

# Chapter 6

## Membrane Vesicles from Plant Pathogenic Bacteria and Their Roles During Plant–Pathogen Interactions



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**Abstract** Membrane vesicle (MV) release occurs in all forms of life, including Gram-negative and Gram-positive bacteria. Bacterial MVs have been studied mostly in relation to the bacterial lifestyle and regarding their role during mammalian host interactions. Surprisingly, while plants are known to be colonized by pathogenic, mutualistic, and commensal bacteria, the functions of MVs produced by these plant colonizers have only begun to be studied in the past decade. In fact, only a handful of studies have been published on this topic. Nevertheless, it is apparent that this field is gaining increasing attention, as does the role of plant and fungal extracellular vesicles (EVs) during plant–pathogen interactions. In this chapter I will review the current literature on plant-associated bacterial MVs and their interactions with plants. I will focus on MV cargo with emphasis on virulence-related proteins and on MVs' function during host colonization including interactions with the plant immune system. I will further provide a view of the possible, yet unexplored, roles of MVs in plant–bacteria interactions, and highlight important questions and limitations in the study of MVs.

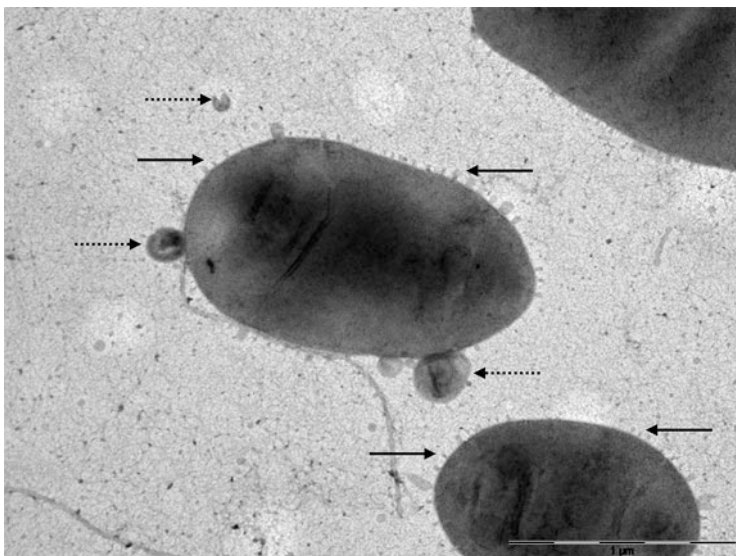
### 6.1 Background

Extracellular bacterial membrane vesicles (MV) are spherical nanostructures originating from the cell envelope and released into the extracellular environment. The initial bulging of MVs can be observed using transmission electron microscopy, as small blebs projecting from the outer membrane (OM) along the cell periphery (Fig. 6.1). One of the first descriptions of bacterial MVs was over 50 years ago by Knox et al. (1966), yet, the following three decades saw only a handful of publications that further developed this topic. The slight interest in MVs in the first decades can be partly explained by the common conception of MVs being an artifact of cell growth or a result of cell breakage and death (Haurat et al. 2014). However, in recent

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**Fig. 6.1** Membrane vesicle formation in the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*). A *Xcc* (strain 33913) culture grown on nutrient agar plate for 48 h was washed off the plate, diluted and negatively stained using 4% uranyl acetate on Cu-400FC grids. Specimens were analyzed using a JEM-1400Plus Transmission Electron Microscope at 40 K magnification. In the micrograph, few *Xcc* cells are shown with small membrane blebs forming along the margins of the cell (solid-line arrow). A few larger membrane vesicles, that appear to have already formed and dissociate from the cells are also seen (dashed-line arrows). Size bar indicates 1  $\mu\text{m}$

years, there is a significant increase in the number of publications on extracellular vesicles (EVs) in general and on bacterial MVs specifically, indicative of the growing interest the scientific community has in this field.

Similar to the progress in the general field of MVs, the first studies on MVs released by plant pathogenic bacteria were late to arrive and were published more than 40 years after the work of Knox et al. (1966). Here too, the scientific community was slow to take up this topic and very few research papers have since been published on MVs of plant-associated bacteria. Nevertheless, the recent 5 years saw a significant increase in published papers studying and reviewing the role, or involvement, of MVs of important plant pathogenic bacteria such as *Xanthomonas campestris* pv. *campestris* (*Xcc*), *X. euvesicatoria*, *Pseudomonas syringae* pv. *tomato* (Pst), and *Xylella fastidiosa*, in plant–microbe interactions (Bahar et al. 2016; Baldrich et al. 2019; Ionescu et al. 2014; Katsir and Bahar 2017; Mendes et al. 2016; Nascimento et al. 2016; Regente et al. 2017; Rutter and Innes 2017, 2018; Rybak and Robatzek 2019; Solé et al. 2015).

Bacterial MVs have been implicated in multiple functions such as virulence, host immune modulation, surface adherence and biofilm formation, cell–cell communication, genetic material transfer and more (Schwechheimer and Kuehn 2015) (see

also Chaps. 1, 2 and 5). Owing to this wide range of activities, the cargo of MV is rich and diverse, containing membrane lipids, lipopolysaccharides, membrane proteins, soluble proteins, nucleic acids, and peptidoglycan. Most of our knowledge on the cargo and functions of bacterial MVs comes from studies involving mammalian pathogens. In this chapter, I will try to summarize the main studies and findings related to MV cargo and function in plant pathogenic bacteria. More specifically, this chapter will focus on proteomic studies of plant pathogenic bacterial MVs, the presence of virulence factors in MVs and their possible functions, and the role of bacterial MVs during host colonization including MV interactions with the plant immune system.

## 6.2 Characterization of the Molecular Cargo of Bacterial Plant Pathogens MVs

Bacterial MVs are not de novo synthesized and are rather formed from preexisting cell structures such as the outer membrane (OM). While, it has become widely accepted that the formation of MVs is not a random process, the mechanisms that govern MV cargo sorting have remained largely elusive (Haurat et al. 2014). Considering that cargo sorting into MVs is a regulated and deliberate process, it is still reasonable to assume that at least some of the molecular cargo associated with MVs is a result of their presence in the preformed structures from which the MV originates, i.e., the OM and the periplasmic space. With this assumption in mind, one of the challenges en route to understanding the role MVs play during plant colonization is to be able to distinguish MV molecules that have specific roles in planta from molecules that are merely associated with MVs.

One way to address this challenge would be to purify bacterial MVs that are formed during plant colonization and compare their molecular cargo with MVs of the same organism, that were produced in a rich artificial medium. Such an approach has been used to identify genes and/or proteins that are specifically expressed in planta using transcriptomic and proteomic approaches (Andrade et al. 2008; Jacobs et al. 2012). However, since the study of plant pathogenic bacterial MVs is only in its infancy, methods to purify MVs from infected plants have not yet been optimized and published. To overcome this limitation, attempts were made to purify and characterize MV proteins following growth in culture media that mimics the plant environment.

*Xcc* is a Gram-negative bacterium that belongs to the family Xanthomonadaceae and is the causal agent of “black rot” disease of crucifers. Similar to some mammalian bacterial pathogens, *Xcc* depends on a functional type 3 secretion system (T3SS) for pathogenicity (Ryan et al. 2011). To identify MV proteins that are more likely to have a role during plant colonization, Sidhu et al. (Sidhu et al. 2008) purified MVs from *Xcc* cultures grown in two different minimal media: M9 and XVM2 and characterized their protein cargo using liquid chromatography mass spectrometry

(LC-MS/MS). XVM2 media was shown in the past to induce the expression of T3S-genes, which are thought to be induced strictly during plant colonization, and hence could serve as a proxy for the plant environment (Wengelnic and Bonas 1996). This first proteomic characterization of a plant pathogenic bacterium MVs revealed several interesting insights. First, the fact that certain proteins are enriched in the MV fraction compared with the OM fraction. This result suggests the existence of a protein sorting mechanism that directs proteins specifically to MVs and is also supported by previous studies with mammalian bacterial pathogens (Haurat et al. 2011). Second, that culture media affects the composition of MV proteins; and third, the association of virulence-related proteins with MVs. The association of virulence factors with MVs of plant pathogenic bacteria was also demonstrated for the tomato pathogen—*Pst* T1 strain (Chowdhury and Jagannadham 2013).

In the study by Sidhu et al. (2008), structural T3S-proteins, T3S-regulators, and T3S-effectors were found in association with secreted MVs. However, these structural proteins were not specifically expressed in the plant-mimicking media as they were also expressed in the control media, highlighting the limitation of this approach in finding plant-induced MV proteins. Additional virulence-related proteins that were found in the MV proteome included plant cell wall degrading enzyme such as cellulase and xylosidase (Sidhu et al. 2008). T3S-effectors such as HopI1 (suppressors of T3E-triggered death in *Nicotiana benthamiana*) and *avrA1* were also identified in the MV fraction of in vitro-grown *Pst* (Chowdhury and Jagannadham 2013). Additional virulence-related proteins found in *Pst* MVs included hydrolytic enzymes such as chitinase and phytase. Here too, these virulence-related proteins were detected in MVs although bacteria were grown in a rich medium, which does not simulate the plant environment.

When attempting to identify MV proteins with specific role in planta, it is important to consider that the MV secretion pathway also serves as a disposal machinery for the cell. Hence, MVs could be associated with a variety of disposed proteins that do not serve a specific function in MVs (McBroom and Kuehn 2007; Schwechheimer and Kuehn 2013). This, obviously, further complicates the task of identifying proteins with strict function in MVs. With this in mind, even a successful proteomic analysis of in planta produced bacterial MVs would still be difficult to interpret and would not necessarily allow us to distinguish MV-functional and nonfunctional proteins.

These two studies (Sidhu et al. 2008; Chowdhury and Jagannadham 2013) were the first proteomic analyses of plant pathogenic bacterial MVs. Interestingly, in both cases, a relatively low number of proteins was identified (30–40 proteins in Sidhu et al. 2008, 139 proteins in Chowdhury and Jagannadham 2013), compared with MV proteomic studies of other bacteria, such as *P. aeruginosa*, where several hundreds of proteins were identified by LC-MS/MS (Choi et al. 2011). The isolation of MVs is a step of the utmost importance in the study of MV function and cargo and should therefore be given careful attention. In a position statement by the International Society for Extracellular Vesicles, Lötvalld et al. (2014) discusses important caveats and recommendations in eukaryotic extracellular vesicle (EV) isolation. These

recommendations should also be adopted in MVs research in the plant–microbe interactions community, to standardize, where possible, the methodology and quality of MVs isolation.

Additional studies that describe the association of plant pathogens virulence factors with MVs include the study of Solé et al. (2015). Aiming to identify T2S-virulence factors from *X. euvesicatoria*, the authors found a predicted protease, a lipase, and two xylanases in *X. euvesicatoria* supernatants. Interestingly, while these enzymes were thought to be secreted by the T2SS; they were found in the extracellular milieu of *X. euvesicatoria* even in the absence of a functions T2SS (Solé et al. 2015). Immuno-gold labeling coupled with transmission electron microscopy observations revealed that these enzymes are present in MVs released by *X. euvesicatoria*. These results suggested that MVs could serve as an additional or alternative secretion pathway for T2S-enzymes by plant pathogens.

Interestingly, the secretion of cellulytic and hemicellulolytic enzymes via MVs was described nearly 40 years ago with the bacterium *Fibrobacter succinogenes* (Forsberg et al. 1981 (formerly *Bacteroides succinogenes*); Montgomery et al. 1988). The authors showed that this bacterium, which colonizes the rumen of cattle, when given cellulose as a carbohydrate source, releases much of its synthesized cellulytic enzymes to the medium. They further showed that over 50% of the cellulytic activity in the medium was associated with sedimentable subcellular MVs. The secreted MVs were observed adhering to cellulose and also free in the culture and exhibited endoglucanase, xylanase, and cellulase activities. Considering that plant pathogenic bacterial MVs are associated with cellulytic enzymes, these results may suggest that MV released during plant colonization may facilitate plant cell wall decomposition. Further examples for secretion of virulence factors in association with MVs from plant pathogens include the secretion of the lipase/esterase LesA by *Xylella fastidiosa*. This lipase esterase is a homolog of the *X. euvesicatoria* lipase (69% identities over 99% coverage) and was also shown to be associated with *Xylella* MVs and to contribute to Pierce’s Disease symptoms and to *X. fastidiosa* virulence (Nascimento et al. 2016). In summary, virulence-related proteins are associated with bacterial plant pathogen MVs, as they are with mammalian bacteria pathogens MVs. Nevertheless, studying the function of these MVs-enclosed, or associated virulence factors during plant colonization will be a feat much more challenging to achieve and will most definitely be occupying this field in the near future.

### 6.3 Functions of MVs During Plant Colonization

Very few studies have been conducted thus far to examine the role bacterial MVs play during plant colonization. Interesting insights were gained from the work of Ionescu et al. (2014) that investigated the role of *Xylella fastidiosa* MVs during xylem vessel colonization. *X. fastidiosa* is a most serious crop-threatening pathogen (Mansfield et al. 2012) known to infect more than 300 different plant species (EFSA

Panel on Plant Health 2015). It is well-known for causing Pierce's Disease in grapevines, citrus variegated chlorosis, and more recently, olive quick decline syndrome in southern Italy (Almeida and Nunney 2015). *X. fastidiosa* is a Gram-negative bacterium transmitted to plants by insect vectors. In the plant, *X. fastidiosa* resides exclusively in the water conducting elements of plants, the xylem, hence the name *Xylella*.

Beautiful scanning electron microscopy images show that *X. fastidiosa* is a potent producer of MVs in vitro (Ionescu et al. 2014). The authors further showed that a cell–cell signaling system, mediated by a diffusible signal factor (DSF), significantly influences the levels of MV release. Interestingly, knocking out the gene responsible for DSF synthesis (regulation of pathogenicity factors, *rpffF*) and abolishing its cell–cell signaling function, resulted in enhanced release of MVs compared with the wild-type strain. To monitor MV production in grapevines, Ionescu et al. (Ionescu et al. 2014) first collected xylem fluids from *X. fastidiosa*-infected and healthy grapevine plants. They then used the XadA as an MV protein marker and nanoparticle tracking analysis to monitor the presence of *X. fastidiosa* MVs. With both approaches the authors were able to show that MVs are released by *X. fastidiosa* during xylem colonization. Moreover, similarly to in vitro conditions, the mutation in the *rpffF* gene led to higher levels of MVs in the xylem sap. As for the function of MVs, Ionescu et al. (2014) found that *X. fastidiosa* MVs act as an anti-adhesive extracellular factors, limiting the adherence of *X. fastidiosa* cells to a glass slide. The adherence of *X. fastidiosa* was also impaired in the presence of MVs when tested in a microfluidic device and in grapevine stems. These results led the authors to suggest a model whereby MVs regulate the transition of *X. fastidiosa* from an aggregated and sticky form to a free-swimming form, which supports bacterial spread through the plant and virulence (Ionescu et al. 2014). One mechanism suggested to explain these results was that binding of MVs to xylem cell walls could restrict the binding of *X. fastidiosa* cells, thereby limiting the number of attachment sites and driving the bacteria more into the free-swimming form over the surface adherent and aggregated form.

How exactly MVs bind to surfaces and block *X. fastidiosa* binding is not clear. Cell surface appendages, like type I pili in *X. fastidiosa*, and other surface molecules like lipopolysaccharides (LPS) were shown to facilitate bacterial cell adherence and biofilm formation (Abu-Lail and Camesano 2003; De La Fuente et al. 2007). Since MVs are basically a microcosm of the bacterial cell wall and carry many of these surface molecules, it is possible that their presence on the MV surface can facilitate MV binding despite being disconnected from the bacterial cell.

Another interesting aspect in relation to *X. fastidiosa* MVs is the theoretical ability to use MVs as a vehicle to transport the hydrophobic cell–cell signaling molecule DSF. Previous studies have shown that *P. aeruginosa* exploits MVs to carry and deliver cell–cell communication molecules of a hydrophobic nature, such as the *Pseudomonas* quinolone signal (PQS) (Mashburn and Whiteley 2005). Hence, it is tempting to speculate that *X. fastidiosa*, and other Xanthomonas in general, may utilize MVs to mediate DSF cell-to-cell signaling. This speculation was recently supported by the work of Feitosa-Junior et al. (2019), which showed that DSF molecules are associated with purified MVs from *X. fastidiosa* cultures.

## 6.4 Bacterial MVs and the Plant Immune System

Bacterial MVs are known immune modulators in mammalian systems. The presence of endotoxin and other bacterial surface molecules in MVs render them carriers of immunogenic material, which interacts with the host immune system (Kaparakis-Liaskos and Ferrero 2015). In mammals, both LPS and the protein cargo of MVs have been shown to induce immune responses (Ellis et al. 2010).

Plants possess a similar innate immune system to that of mammals, composed of surface receptors that interact with conserved microbial determinants and mediate the elicitation of an immune response (Ronald and Beutler 2010). These receptors are termed pattern recognition receptors (PRRs) and their respective microbial elicitors are customarily termed microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs).

Therefore, it was not surprising that MVs purified from plant pathogenic bacteria interacted with the plant immune system (Bahar et al. 2016). Challenging *Arabidopsis* seedlings with plant pathogenic bacterial MVs resulted in the production of a reactive oxygen species (ROS) burst and elevation in the expression of immune marker genes, both represent typical outputs of innate immune system activation (Bahar et al. 2016). These responses were shown to be partially mediated by membrane-bound co-receptors, such as brassinosteroid-insensitive 1-associated kinase (BAK1). BAK1 is a leucine-rich repeat (LRR)-receptor-like kinase (RLK), which forms a complex with multiple primary immune receptors immediately after ligand binding. These interactions stabilize this immune complex and lead to auto- and transphosphorylation of the intracellular kinase domains of the primary and co-receptors, initiating downstream signaling (Schwessinger et al. 2011; Schwessinger and Rathjen 2015). The absence of a functional BAK1 co-receptor would therefore lead to the impairment of multiple primary immune receptors. Together with proteomic and biochemical studies of MVs, these results suggested that MVs carry multiple immune elicitors, which may interact simultaneously with multiple plant immune receptors. This hypothesis may also explain why single primary immune receptor knockouts in *Arabidopsis* plants do not show any reduction in the response to MVs, while knocking out co-receptors, which are important for the functionality of multiple PRRs, leads to a reduced immune response to MVs (Bahar et al. 2016).

Boiling and protease treatments applied to MVs did not appear to alter their immunogenic properties suggesting that similar to the mammalian immune system, both the protein and the nonprotein cargo of MVs elicit the plant immune response. Interestingly, one of the well-known bacterial MAMPs in plants, elongation factor—thermo unstable (EF-Tu) (Kunze et al. 2004) was found in association with MVs, hinting of its possible involvement in immune induction via MVs delivery (Bahar et al. 2016). While EF-Tu itself is heat unstable, its immunogenic properties in plants appeared not to be affected by boiling (Kunze et al. 2004). Since the immunogenic activity of EF-Tu does not depend on a functional protein, but rather on the conserved 18 amino acid epitope, elf18, it is not surprising that EF-Tu remained immunogenic despite the heat treatment. Immune marker



activation assays, conducted with the *Arabidopsis* EF-Tu receptor (EFR) (Zipfel et al. 2006) knockout line, further supported the abovementioned notion that MVs induce plant immunity via multiple receptors, as the EFR knockout line and its wild parent responded similarly to MVs (Bahar et al. 2016).

Differently from mammalian hosts, plant cells possess a cell wall composed primarily of oligosaccharides such as cellulose, hemicellulose, and pectin. An intriguing question is how exactly do bacterial MAMPs come into contact with membrane-bound receptors? PRRs most often possess a LRR domain projecting from the plasma membrane into the cell wall. This protein domain is far shorter than the depth of the cell wall (0.2–1.0  $\mu\text{m}$ ) in which it is embedded. Hence, the LRR domain is not exposed to the extracellular space but rather engulfed by the cell wall matrix. The pore size of the sugar-based mesh of the cell wall was evaluated to be  $\sim 50$  angstroms (Carpita et al. 1979). This is 1000-fold smaller than the diameter of a small bacterial MV, and  $\sim 10,000$ -fold smaller than the size of a bacterial cell. This indicates that intact bacterial cells or MVs most probably do not come into direct contact with the plant cell plasma membrane or the membrane-bound receptors as long as the plant cell wall is intact. One possible explanation of how bacterial immune elicitors come into contact with immune receptors is the lysis or breakdown of intact pathogen cells or of MVs into smaller pieces, which can diffuse through the cell wall pores. Another possibility is that plant cell wall degrading enzymes, such as those seen in association with MVs and discussed earlier in this chapter (i.e., cellulases and pectinases) facilitate the breaking down of the cell wall envelope, thereby exposing the plant cell plasma membrane and its embedded immune receptors to direct contact with bacterial cells or MVs. Further research will be needed in order to better understand the mechanisms by which bacterial MVs interact and activate the plant immune system.

## 6.5 Future Prospects and Major Questions

One of the most intriguing questions regarding the functions of MVs during plant colonization is whether MVs deliver specific cargo into host cells to facilitate infection. MV-mediated delivery of signal molecules, toxins, etc. is known to occur between bacterial cells (Li et al. 1998; Mashburn and Whiteley 2005) and mammalian host cells. These include the delivery of DNA, sensed by the mammalian toll-like receptor 9 (Laura et al. 2010), or the delivery of toxins to mammalian host cells to facilitate infection (Kesty et al. 2004). A critical question in this regard is whether and how MVs overcome the plant cell wall to interact with the plasma membrane. If indeed they do so, perhaps by the use of cell wall degrading enzymes as discussed above, is the interaction with the plant cell mediated by membrane receptors? Are the MVs endocytosed by plant cells, or are the MVs integrated into the plant cell plasma membrane?

Bacterial secretion systems have always drawn a lot of attention from the scientific community. Yet, the MVs secretion pathway in plant-associated microbes



has barely been studied and most certainly holds many interesting discoveries yet to be made. Bacterial MVs have already been suggested to function as a complementary secretion system for T2S virulence factors. It would be very interesting to see whether MVs can also complement the functions of the T3SS, whose contribution to pathogenicity is paramount. First evidences for T3S-effectors in MV have already been given; however, the question of whether they serve a functional role in this form remains to be answered.

Other possible functions of MVs during plant colonization include the facilitation of long-distance cell–cell communication, quenching of antimicrobial molecules produced by the plant defense system, promotion of cell surface adherence and/or biofilm formation (or limiting it as seen with *X. fastidiosa*), acquisition of nutrients, competition with other plant-associated microbes and more. Future research on this unique and scarcely explored secretion system will most likely help to shed more light on its functions during plant–microbe interactions.

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