



Characterization and function of membrane vesicles in Gram-positive bacteria

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Abstract

In recent years, extracellular vesicles have gained more attention. However, studies on membrane vesicles in Gram-positive bacteria were carried out relatively late because of the thick bacterial wall and the low production of membrane vesicles. Thanks to the research in recent years, the cognition of the composition and function of the membrane vesicles of Gram-positive bacteria has made significant progress. Membrane vesicles are spherical in shape comprising bilayer membranous structures with a diameter of 20–400 nm. Components of membrane vesicles are diverse, including proteins, nucleic acids, lipids, and metabolites. It also has been reported that membrane vesicles are involved in various pathophysiological processes and serve as communication tools in pathophysiological activities between the bacteria and the host. This review provided the current understanding of components and functions of membrane vesicles in Gram-positive bacteria. The findings might facilitate further research in the emerging field of membrane vesicles in Gram-positive bacteria.

Key points

- Membrane vesicles in Gram-positive bacteria contain proteins, nucleic acids, lipids, and metabolites, suggesting their biological significance.
- Membrane vesicles in Gram-positive bacteria are thought to be involved in stress response, biofilm formation, immune regulation, and so on.

Keywords Characterization · Function · Gram-positive bacteria · Membrane vesicles

Introduction

In recent years, extracellular vesicles (EVs) have gained more attention (El Andaloussi et al. 2013; van Niel et al. 2018). EVs were first discovered in eukaryotes as vesicles derived from the plasma membrane carrying proteins, nucleic acids, and lipids. They are mainly divided into three categories according to their size: exosomes, microvesicles, and apoptotic bodies (Raposo and Stoorvogel 2013). Then, EVs were also found in prokaryotes such as bacteria (Brown et al. 2015). Bacteria are

divided into Gram-negative and Gram-positive bacteria according to their membrane structure. The Gram-negative bacteria have two membrane layers separated by the periplasm. The membrane in Gram-positive bacteria is very different from that in Gram-negative bacteria with only one membrane and one thicker layer of peptidoglycan. The EVs in Gram-negative bacteria are called outer membrane vesicles (OMVs), while EVs in Gram-positive bacteria are called membrane vesicles (MVs) (Avila-Calderón et al. 2015). Both OMVs and MVs are subclasses of microbial EVs. Research on OMVs has been extensive, with many reviews highlighting their functions (Jan 2017; Tan et al. 2018). Many people have questioned the production of vesicles in Gram-positive bacteria in the past because it is relatively low. However, in the last decade, a great deal of research has been conducted on the role of Gram-positive MVs.

MVs have been observed in all pathogenic and nonpathogenic Gram-positive bacteria under different growth conditions and different natural environments. The secretion of

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MVs is a universal and widespread phenomenon (Toyofuku et al. 2019). With the increase in the number of studies on Gram-positive bacteria, such as *Staphylococcus aureus*, *Bacillus anthracis*, and *Streptococcus mutans*, it was found that MVs in Gram-positive bacteria had a 20- to 400-nm bilayer spherical structure (Jeon et al. 2016; Liao et al. 2014; Rivera et al. 2010).

In this review, the current knowledge on content characterization and functions of MVs in Gram-positive bacteria was discussed (Fig. 1).

Composition of MVs

The mechanism of MV biogenesis in Gram-positive bacteria has been reviewed in some studies (Briaud and Carroll 2020; Brown et al. 2015). A few genes found in certain bacteria have been reported to regulate MV biogenesis. For example, the transcription factor σ^B regulates MV formation in *Listeria monocytogenes* (Lee et al. 2013). A two-component regulator CovRS impacts MV production in Group A Streptococcus (Resch et al. 2016). However, a unified gene that can be applied to all Gram-positive bacteria is still lacking. MVs in Gram-positive bacteria are produced when a section of the cytoplasmic membrane protrudes and buds off selectively encapsulating various components, and then passes through the cell wall. Therefore, the whole process of MV biogenesis is affected by many pathways. Turgor pressure, protease

enzymes, and protein channels are proposed hypotheses that explain how MVs traverse thick cell walls (Brown et al. 2015). In summary, all these studies showed that MV production is regulated by a complex gene network. Whether Gram-positive bacteria share a conserved general mechanism for MV biogenesis is currently unknown.

For MV composition analysis, sample concentration is critical. The separation of purified MVs is the key to mass spectrometry. MVs can be isolated from bacterial cultures or biofilms. Generally speaking, MVs can be obtained by ultra-centrifugation after the supernatant culture is filtered through a filter with a pore diameter of 0.22–0.45 μm (Chutkan et al. 2013). It is worth noting that a typical filter with 0.22- μm diameter might lower the yields up to approximately 50% when the actual size of MVs is more than 200 nm under a certain environment (Gorringe et al. 2005). However, ultra-centrifugation is not sufficient to purify MVs from protein aggregates or membrane fragments (Choi et al. 2013). Larger macromolecules such as fimbriae and flagella should be included in the MV pellet during the ultracentrifugation method. In addition, sucralose concentration ultracentrifugation can also be used to extract bacterial MVs, but sucrose does not fully preserve the size of MVs in the gradient. In short, the aforementioned methods cannot remove pollutants other than MVs. To characterize a pure population, density gradient ultracentrifugation is one of the best methods to isolate bacterial MVs (Chutkan et al. 2013; Klimentova and Stulik 2015). At present, a procedure for isolating MVs,

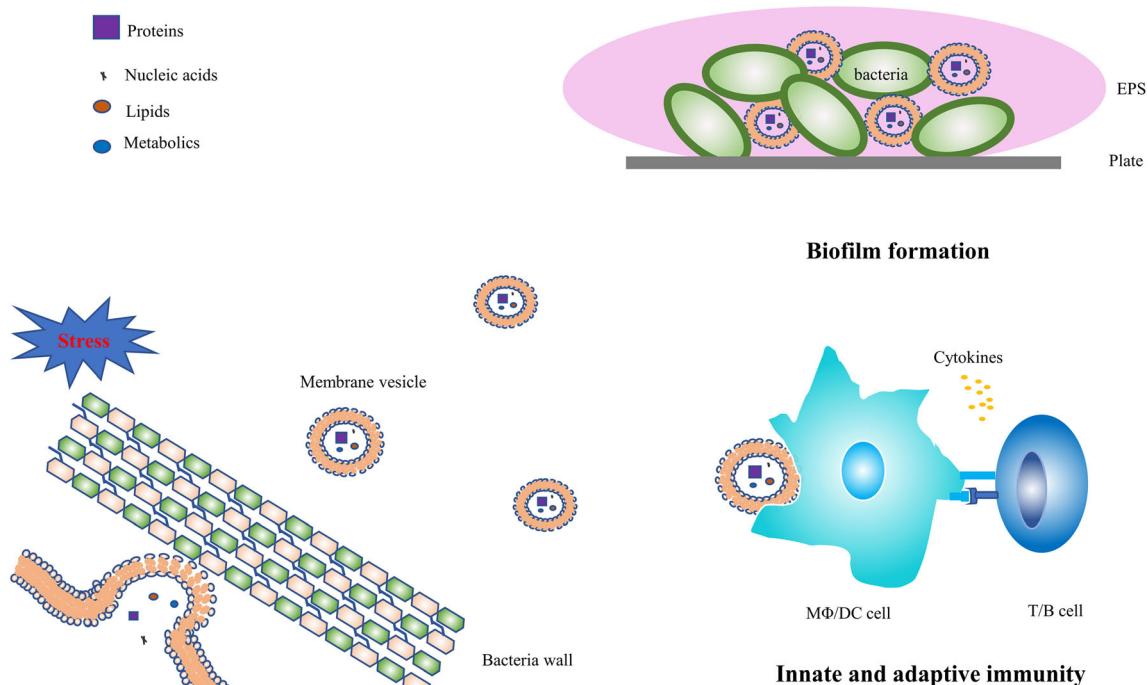


Fig. 1 Model of the function of Gram-positive MVs

consisting of multiple centrifugation, filtration, and density gradient ultracentrifugation, has replaced original methods such as gel filtration and is used to guide the separation of bacterial MVs (Klimentova and Stulik 2015).

With the development of omics technology, various contents have been discovered, indicating the diversity of vesicle functions. Studies on different bacteria under different conditions have shown that MVs in Gram-positive bacteria contain proteins, genetic material, lipids, and metabolites (Coelho et al. 2019; Rivera et al. 2010). The following sections discuss the current understanding of the components of MVs.

Proteins

Experiments have shown similarities and differences in protein components between bacteria and MVs. For example, the analysis of the protein composition of *Listeria monocytogenes* found that 296 proteins were present in both whole cells and 16 proteins were present only in the MVs, including PI-PLC, autolysin, uncharacterized protein yabE, competence protein ComEC/Rec2, flagellar proteins, and other uncharacterized proteins (Karthikeyan et al. 2019). Protein localization showed differences in the sources of vesicles. PSORTb 3.0 software was commonly used for protein localization, and the composition of the protein localization was found to be different from that of Gram-positive bacteria. Most MV-related proteins were predicted as cytoplasmic or plasma membrane proteins, while a few were predicted as cell wall or extracellular binding proteins (Rivera et al. 2010). The unique protein composition that MVs have proteins which were only present in MVs and that MVs can selectively be enriched with certain proteins suggested that MVs had special functions.

MVs allow the bacteria to disperse bacterial products into the surrounding environment. For pathogenic Gram-positive bacteria, many MV proteins are virulent factors and toxins, which help the bacteria exert their actions. MV-related virulence factors vary according to the bacteria. For example, the virulence factor of *L. monocytogenes* MVs is a toxin that can interact with host cells and contribute to the pathogenesis of *L. monocytogenes* during infection (Karthikeyan et al. 2019). The virulence factors of *S. mutans* MVs are glucosyltransferases, which help *S. mutans* colonize (Rainey et al. 2019). For nonpathogenic Gram-positive bacteria, for example, MV proteins have a variety of protective effects as probiotics. In the study of *Lactobacillus casei*, researchers identified MV proteins as mediators of probiotic effects of *L. casei*, which are involved in the bacteria–gastrointestinal cell interface (Dominguez Rubio et al. 2017).

The composition of MV proteins in Gram-positive bacteria is different in different strains and environments. Many researchers have performed the proteomic analysis of different clinical strains under different culture conditions and found that the composition of MV proteins had many common and

unique characteristics (Wagner et al. 2018). The growth medium affects gene expression in bacteria, thereby altering the number and/or content of MVs.

Nucleic acids

In Gram-negative bacteria, DNA of OMVs can be horizontally transferred between the same and different species, leading to the transmission of bacterial resistance (Domingues and Nielsen 2017). The related studies on Gram-positive bacteria were carried out late. Previous studies showed that genetic materials, including DNA and RNA, were present in MVs isolated from Gram-positive bacteria.

DNA in Gram-positive bacteria was first reported in cellulolytic *Ruminococcus* spp. The study reported that MVs from wild-type bacteria could continuously “rescue” the mutants and cause them to degrade crystalline cellulose (Klieve et al. 2005). Further studies found that *Clostridium perfringens* also contained partial gene-fringed DNA (Jiang et al. 2014). In addition, *S. mutans* released MV-related eDNA to improve biofilm formation (Liao et al. 2014; Senpuku et al. 2019).

The differentially enriched intragenic RNAs in MVs, mostly rRNAs and tRNAs, were first reported in Group A *Streptococcus* (Resch et al. 2016). However, no studies explored the possibility of RNA-level transfer mediated by MVs in Gram-positive bacteria, which was contrary to the RNA content of mammalian exosomes.

Lipids

Lipids are important components of MVs. The identification of key lipid biomarkers in metabolic regulation can reveal the mechanism underlying various life activities.

Studies have shown that MVs contain lipids, but they also show some specific aggregation. For example, the lipids identified from *Bacillus anthracis* and MVs were mainly palmitic acid and stearic acid, but considerable differences were detected in the composition of secondary lipid components. MVs were rich in myristic and palmitic acids (Rivera et al. 2010). The study on *Streptococcus pneumoniae* found that MVs had a different fatty acid composition and were enriched in short-chain saturated fatty acids (Olaya-Abril et al. 2014). The study on *L. monocytogenes* found that unsaturated fatty acids such as phosphatidylethanolamine and sphingolipids were more abundant in MVs (Coelho et al. 2019). These studies showed that the lipid components of MVs did not replicate in the bacterial membrane, but had unique aggregation patterns. The result suggested that MVs were produced via certain mechanisms. One possible explanation is that short-chain saturated fatty acids and unsaturated fatty acids, which are selectively enriched in Gram-positive MVs, affect membrane curvature and membrane fluidity. Since the membrane is more fluid with shorter and more unsaturated fatty acids (Mercier

et al. 2012), selective enrichment makes it easier for Gram-positive bacteria to form and release MVs. However, the specific role of these lipid components needs further exploration.

Metabolites

The changes in metabolites indicate the influence of disease, toxicity, gene modification, and environmental factors. The study on bacterial vesicle metabolites is an emerging research field (Bryant et al. 2017; Zakhazhevskaya et al. 2017). Comparative metabolomic analyses were conducted on vesicles isolated from virulent strains of *Bacteroides fragilis* and nonvirulent strains, and metabolic pathways of OMVs were reconstructed (Zakhazhevskaya et al. 2017).

Compared to the studies on the Gram-negative bacteria, few studies explored the metabolomics of Gram-positive bacteria. The metabolites carried by MVs include ornithine, citrate, inositol, phenylalanine, citric acid, and the key intermediate metabolite pyruvate (Coelho et al. 2019). The metabolic composition of MVs varies under different environments. Our study compared the differences in metabolites in *S. mutans* under acidic conditions and obtained 35 different metabolites. The upregulated MV metabolites, such as betaine, trehalose, and L-carnitine, were stress protectors, providing new information for assessing the survival and proliferation of Gram-positive bacteria under environmental pressure (Cao et al. 2020). The characteristics of metabolites in different types of bacteria are not clear, which is worth further exploration.

Function of MVs

The benefits must be sufficient considering the huge energy costs involved in producing MVs. MVs are likely to have important biological functions based on their metabolic cost and interesting contents (Brown et al. 2015). In recent years, thanks to intensive research, it was soon discovered that MVs were vital in bacterial physiology and pathogenesis. They were thought to be involved in stress response, biofilm formation, intraspecies and interspecies communication, immune regulation, and so on. In the following sections, the current understanding of the function of MVs was presented.

Stress response

Bacteria are exposed to a variety of pressures, such as malnutrition, antibiotics, and oxidative stress, under both physiological and clinical conditions. The bacteria increase the pressure response, which usually includes activation of pressure sensors, changes in transcription levels, and downstream changes in bacterial envelope composition, to counteract the effects of environmental damage on their structures (Ahn et al. 2006). Current studies suggest that vesicle involvement in pressure

regulation is related to lipid involvement in changes in membrane curvature and secretion of misfolded proteins and metabolites.

OMVs are used as part of the general stress response mechanism to improve the chances of bacterial survival in Gram-negative bacteria (Atashgahi et al. 2018; Baumgarten et al. 2012). The production of OMVs is also considered as a stress response, which can eliminate bacterial misfolded proteins and provide an external bait to absorb antimicrobial agents on the cell surface (Schwechheimer and Kuehn 2015).

However, MVs in Gram-positive bacteria have been less explored. *L. monocytogenes* can survive under extreme environmental stress conditions, and the transcription factor sigma B is involved in this survival ability (Wiedmann et al. 1998). Studies found that a wild-type strain produces ninefold more MVs compared with sigB mutant (Lee et al. 2013). Studies found that the extrusion of the polar growth tissue at the hypha tip led to the formation of MVs, which was related to the survival of bacteria in *Streptomyces venezuelae* (Frojd and Flardh 2019). In the present study, acidic conditions induced *S. mutans* to produce more and smaller vesicles containing different proteins and metabolites. The expansion of the MVs may serve as a defensive response to acid pressure, increasing the chances of bacterial survival (Cao et al. 2020). The forementioned studies suggested that MV formation helps Gram-positive bacteria to better adapt to environmental stress and prevent the fatal effects of adverse environments. On the one hand, the release of MVs can maintain the stability of the membrane; on the other hand, MVs can carry some stress substances out of the bacteria. MV formation is a strategy for Gram-positive bacteria to respond to environmental changes. Hence, the role of MVs in the stress response of Gram-positive bacteria deserves further investigation.

Biofilm formation

Biofilm is considered as the main state of microorganisms in the environment, and the formation of biofilms is an important issue in clinic. Biofilms protect microbial growth from surface detachment and interference by antimicrobial substances (Kumar et al. 2017). The involvement of vesicles in biofilm formation is related to vesicle cargos, such as eDNA as a biofilm substrate, lipids as hydrophobic surface providers, and adhesion-related proteins as adhesive mediators. OMVs are crucial in the formation, communication, nutrition acquisition, and defense of biofilms and are the key substances of biofilms (Brown et al. 2015; Schooling and Beveridge 2006).

The effects of MVs in Gram-positive bacteria on biofilms have also been reported. On the one hand, MVs can be used as the substrate of biofilms. On the other hand, adhesion-related enzymes in MVs are actively involved in biofilm formation. In the *Bacillus subtilis* biofilm, MVs exist in the stroma and emerge from embedded cells in the biofilm (Brown et al.

2014). In the *Mycobacterium ulceris* biofilm, MVs are confined in the extracellular matrix surrounding the outer part of the bacterial community, rather than distributed throughout the complex biofilm structure. MVs isolated from planktonic *L. monocytogenes* contain protein components of biofilms. In *S. mutans*, MVs contain eDNAs and adhesive proteins that contribute to the formation of biofilms. In particular, MVs are easily dispersed and hence make local use of sucrose in the surrounding environment, facilitating the colonization of bacteria (Cao et al. 2020). MVs in *S. mutans* not only benefit the formation of their own biofilms but also contribute to the development of *Candida albicans* biofilm (Wu et al. 2020).

Immune regulation

The role of MVs in host immune regulation has been increasingly recognized because MVs contain many immune-related molecules and can be accepted by host cells. These immune-related molecules include lipoproteins and toxins which could trigger innate and adaptive immunity.

MVs in both pathogenic and nonpathogenic Gram-positive bacteria are involved in innate immunity, in which macrophages, dendritic cell, and Toll-like receptor 2 (TLR2) are the key molecules. A large number of MVs in pathogenic bacteria can activate the host innate immune response. For example, MVs in *C. perfringens* induce the release of inflammatory cytokine interleukin-6 (IL-6) through the TLR2 signaling pathway (Jiang et al. 2014). *Staphylococcus aureus* enhances the production of protein inflammatory mediators, such as tumor necrosis factor (TNF-), IL-6, and IL-12, through the expression of co-stimulatory molecules via the TLR2 signaling pathway (Choi et al. 2015; Gurung et al. 2011). MVs in *Streptococcus suis* also activate the nuclear factor kappa B signaling pathway in single cells and macrophages, inducing the secretion of pro-inflammatory cytokines (Haas and Grenier 2015). In a study on MVs in nonpathogenic bacteria, MVs derived from *Lactobacillus sakei* subsp. *sakei* NBRC15893 enhanced immunoglobulin A production by activating TLR2 signaling (Yamasaki-Yashiki et al. 2019). The cell wall components of Gram-positive bacteria contain large numbers of TLR2 ligands, such as lipoteichoic acid, teichoic acid, peptidoglycan, and lipoprotein (Kaji et al. 2010; Matsuguchi et al. 2003; Shida et al. 2009; Zeuthen et al. 2008). Thus, MVs in Gram-positive bacteria which also contain cell wall components could work together with bacteria to exert innate immunomodulatory effects via TLR2. To date, lipoproteins contained in MVs have been reported to be involved in immunomodulatory responses in a variety of Gram-positive bacteria (Prados-Rosales et al. 2011; Yamasaki-Yashiki et al. 2019).

MVs are also vital in adaptive immunity, which has also been confirmed by relevant studies. Mice inoculated with MVs in *Streptococcus pneumoniae* produced specific

antibodies (Olaya-Abril et al. 2014). *S. aureus* induced Th1, Th17, and Th2 cells as well as IgG antibody responses (Choi et al. 2015). These findings indicated an effective vaccination efficacy of MVs in Gram-positive bacteria.

Conclusions and perspectives

The discovery of EVs is a breakthrough and provides a new mechanism for the release of components into the extracellular environment (Avila-Calderón et al. 2015; Nagakubo et al. 2019). Studies on MVs in Gram-positive bacteria are relatively few, lagging behind those on eukaryotes and Gram-negative bacteria, due to the thick cell walls and low production of vesicles (Brown et al. 2015). However, these MVs deserve further exploration owing to the importance of their functions.

The enrichment and selection of specific factors associated with Gram-positive MVs indicated a regulatory mechanism for MV cargo. Genetic and environmental factors also influence MV contents and regulate their release. The molecular mechanisms underlying MV release and cargo are still unknown, but will surely be elucidated in recent years.

Besides the analysis of individual omics, the combined analysis of multiple omics has become more important. In previous studies, the analysis of single omics data was often carried out; the relationship between the omics data and biological processes was explored through the independent analysis of each omics to explain the single information of some species. However, single omics is far from enough for the regulation of complex biological processes. Researchers began to integrate and analyze multiple sets of data (Biagini et al. 2015; Coelho et al. 2019; Olaya-Abril et al. 2014). In the field of MV research, multiomics is beginning to be applied and some important conclusions have been drawn.

The diversity of MV composition indicates the diversity of their functions. This review briefly introduced the role of MVs in stress resistance, biofilm formation, and immunity, but more roles are worth exploring. In particular, as OMVs in Gram-negative bacteria have been used as vaccines (Jiang et al. 2019; Pastor et al. 2021), there is great potential for the application of MVs in Gram-positive bacteria, too.

The findings of this review might stimulate the exploration of MVs in Gram-positive bacteria.

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Declarations

Ethics approval None required.

Conflict of interest The author declares no competing interests.

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