Pathogenesis and Vaccines

The finding that many microbial MVs packs a suite of virulence factors means that vesicles have a role in microbial pathogenesis. For example, the finding that such toxigenic Gram-positive bacteria as *B. anthracis* (Rivera et al. 2010), *L. monocytogens* (Coelho et al. 2019), and *C. perfringes* (Jiang et al. 2014) pack their toxins into MVs is now an important factor to consider when incorporating vesicles into concepts of pathogenesis. MVs allow microbes to deliver a concentrated punch to a target such as predatory amoeba or a macrophage without concern about dilution during diffusion from the microbial surface. MVs also affect and induce immune responses (Kuipers et al. 2018). MVs from mycobacteria (Prados-Rosales et al. 2011, 2014b), *S. aureus* (Choi et al. 2015; Wang et al. 2018), and *S. pneumoniae* (Choi et al. 2017) elicit immune responses with vaccine potential. Hence, MV secretion poses new questions on how microbes damage host cells as well as providing a new set of components that can be used in vaccine design.

3.9 Concluding Remarks

A decade plus since the first reports of MVs in cell-walled microorganisms, this topic has emerged as an exciting area of study that poses new scientific problems that will undoubtedly produce new insights into prokaryotic and eukaryotic cell biology when solved. The production of MVs by cell-walled microorganisms places them in a continuum that includes plant and animal cells and Gram-negative bacteria, each of which uses vesicles to package cellular components for use in a wide array of functions that range from communication to modification of their environment. In fact, MV production may be a universal feature of cells irrespective of whether they have cell walls. One can anticipate that as additional investigators spin the culture supernatants of their favorite cell wall microorganisms, additional species will be associated with MV production, which in turn will lead to new research projects.

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