Membrane vesicles of *Clostridioides difficile* and other Clostridia

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Abstract
Membrane vesicles are secreted by growing bacterial cells and are important components of their secretome in delivering effector molecules that ultimately enable bacterial survival. Membrane vesicles of *Clostridioides difficile* likely contributes to pathogenicity, and is a new area of research but with very limited information. This chapter presents current knowledge of membrane vesicle formation, content, methods of characterisation and functions in Clostridial and Gram positive species for discussions on relevance to *C. difficile*.

1. Introduction to membrane vesicles

Extracellular vesicles (EVs) are natural lipid-lined nanoparticulate products of actively growing eukaryotes and prokaryotes. EVs produced by Gram negative bacteria are known as outer membrane vesicles (OMVs), while EVs from Gram positive bacteria are commonly known as membrane vesicles (MVs). There is greater understanding of OMVs because they were described first, however studies on MVs from Gram positive bacteria are increasing because of the diverse roles that they play in bacterial fitness and pathogenesis, and their numerous potential applications in medicine and biotechnology (Brown et al., 2015, Kaparakis-Liaskos and Kufer, 2020) (Bali et al., 2022). For instance, studies of MVs from Gram positive bacteria such *Staphylococcus aureus*, *Bacillus anthracis*, *Bacillus subtilis*, and *Clostridium perfringens* indicate functions in biofilm formation (He et al., 2017), survival against host cell killing and antibiotic activity (Andreoni et al., 2019, Askarian et al., 2018), toxin secretion (Rivera et al., 2010, Jiang et al., 2014), phage infection (Tzipilevich et al., 2017), and immunogenicity (Jiang et al., 2014) (Bitto et al., 2021a). There are many more studies describing Gram positive MV contents, which imply MV involvement in horizontal gene transfer, antimicrobial resistance transfer, and quorum sensing (Lee et al., 2009, Kim et al., 2019, Afonina et al., 2021, Ichikawa et al., 2021) (Bitto et al., 2021a).

In *Clostridioides difficile*, the potential functions of MVs are secretion of toxins, horizontal gene transfer, phage infection, immunogenicity, and biofilm formation. As there has only been two studies of *C. difficile* MVs by Nicholas et al., 2017, and Lopes et al., 2018, the rationales for these will be presented in the context of current knowledge on MVs from Clostridial species (Table 1). Two studies done in the 1980s in *Clostridium acetobutylicum* and *Clostridium thermoautotrophicum* are recorded in Table 1, however the MVs were artificially created by osmotic lysis for the purpose of investigating transport of molecules across cell membranes. As such, these two reports will be excluded from further discussion. Knowledge on MVs from model Gram positive species and OMVs from Gram negative species will be drawn upon where appropriate, as they are more extensively studied.

2. Vesiculogenesis

2.1 Mechanisms in Gram positive bacteria

MV biogenesis in *C. difficile* and Clostridia have not yet been studied, but it has in model Gram positive organisms. MV formation in *S. aureus* and *S. epidermidis* was recently examined using super-resolution stochastic optical reconstruction microscopy (STORM), in addition to TEM and SEM (Jeong et al., 2022). MVs were formed either through membrane blebbing or explosive cell lysis, both of which were first described for OMVs (Schwechheimer and Kuehn, 2015) (Turnbull et al., 2016). A new observation for membrane blebbing was that MV precursors were seen in the periplasmic space of *S. aureus* with intact peptidoglycan. Another recent study on *Lacticaseibacillus casei* also observed MVs in the periplasmic space (da Silva Barreira et al., 2022). Jeong et al (2022) found that MV precursors were released either from localised cell wall lysis or less commonly after coating with peptidoglycan. Localised cell wall lysis could be due to cell wall restructuring by modifying enzymes (e.g. hydrolases) during cell division. As for explosive cell lysis, this resulted from an expanded periplasmic space that ruptured the cell wall leading to release of cell debris and MV formation external to the cell (Jeong et al., 2022). MVs formed in different ways had different sizes and varied in surface components. Although MV biogenesis has not been investigated in Clostridia, it is likely that MVs from Clostridial species, including *C. difficile*, are formed in a similar, if not the same way.

2.2 Regulation of vesiculogenesis

Regulation of MV formation is less understood. In the Gram positive bacteria *Streptococcus pyogenes*, strain to strain variation in MV production was associated with a two-component system control of virulence regulator sensor operon (*covRS*) (Resch et al., 2016). In *Clostridium perfringens,* deletion of the sporulation master regulator gene, *spo0A,* and orphan sensor histidine kinase genes *CPE1316* or *reeS* that phosphorylate SpoA*,* significantly reduced MV production. However, deletion of *sigF*, which is essential for sporulation did not affect MV production (Obana et al., 2017). This suggests that phosphorylation of Spo0A rather than sporulation itself is important for MV formation. In *C. difficile*, phosphorylated Spo0A has a global effect in addition to sporulation, and has been shown to indirectly control toxin production, cell envelope structure, flagellar formation, butyrate production (Pettit et al., 2014). Spo0A was shown to negatively regulate the *sinRI* operon, which regulates sporulation, toxin production, motility, and biofilm formation (Dhungel Babita and Govind, 2020). Hence it may be possible for Spo0A to influence vesiculogenesis in *C. difficile*.

2.3 Phage and antibiotics

In *B. subtilis*, *S. aureus*, and *L. casei*, prophage induction and endolysin activity aided MV release (Toyofuku et al., 2017) (Andreoni et al., 2019) (da Silva Barreira et al., 2022), indicating prophages can regulate MV formation. In *Enterococcus faecalis* however, phage did not appear to be involved with MV production (Afonina et al., 2021). In *C. difficile* (Nicholas et al., 2017) and other Gram positive bacteria, proteomic analysis of MV contents have found phage structural proteins as major products (Resch et al., 2016, Afonina et al., 2021, Champagne-Jorgensen et al., 2021) (da Silva Barreira et al., 2022). Whole phage virions were observed within *B. subtilis* MVs (Toyofuku et al., 2017), phage DNA was detected in *Lacticaseibacillus rhamnosus* MVs (Champagne-Jorgensen et al., 2021), and phage RNA was detected in *S. pyogenes* (Resch et al., 2016). Hence there is ample data indicating phage can be involved with MV formation.

Antibiotics can affect MV formation. This is not only been observed in OMVs (Bos et al., 2021) but also for MVs of *Enterococcus faecium* (Kim et al., 2019), and *S. aureus* (Andreoni et al., 2019). This may be the combined effects of antibiotic activity on the cell wall (for cell-wall targeting antibiotics), and antibiotic-induced stress responses leading to prophage-induced MV formation and regulation of genes involved in MV production.

3 MV contents

3.1 Variability in content

MVs serve as a secretory system and are selectively packaged, i.e. only certain proteins produced within bacterial cells are found in MVs, and the composition of MVs can be altered. For instance, in *Streptococcus mutans*, *srtA*-deficient mutants produced as much MVs as wild type cells but with protein profiles that differed (Liao et al., 2014). Consistent with selective packaging, MV content is dependent on growth phase (Obana et al., 2017) (Zavan et al., 2019, Jeong et al., 2022)., growth state (i.e. planktonic or sessile) (Grande et al., 2017), growth medium (Askarian et al., 2018, Kim et al., 2019), and different mechanisms of biogenesis (Jeong et al., 2022), all of which leads to different biological functions of MVs.

3.2 Virulence factors

MVs of many Gram positive species contain virulence factors, such as anthrax toxins in *B. anthracis* (Rivera et al., 2010), alpha-toxin, enterotoxin B, and virulence-associated factors in *S. aureus* MVs (Thay et al., 2013, Jeong et al., 2022, Askarian et al., 2018). Toxins were not found in *C. difficile* VPI 10463 MVs (Nicholas et al., 2017), but TcdA was apparently detected in *C. difficile* R20291 MVs by western blot (Lopes et al., 2019). Since MV cargo is dependent on strain, growth conditions and phase, such differences in the two studies could explain these observations (Table 1). Phospholipase C and beta2 toxins were found in *C. perfringens* MVs (Jiang et al., 2014) (Obana et al., 2017), but neurotoxins were not detected in *C. botulinum* nor *C. sporogenes* MVs (Kobayashi et al., 2022). The other Clostridia in Table 1 are not known to be toxigenic.

3.3 Fitness factors

MVs can contain enzymes that improve fitness. For instance, *S. aureus* MVs were found to contain beta lactamase, which mediated ampicillin-resistance of other bacterial species (Lee et al., 2013). *C. difficile* MVs contained VanZ (Nicholas et al., 2017) which may mediate teicoplanin resistance, *C. thermocellum* MVs contained cellulosomes which degrade cellulose (Ichikawa et al., 2019), enabling the bacterium to degrade plant matter and survive in diverse natural environments. Cellulosomes are valuable for bioprocessing and creating biofuels from plant biomass.

3.4 Nucleic acids

MVs can contain nucleic acids. *C. perfringens* MVs contained 16S rRNA, alpha-toxin gene *plc*, and perfringolysin O gene *pfoA* that may be transferrable (Jiang et al., 2014), *S. mutants* MVs contained eDNA (non-selectively packaged) which aided early stages of biofilm formation (Liao et al., 2014). *Lactobacillus reuteri* MVs from planktonic cells and biofilm cells were compared for eDNA abundance, and MVs from biofilm cells were found to contain significantly more eDNA perhaps for aiding biofilm maintenance (Grande et al., 2017). *S. pyogenes* MVs contained RNA which were differentially abundant compared to RNA within bacterial cells, perhaps to enable rapid adaptive responses to environmental changes (Resch et al., 2016).

3.5 Cell envelope

MVs contain cell envelope components such as cell membrane, peptidoglycan, and surface layer (S layer) proteins. *C. difficile* has an S layer (Fagan and Fairweather, 2014), which may be incorporated into MVs. Current models of vesiculogenesis do not appear to specify or take into consideration the S layer, hence this could be an interesting area of investigation. SlpA, Cwp8, Cwp10, Cwp66, were found in the proteome of *C. difficile* VPI 10463 MVs (Nicholas et al., 2017). Other Gram positive species which have an S layer and known to form MVs are *C. thermocellum* (Ichikawa et al., 2019), *C. botulinum* (Kobayashi et al., 2022), and *B. anthracis* (Rivera et al., 2010), although S layer presence was not investigated. S layer was apparently observed morphologically on OMVs of the Gram negative bacterium *Campylobacter fetus* (Farace et al., 2022). Peptidoglycan appears to be a major component in MVs of *C. perfringens* (Obana et al., 2017) and *C. butyricum* (Morishita et al., 2021).

4. MV preparation and quantification

4.1 General method of separation, concentration, and purification

Preparation methods of MVs from Clostridial species are very similar to other bacteria and involves firstly the separation of MVs from bacterial cells in liquid culture by centrifugation to pellet bacterial cells, followed by supernatant filtration through 0.22 μm or 0.45 μm filters. MVs in filtrates are then concentrated either by ultrafiltration or pelleted by ultracentrifugation. Finally, MVs are washed and purified, most commonly through a density gradient such as Optiprep™ (Table 1). To obtain purified MVs from Clostridial species, usually 0.4 -1L of bacterial culture were used. Depending on the purpose of study, MVs were not always purified. It is widely acknowledged by EV researchers that non-vesicular entities (e.g. phage, lipoproteins, extracellular proteins) cannot be fully separated from EVs by the common methods described above (Théry et al., 2018). Hence to assign effects to EVs, consideration should be given to demonstrating a lack of effect in the remaining EV-depleted sample where possible (Théry et al., 2018). Otherwise, using a combination of purification methods, and developing EV-selective methods (Nakao et al., 2014) to achieve as high purity of EVs as possible should be employed.

4.2 Common quantification methods

MVs can be quantified by i) protein content of vesicles using either BCA, Bradford, or Qubit assays; ii) particle number using light scattering techniques such as nanoparticle tracking analysis (NTA), and iii) fluorescence labelling with lipophilic dyes followed by detection, such as flow cytometry or NTA. Although quantification by protein content is the most commonly used method of MV quantification in bacterial EV studies (including for Clostridial species, see Table 1), perhaps because of convenience, this method could overestimate co-purified protein contaminants, or underestimate MV protein content if detergent was not used to disrupt MVs to release protein prior to quantification, or if the sample was not purified. Importantly, protein content will not correlate with MV particle number, potentially affecting experimental findings (Théry et al., 2018, Bitto et al., 2021b, Steć et al., 2022).

A lipid dye FM1-43 has been used for quantifying MVs of *Streptococcus pyogenes, Bacillus subtilis, and S. aureus* (Resch et al., 2016) (Toyofuku et al., 2017) (Andreoni et al., 2019), and

was recently used to track MV release from *E. coli* cells in real-time (Bos et al., 2021). FM1-43 was validated with the Bradford assay for MV protein mass in one study (Resch et al., 2016). We have developed a fluorescence-based semi-quantitative assay using FM1-43 for quantifying MVs from *C. difficile*, and validated its use against MV particle counts from NTA (unpublished). The advantages of this method is that it is requires less sophisticated equipment for quantification (fluorescence plate reader compared to a nanoparticle tracker or flow cytometer), is quicker and easier to obtain a measurement compared to an NTA which has a small tolerance for appropriate sample concentration for optimal counts; i.e. several dilution series must be tried to achieve 2 – 20 x 10^8 particles/mL (Szatanek et al., 2017), and background MV counts are lower than NTA. The disadvantage is that for semi- quantification, a standard curve of RFU vs particle count is required and that will require some form of particle counting to start with. However, once that has been established it is a relatively rapid fluorescence-based, medium-throughput assay for determining MV concentration in samples in 96-well plates.

4.3 Clostridial MV characteristics

The most common parameters of MV characterisation are particle size distribution, morphology by TEM, and protein content. Amount and size of bacterial MVs, and protein quantity of MV are species and strain dependent (Bitto et al., 2021b) (Kobayashi et al., 2022), as well as growth phase and growth medium dependent (Askarian et al., 2018, Jeong et al., 2022). MVs of *C. difficile* VPI10463 and R20291 were 20 – 400 nm, possibly with larger MVs forming in stationary phase of R20291 compared to log phase of VPI 10463 growth. MVs produced at stationary phase of other Clostridia have a similar size range (Table 1). In *C. perfringens*, MVs produced at different time points were found to vary in yield, size, and composition, where MVs from stationary phase cultures were larger than from log phase cultures (Obana et al., 2017).

C. difficile MVs appeared spherical with a lipid bilayer of uniform thickness (Nicholas et al., 2017), similar to MVs from *C. thermocellum* (Ichikawa et al., 2019). However samples composed of some MVs having a thicker lipid bilayer were observed in *C. perfringens* (Jiang et al., 2014, Obana et al., 2017), *C. botulinum*, *C. sporogenes*, *C. scindens* (Kobayashi et al., 2022), and *C. butylicum*. The significance of a thicker membrane is unknown; it could be a staining artefact, or associated with spontaneously self-assembled vesicles from bacterial debris external to bacterial cells (Huang et al., 2017). For protein content, see 3.1-3.5.

5. Potential functions of *C. difficile* MVs

5.1 Toxin secretion

While there is evidence of toxin secretion mechanisms in *C. difficile* mediated by TcdE (Govind and Dupuy, 2012, Govind et al., 2015) and Cwp19 (Wydau-Dematteis et al., 2018), the absence of these proteins did not always abolish toxin secretion (Olling et al., 2012, Wydau-Dematteis et al., 2018), indicating the existence of other secretory pathways. The first study on *C. difficile* MVs did not find toxins as a constituent (Nicholas et al., 2017), however it is known that MV cargo is dependent on growth phase or conditions (perhaps affecting MV biogenesis pathways), and this has not yet been investigated.

5.2 Horizontal gene transfer

A DNase- sensitive mechanism for DNA transfer requiring the presence of viable donors was demonstrated in *C. difficile* (Khodadoost et al., 2017). Hence, MVs may be involved in such a transformation-like DNA transfer known as "vesicle-mediated gene transfer" or VMGT (Fulsundar et al., 2014). VMGT has been shown in Gram negative bacteria. OMVs can carry plasmid DNA capable of gene transfer within (e.g. *Acinetobacter baumannii*) (Rumbo et al., 2011) and between species (Fulsundar et al., 2014), where interspecies transfer was not entirely dependent on the relatedness of OMV donor and recipient (Tran and Boedicker, 2017). OMVs were actively associated or loaded with DNA in growing bacterial cells (Tran and Boedicker, 2017), and DNA could be carried externally and internally, hence DNase treatment reduced transfer frequency (Bitto et al., 2017). In Gram positive bacteria such as *S. aureus* (Bitto et al., 2021a) and *S. mutans* (Liao et al., 2014), MVs were shown to carry DNA externally and internally. Two mechanisms of VMGT have been proposed, based on OMV-mediated transfer of plasmid borne *bla* in *Acinetobacter baylyi*: either dependent on competence factor uptake of DNA external/internal of the MV, or internalisation of MV and release of vesicular DNA (Fulsundar et al., 2014). This is an area that requires more investigation, particularly for Gram positive MVs.

5.3 Phage infection

OMVs are known to carry phage receptors which enable phage adsorption and DNA ejection (e.g. *Salmonella typhimurium* OMVs and P22 phage) (Manning and Kuehn, 2011) (Stephan et al., 2020) (Bali et al., 2022). Recently in the Gram positive organism *B. subtilis*, MVs carrying phage receptors were shown capable of sensitising normally phage-resistant cells to phage infection (Tzipilevich et al., 2017). This phenomenon would lead to increased dissemination of phages in a cell population, and could be a naturally occurring mechanism for phage expansion of host ranges in some species but not others (Augustyniak et al., 2022). On the other hand, OMVs carrying phage receptors have been shown to act as decoys to protect bacteria against phage infection (Reyes-Robles et al., 2018, Stephan et al., 2020) (Augustyniak et al., 2022). *C. difficile* phages identified so far have relatively narrow host ranges, in part defined by phage receptor-binding proteins recognising a cell surface layer protein, SlpA, as a phage receptor on bacterial cells (Royer et al., 2022). Proteomics analysis of *C. difficile* MVs by Nicholas et al., 2017 found SlpA, suggesting *C. difficile* MVs may be capable of transferring phage receptors or acting as decoys for phage infection.

5.4 Immunogenicity

MVs are known to contain cell wall, cell membrane and cytoplasmic content which are immunogenic. Nicholas et al., 2017 reported that *C. difficile* MVs from a toxigenic strain stimulated expression of pro-inflammatory cytokine genes for IL-1β, IL-6, IL-8, MCP-1 in CaCo-2 cells, an intestinal cell line. Although toxins were not detected in MVs, CaCo-2 cells treated with >1 μg/mL MVs led to cytotoxicity (Table 1). MVs of other Clostridia were also shown to be immunogenic. *C. perfringens* MVs induced secretion of proinflammatory cytokines IL-6 and TNF-α in J774.1 cells, a macrophage cell line, via TLR2 signalling (Obana et al., 2017). Similar observations were reported by Jiang et al., (2014) in RAW264.7 cells, a macrophage cell line, and sera of MV-immunized mice, in addition to G-CSF production. *C. botulinum*, *C. sporogenes*, and *C. scindens* MVs induced expression of inflammatory cytokine genes for IL-1-β, IL-6, TNF in

CaCo-2, IL-6, CXCL2, CCL2 in CMT-3 (an intestinal cell line), and IL-6, IL-8, and CCL2 in RAW264.7 cell lines (Kobayashi et al., 2022). *C. butyricum* induced expression of inflammatory cytokine genes for IL-6, TNF-α, and TGFβ-1 in RAW264.7 cells (Morishita et al., 2021). *C. butyricum* was shown to upregulate gene expression of gut barrier-related proteins (MUC2, ZO-1, Arg1, IL-10) in mice, and induced polarization of anti-inflammatory M2-type macrophages (Liang et al., 2022).

Exploring the use of MVs as vaccines for protection against *C. perfringens* infection was disappointing; MVs were found not to protect mice from a *C. perfringens* challenge (Jiang et al., 2014). However, MVs as disease intervention agents was promising; *C. butyricum* MVs protected mice from ulcerative colitis through M2 macrophage transformation and gut microbiome modulation (Liang et al., 2022). Vaccines being developed for *C. difficile* have focused on antigenic toxin components, cell wall and flagellar proteins (Nibbering et al., 2021, Razim et al., 2021). As non-toxigenic *C. difficile* isolates are protective against disease caused by toxigenic strains, vaccine development should include proteins other than toxins (Nibbering et al., 2021). In this regard, MVs from non-toxigenic *C. difficile* could be effective vaccines if their contents can be controlled to exclude cytotoxic agents.

5.5 Biofilm formation

MVs were found to be involved in biofilm formation in *S. aureus*, where the presence of MVs derived from MRSA culture in vancomycin significantly increased cell attachment and aggregation (He et al., 2017). MVs can be produced by cells within a biofilm, as demonstrated in *L. reuteri*. MVs from planktonic cells (pMV) or biofilms (bMV) were compared and bMVs contained more eDNA but less protein compared to pMVs (Grande et al., 2017). This may indicate an important role of vesiculated eDNA in maintaining biofilms. Biofilm formation by *C. difficile* depends on multiple factors as reviewed by Frost *et al* (Frost et al., 2021). Spo0A is important for biofilm formation in *C. difficile* (Dawson et al., 2012), similar to *C. perfingens* (Huang et al., 2004), which also depends on Spo0A for efficient MV formation (Obana et al., 2017). It may be possible that both MV and biofilm production in *C. difficile* are regulated by Spo0A and that MVs contribute to biofilm formation as seen in *S. aureus*. Quorum sensing (QS) also contributes to biofilm formation in *C. difficile*. A mutant unable to synthesize the QS signalling molecule AI-2 produced significantly less biofilm containing less eDNA, and had reduced expression of prophage genes (Slater et al., 2019). The possible relationship between AI-2 and prophage induction may extend to MV production, since prophage induction can increase vesiculogenesis (see 5.3), a proportion of which may contain vesiculated eDNA as mentioned in 3.4 and 5.2.

6. Concluding remarks

Membrane vesicles are increasingly recognised to play important roles in bacterial survival. Research in MVs is catching up to OMVs, and MVs in *C. difficile* deserve to be investigated because they potentially have multiple functions and could interact broadly intra- and interspecies. Although this chapter proposed many things which are based on related Clostridia due to very limited knowledge on *C. difficile* MVs at the moment, many findings on MVs so far apply to both Gram positive and Gram negative bacteria, hence some aspects of MVs could be conserved in prokaryotes. On the other hand, it is worth noting that although *C. difficile* is in the same genus as other Clostridia mentioned here (i.e. *C. botulinum, C. sporogenes, C. scindens, C.*

perfringens, C. butylicum), it is distantly related to them phylogenetically hence recently re-classified as Clostridioides in its own cluster (XIa) separate from the rest (cluster I, sensu stricto) (Lawson et al., 2016) (Cruz-Morales et al., 2019). Therefore, some differences between MVs from *C. difficile* and those of other Clostridial species would be expected.

The choice of materials or methods for EV separation, concentration and purification has been shown to significantly impact their characterisation (Vergauwen et al., 2017, Théry et al., 2018, Bitto et al., 2021b, Steć et al., 2022). The International Society for Extracellular Vesicles (ISEV) has published Minimal Information for Studies of Extracellular Vesicles (MISEV 2018) with suggestions for preparation, purification, and characterisation (Théry et al., 2018). Although MISEV 2018 is focused on EVs of mammalian cells, many suggestions are relevant to OMV and MV research and should be more frequently adopted by the bacterial research community. Currently there are no similar guidelines for bacterial EVs, but there is a desire by various research groups to have a standardized approach for preparing and analysing OMVs and MVs (Bitto et al., 2021b).

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