FEMS Microbiology Reviews, fuaa047, 45, 2020, 1–26

doi: 10.1093/femsre/fuaa047
Advance Access Publication Date: 14 September 2020
Review Article

REVIEW ARTICLE

Eco-evolutionary feedbacks mediated by bacterial membrane vesicles

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One sentence summary: This review aims to highlight the role of the bacterial membrane vesicles in different eco-evolutionary processes in which bacteria are subjects and operators.

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ABSTRACT

Bacterial membrane vesicles (BMVs) are spherical extracellular organelles whose cargo is enclosed by a biological membrane. The cargo can be delivered to distant parts of a given habitat in a protected and concentrated manner. This review presents current knowledge about BMVs in the context of bacterial eco-evolutionary dynamics among different environments and hosts. BMVs may play an important role in establishing and stabilizing bacterial communities in such environments; for example, bacterial populations may benefit from BMVs to delay the negative effect of certain evolutionary trade-offs that can result in deleterious phenotypes. BMVs can also perform ecosystem engineering by serving as detergents, mediators in biochemical cycles, components of different biofilms, substrates for cross-feeding, defense systems against different dangers and enzyme-delivery mechanisms that can change substrate availability. BMVs further contribute to bacteria as mediators in different interactions, with either other bacterial species or their hosts. In short, BMVs extend and deliver phenotypic traits that can have ecological and evolutionary value to both their producers and the ecosystem as a whole.

Keywords: bacterial membrane vesicles; ecology; evolution

INTRODUCTION: 'EVERYTHING IS EVERYWHERE, [BUT] THE ENVIRONMENT SELECTS'

What goes on in nature, according to the father of comparative biochemistry Albert Jan Kluyver, can be explained by the rules established by the founder of the Delft School Martinus Willem Beijerinck (Van Iterson, De Jong and Kluyver 1940; Kluyver 1953). These rules, summarized by another Delft scholar, Baas Becking, state that 'everything is everywhere, [but] the environment selects' (Becking 2016 [1934]). The rules represent the relationship of bacteria with their *milieu cosmique* as a result of their different trophic modes, combined with different adaptive traits (i.e. phenotypes whose values increase the fitness of the bacteria in a given environment). Not only has this combination allowed bacteria to inhabit virtually all the ecosystems of our planet, but

Received: 31 January 2020; **Accepted:** 11 September 2020

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it has also provided bacteria with the capacity to modify the structure and function of the different ecological systems they inhabit (Van Iterson, De Jong and Kluyver 1940; Kluyver 1953; Becking 2016 [1934]).

Based on this relationship, Kluyver suggested that the bacterial adaptation to a change in environmental conditions or nutrients is 'an evaluation of the actual situation as [it results from] evolution' (Kluyver 1953). If inherited, that change will lead to a stepwise evolution due to mutation and selection; in other words, he suggested the link between bacterial adaptation and the causes of evolution (Kluyver 1953). The phenotypic and genetic diversity found among different taxa, the short generation time and the existence in vast populations all provide bacteria with the opportunity for rapid evolutionary changes that can occur at contemporary time scales (Bohannan and Lenski 2000; Hansen *et al.* 2007; Heilmann, Sneppen and Krishna 2010; Andrade-Dominguez *et al.* 2014). Bacteria can also enter a non-evolving state in the form of persisters, spores and viable but non-culturable bacteria (Bohannan and Lenski 2000; Lewis 2010; Oliver 2010). The fast-evolving and non-evolving states of bacteria can be a direct, simultaneous result from both ecology affecting evolution (i.e. the survival of the conditionally fittest) and evolution affecting ecology (i.e. the structure of ecosystems), collectively referred to as eco-evolutionary (or 'eco-evo') feedbacks. Besides being subject to eco-evolutionary cycles, bacteria can also be the operators of these cycles. A group of examples demonstrates the importance of the microbiota as drivers of the eco-evo developmental processes of the multicellular organisms: symbionts can help with morphogenesis and species maintenance, provide selectable allelic variation or potentiate reproductive isolation (Gilbert, Bosch and Ledon-Rettig 2015). Finally, whether the bacterial symbionts may also trigger contemporary transitions toward multicellularity, such as in the Choanoflagellates (Alegado *et al.* 2012; Gilbert, Bosch and Ledon-Rettig 2015), is part of a much bigger and unsettled question on the role of the symbionts in 'evolutionary progression' from unicellular organisms to multicellularity.

The extracellular bacterial membrane vesicles (BMVs) hold an interesting place in biology. They are currently viewed as extracellular spherical structures, with an average diameter between 20 and 250 nm, in which a hydrosol is enclosed by one or two biological membranes. Their structure is indirectly encoded and is the result of the assembly of their biological membrane, a phospholipid (PL) bilayer carrying embedded membrane proteins, and the accumulation of the vesicular cargo. Not only can bacteria release these subcellular structures, but they can also integrate naturally selected functionalities in their vesicles as a response to environmental (extrinsic) stimuli or to intrinsic factors. Thus, the secreted BMVs extend and deliver 'phenotypic traits' that can have adaptive value to the individual organism, its population and to the ecosystem as a whole.

This review, which follows the same approach outlined by these Delft-based scholars, presents the role of BMVs in different eco-evo dynamics. The specialized functions mediated via BMVs depend on the type of bacteria and their evolutionary history, as well as on the bacterial population's specific state and ecological niche. For this reason, we will first briefly outline certain evolutionary aspects; in the second part of the introduction we will then consider the different aspects of BMV biogenesis.

Lessons from the 'pre-cells' and various evolutionary aspects linked to BMVs

Beijerinck's rules bear another deep meaning: the environment was what also selected when, where and how life once began. According to the notable biochemist Alexander I. Oparin, life on earth began with the formation of coacervates (the original spelling referring to Oparin´s work was "coazervates"), or 'pre-cells': vesicles with a certain degree of individuality due to their spatially concentrated organic substance, isolated from the surrounding environment by a semi-permeable membrane (Oparin 1961 [1924]). This membrane has opened the door to the existence of unique assemblies of organic and inorganic molecules, and for their possibility to interact with each other (Oparin 1961 [1924]). To achieve this degree of individuality, the coacervates had to obey the laws of physics and chemistry, as well as those of biology (Oparin 1961 [1924]). And since life presumably started with the formation of vesicles, the logical continuation of this supposition is that life is to be also maintained by vesicles.

An interesting parallel may be drawn between BMVs and coacervates, because both exist on the border of living and non-living biological structures. We will present the basic characteristics of BMVs through previous studies obtained from evolutionary experiments on lipid vesicles that lack the protein component of the biological membrane. These characteristics both provide clues about the origin of life and explain the functionalities brought by the lipid component of the secreted BMVs. To self-maintain in the environment, the pre-cells have a certain degree of stability because of their membranes' composition of mixed amphiphilic and hydrophobic lipids (Namani and Deamer 2008; Jordan *et al.* 2019). The evolutionary experiments that Szostak and Luisi conducted in their respective laboratories have demonstrated that the fatty acid vesicles are capable of primordial quasi-biological processes (such as growth, division, environmental interaction and intra- and intervesicular competition for resources), which eventually may have led to primeval natural selection for stable vesicle populations with heterogeneous lipid membranes over others with homogeneous membranes (Berclaz *et al.* 2001; Cheng and Luisi 2003; Hanczyc, Fujikawa and Szostak 2003; Chen and Szostak 2004; Chen, Roberts and Szostak 2004; Luisi *et al.* 2004). Researchers have also suggested that the osmotic pressure exerted by the vesicular aqueous phase and its content on the composite membrane led to the redistribution of the lipid species that define membrane asymmetry, and size and curvature of the vesicle (Fig. 1A, Box 1)—all of which represent important factors for lipid-facilitated vesicular fusion and fission (Cheng and Luisi 2003; Chen, Roberts and Szostak 2004). As with any physical object, the membrane vesicles are characterized by properties, including size, shape and the total energy of the system, which can be transformed for processes such as fusion and fission (Fig. 1A). In Box 1, we briefly outline the physical properties of the vesicles to help demonstrate the bacterial vesicle release as a spontaneous, indirectly energy-dependent process that can be further controlled by the state of the bacteria and the external environment.

Box 1.
Physical properties of the vesicles

For most of the history of science, physicists have been fascinated by the way nature integrates complex physiological processes in simple figures (Thompson 1992 [1917]). The

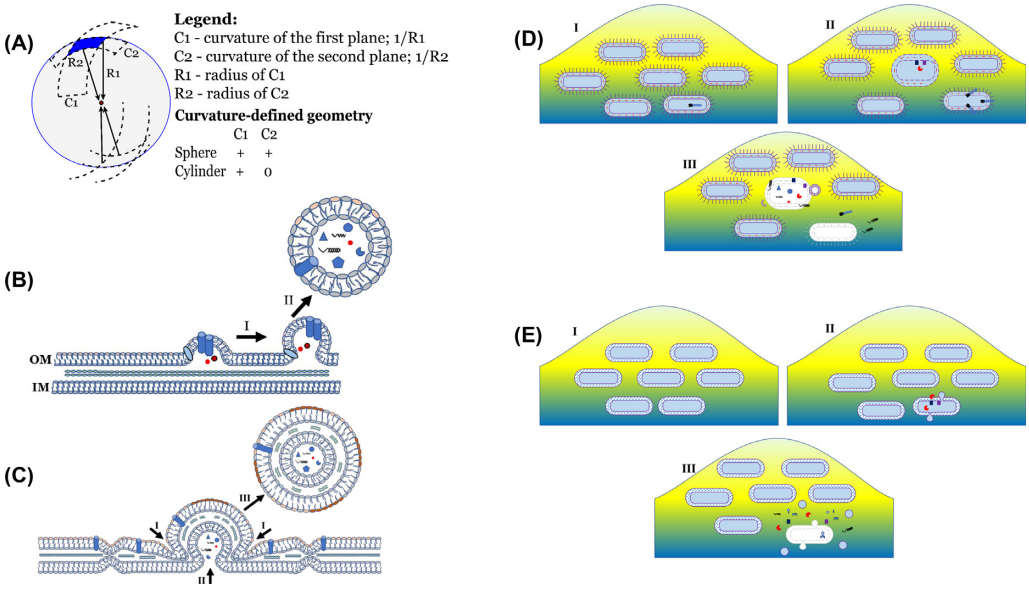

Figure 1. Physical properties of the vesicles **(A)** (see also Box 1) and the proposed mechanisms for vesicle genesis—single cell-based vesicle release **(B, C)**, and cell death-mediated vesicle release **(D, E)**: (A) the radii (R1, R2) and the curvatures (C1, C2) of a centrally symmetric spherical system. (B) The process of vesicle release (modified from Kulp and Kuehn 2010). Phase I represents the bulging of the outer membrane (OM) due to membrane-bending proteins (blue cylinder), to the presence of non-bilayer lipids (blue eclipse) or to a change in the osmotic pressure in the presence of selected compounds (red circles). Phase II represents the release phase in which the membrane tension and the osmotic pressure of the bilayer and the vesicle are balanced. (C) A hypothetical model for the outer-inner membrane vesicle (O-IMV) biogenesis based on Kadurugamuwa and Beveridge (1995) and Pérez-Cruz *et al.* (2013). The bulging region that involves the two membranes is stabilized by the rigidity of the mature lipopolysaccharide (LPS; dark red) and results from a process that involves the formation of close contacts between the IM and OM (step I) surrounding a region on which the cytoplasmic osmotic pressure can act (step II). Upon close proximity of the two zones of close contact, the presence of bilayer-disturbing amphiphiles leads to a 'recombination event' between the PLs of the two zones, which is resolved in the release of an O-IMV (step III). (D) Explosive cell lysis-like mechanism based on Turnbull *et al.* (2016). It takes place within a bacterial community, e.g. biofilm (panel I), and involves the production of a holin/endolysin dyad (panel II, holins are represented with blue and purple squares, while the endolysin is shown as a red packman) that acts upon the cell wall of the bacteria resulting in their rounding (panel II), followed up by their 'explosive' death that results in the release of membrane fragments that can form vesicles (panel III). (E) Prophage-triggered vesiculation based on Toyofuku *et al.* (2017). The mechanism also takes place in a bacterial community (panel I) and involves the production of holin/endolysin dyad (holins are represented with blue and purple squares, while the endolysin is shown as a red packman) that disrupts the membrane potential and osmotic integrity of the bacterial membrane (panel II) resulting in vesiculation without cell rounding and in the death of the bacteria (panel III).

form of the vesicle can be described in a centrally symmetrical system with curvature radii (R1, R2) of the two perpendicular curvatures (C1, C2), as shown in Fig. 1A. When the force lines in all points of the vesicle are equally distributed, the shape becomes spherical (R1 = R2); see Fig. 1A. In terms of physics, membrane vesicles are often thought of as structures in which a 2D membrane encloses a 3D aqueous phase. Membrane vesicles have also been defined as low-energy shapes ($E_{b,min}$) that depend on the bending energy (E_b) constrained by a given area (A), the enclosed volume and the mean curvature. The link between the shape and energy, here for simplicity E_b, can be expressed with the simplified version of Helfrich's equation, where k stands for the static bilayer bending rigidity: $E_b = (k/2) \oint dA(C1 + C2)^2$. For further details, see Seifert *et al.* (1991) and Jarić *et al.* (1995). Overall, the $E_{b,min}$ is maintained by the balanced interaction of the membrane tension and the osmotic pressure of the aqueous phase exerted on the membrane. If a change is introduced (due to molecular crowding or lipid clustering, for example), in order to restore the mechanical equilibrium, the vesicle may enter into another $E_{b,min}$ state by changing shape in this transition from spherical to a dumbbell, pear-like, oval or tubular shape (Seifert *et al.* 1991; Jarić *et al.* 1995). The other option for this system is to change: it can either increase its volume or undergo processes such as fusion or fission, such as by vesicle release (Fig. 1B).

Life likely started under hydrothermal and chemiosmotic conditions (such as those found in black smokers and/or Lost City systems) that consecutively allowed for chemical, biochemical, biological and ecosystem evolution that then developed the tree of life (Baross and Hoffman 1985; Martin *et al.* 2008). Even though some biogenic activities may have existed 4.1 billion years (Byr) ago (Bell *et al.* 2015), based on fossils records, primordial life presumably did not arise until 3.95 Byr ago (Tashiro *et al.* 2017). Since recent phylogenetic studies have placed the eukaryotes in a monophyletic group with **Lokiarchaeota** (Domain Archaea), the tree of life most probably was developed by the prokaryotes—Domain Archaea and Domain Bacteria (Cavalier-Smith 2002; Yutin *et al.* 2008; Williams *et al.* 2012; Spang *et al.* 2015; Eme *et al.* 2018; Williams *et al.* 2020). (Phyla appear in **bold** text in this review.)

The ancient bacteria appeared around 3.5 Byr ago, based on Cavalier-Smith's (2002) theory, the microfossil records and the presence of deep-sea vent phototrophic bacterial life, and are presumed to have been anaerobic green (sulfur) photosynthetic with a double-membrane envelope, porins, acylester phospholipids and a chromosome (Ochman and Wilson 1987; Rasmussen 2000; Cavalier-Smith 2002; Beatty *et al.* 2005; Wacey *et al.* 2011). The lineages of the deep-branching bacteria *Aquifex* and *Thermotoga* may have appeared 3.5–3.3 Byr ago, while the land colonizers (**Actinobacteria**, **Deinococcus-Thermus** and **Cyanobacteria**) branched off 2.5–3 Byr ago from the lineages that also produced the **Proteobacteria** and other bacterial phyla (Feng, Cho and Doolittle 1997; Hedges *et al.* 2001; Sheridan, Freeman

and Brenchley 2003; Battistuzzi, Feijao and Hedges 2004). The prokaryotes maintained life and evolution on their own for almost 2 Byr until the emergence of the eukaryotes, with which they have co-existed ever since (Ochman and Wilson 1987; Schopf 1994).

For all this time, the membrane vesicle release may have remained a common feature of all domains of life (Deatherage and Cookson 2012; Gill, Catchpole and Forterre 2019). And the contribution of how vesicle release evolved—combined with the drastic changes in cell wall structure, membranes and secretion machineries—needs to be considered as a feature of prokaryotic evolution, among other factors (Cavalier-Smith 2002; Makarova *et al.* 2010; Gould, Garg and Martin 2016).

Single cell-based, bona fide, biogenesis and cell death-mediated formation of BMVs

The type of secreted BMVs depends on bacterial physiology and the cell envelope—a mechanical structure dynamically composed of extracellularly sugar-coated biological membrane(s). The cell envelope defines the borders of bacteria as discrete biological units, provides them with physicochemical plasticity and is the frontline of interaction between the cells and the environment. The cell envelope is also where BMV biogenesis takes place.

A typical bacterial cell may have a Gram-positive or Gram-negative organization of its cell envelope. The cellular content of both types is delineated by a cytoplasmic membrane (CM). The lipid bilayer of the CM exists in a dynamic liquid-crystalline state that is often (but not always) maintained by the composition of the phosphatidylethanolamine (PE), lyso-PE, phosphatidylglycerol (PGl) and cardiolipin (CL), as well as by the type of acylesterified fatty acids (Morein *et al.* 1996; Parsons and Rock 2013; Sohlenkamp and Geiger 2016; Rajagopal and Walker 2017). In the case of Gram-positive cell wall, on the outer side the CM carries a relatively thick peptidoglycan (PG) composed of 25–30 layers of linear polysaccharide chains of *N*-acetylglucosamyl-β-1,4-*N*-acetylmuramyl parental unit cross-linked via oligopeptide chains (Schleifer and Kandler 1972; Vollmer, Blanot and de Pedro 2008; Rohde 2019). Recent atomic force microscopy experiments with *Staphylococcus aureus* and *Bacillus subtilis* have shown that the PG of the Gram-positive bacteria displays two distinct architectures: the inner PG layer is denser, while the mature outer layer represents a disordered gel carrying large (around 60 nm in diameter) and up to 23 nm deep pores (Pasquina-Lemonche *et al.* 2020). Exterior to the CM, a typical Gram-negative cell wall is surrounded by an outer membrane (OM) (Beveridge 1981; Silhavy, Kahne and Walker 2010).

Most of the extracellular leaflet of the OM is built by lipid A—the hydrophobic anchor of the lipopolysaccharide (LPS) molecular complex—linked to a glycan polymer via a core oligosaccharide (Nikaido 2003; Kalynych, Morona and Cygler 2014; Putker, Bos and Tommassen 2015; Henderson *et al.* 2016). The inner leaflet of the OM often contains relatively large amounts of PGl, lyso-PE, CL and relatively little PE compared with the IM (White, Lennarz and Schnaitman 1972; Hoekstra *et al.* 1976). Kinetic experiments with radioactively labeled LPS from *Escherichia coli* have demonstrated that the LPS distribution in the OM is non-random: the mature LPS clusters in rigid localized domains, while the newly produced cluster is inserted at specific zones characterized by increased fluidity (Leive 1977). The difference between the inner and outer phospholipid layers of the OM defines the OM asymmetry, which protects the bacteria from the dangerous activity of some lipophilic and polarly charged molecules and allows the passage of the useful molecules (Nikaido 2003; Henderson *et al.* 2016). Upon exposure to membrane-disrupting agents, such as chelators or antimicrobial peptides, bacteria maintain OM integrity by incorporating PLs instead of LPS on the OM (Nikaido 2003). To prevent the incorporation of PLs instead of LPS into the external OM layer and thus to maintain OM asymmetry, bacteria use the Mla (Maintenance of OM/lipid asymmetry) system, which is an ABC transporter that in a retrograde fashion flips the surface-exposed PLs back into the inner leaflet of the OM (Malinverni and Silhavy 2009; Henderson *et al.* 2016).

Experiments with fluorescence probes in *E. coli* have further demonstrated that the OM is more rigid than the IM (Overath and Trauble 1973; Cheng, Thomas and Kulpa 1974; Leive 1977; Nikaido 2003). While the proteins represented in the CM are predominantly α-helical, the OM is embedded with *trans*-membrane β-barrel proteins collectively referred to as outer membrane proteins (OMPs). Most OMPs function as porins, allowing the passive diffusion of different metabolites, ions and other compounds, but they can also have other properties, for example by serving as receptors or facilitating adhesion (Beveridge 1981; Silhavy, Kahne and Walker 2010). The PG layer of Gram-negative bacteria is thin (1–3 layers) and is located between the IM and the OM in an oxidizing, ATP-devoid environment called the periplasmic space (Beveridge 1981; Silhavy, Kahne and Walker 2010). Some OMPs, such as OmpF and OmpC, are embedded only within the OM, while others either interact with the PG layer (e.g. OmpA and Lpp), or, as is the case with the Tol-Pal system, they interact with the PG and the IM (Braun and Sieglin 1970; De Mot and Vanderleyden 1994; Clavel *et al.* 1998; Walburger, Lazdunski and Corda 2002; Cascales and Lloubès 2004; Parsons, Lin and Orban 2006). Electron microscopy (EM) studies with *E. coli* and *Salmonella enterica* demonstrated the existence of membrane adhesion zones where the OM comes in close proximity to the IM, known as 'Bayer bridges' (Bayer 1968, 1991; Crowlesmith, Schindler and Osborn 1978; Smit and Nikaido 1978; Lopez and Webster 1985; Bayer and Bayer 1986; Walderich *et al.* 1988; Leduc and Frehel 1990). The number of these bridges varies between 200 and 400 per cell and increases when the bacteria are challenged with UV irradiation or when they are under attack by certain bacteriophages (Walderich *et al.* 1988; Bayer 1991). Although Kellenberger (1990) has challenged the existence of Bayer bridges, others have suggested that they serve as zones where the transportation of OM components (such as LPS, capsular polysaccharide and OMPs) as well as the attachment and/or release of some bacteriophages, all take place (Smit and Nikaido 1978; Lopez and Webster 1985; Bayer and Bayer 1986; Kellenberger 1990; Henderson *et al.* 2016).

Reasoning from the fact that adaptations for the benefit of a community may markedly differ from adaptations for the benefit of an individual, we employ two approaches to define vesiculation and its mechanisms. The first approach is single cell-based and considers the BMV release by individual free-living bacteria, which eventually leads to the preservation of the integrity of the bacterium and its vesicles (Fig. 1B and C). While the first type of vesiculation implies that the BMV release is not adapted for death of the bacterium, the second approach involves vesiculation that results from a genetically encoded cell death (CD) triggered in a subpopulation sacrificed for the survival of the community (Fig. 1D and E).

Based on previous studies on vesicle release, pre-cell models and membrane biochemistry, the following description is

how we envision vesicle release, i.e. bona fide vesicle biogenesis, according to the single cell-based approach (Fig. 1B and C). For a BMV to be released, there must be an extracellular protrusion of PG-detached membrane areas, as shown in Fig. 1B (Kulp and Kuehn 2010). The protrusion may be triggered by a change in membrane curving due to (i) molecular crowding of membrane-bending proteins, (ii) curve-introducing lipids (e.g. in the presence of non-bilayer lipids such as CL or lipids with small polar heads such as PE) or (iii) a local imbalance between the membrane tension and the osmotic pressure exerted by the aqueous phase of the vesicular lumen that forms, enriched in certain components; see Fig. 1 and Box 1 (Chamberlain *et al.* 2003; Chen, Roberts and Szostak 2004; Yeagle 2016). These events can be linked, or they can take place separately or all together, but eventually they lead to an imbalance between membrane tension and osmotic pressure that can become a driving force of energy for membrane fission (vesicle release) in an ATP/GTP-independent manner, thus preserving cellular and vesicular integrity (Fig. 1 and Box 1). The complexity of the Gram-negative cell wall is transferred onto the types of BMVs released by these bacteria. If the interface for vesiculation occurs between the periplasm and the OM, then the Gram-negative bacteria secrete outer membrane vesicles (OMVs), and if the interface involves the periphery of the cytosol, the IM, the periplasm and the OM, then bacteria can release outer-inner membrane vesicles (O-IMVs) (Kadurugamuwa and Beveridge 1995; Perez-Cruz *et al.* 2013; Sjostrom *et al.* 2015) as depicted in Fig. 1B and C. For clarity, even though most studies have been conducted with OMVs, we will refer to them collectively as BMVs throughout this review; if exceptions exist (e.g. a release of O-IMVs) we will point out which kinds of vesicles a particular bacterium releases.

The outcome of the single cell-based vesiculation is directly related to the maintenance of the integrity of the membrane dynamics. The BMVs can be released to counterbalance the excessive growth of the cell wall during normal bacterial growth, during protein and/or RNA restrictions or during the ingrowth of the division septum (Knox, Vesk and Work 1966; Burdett and Murray 1974; Hoekstra *et al.* 1976; Mug-Opstelten and Witholt 1978; Gamazo and Moriyon 1987; Cushnie, O'Driscoll and Lamb 2016). Bacteria can also employ the BMVs to maintain the asymmetry of their OM when their Mla system malfunctions or is downregulated, for example under iron restriction, leading to the incorporation of PLs in the outer leaflet of the OM (Roier *et al.* 2016; Zingl *et al.* 2020). The bacteria can also release misfolded proteins packed within BMVs as an envelope stress (McBroom and Kuehn 2007). Besides the benefits to the individual cell, the BMVs as a result of single cell-based vesiculation have been found to play a role in bacterial survival during environmental stress, lateral gene transfer (LGT), the regulation of microbial interactions within bacterial communities and bacteria–host interactions (Mayrand and Grenier 1989; Kulp and Kuehn 2010; Schwechheimer and Kuehn 2015).

The CD-mediated mechanisms are the most extreme case of vesiculation since these mechanisms are the result of cell lysis (Turnbull *et al.* 2016; Toyofuku *et al.* 2017) and depicted in Fig. 1D and E. Besides living in solitary conditions, 40–80% of the bacteria, as Flemming and Wuertz (2019) have calculated, also co-exist while organized into matrix-enclosed multicellular communities of differentiated cells, called biofilms (van Gestel, Vlamakis and Kolter 2015; Flemming and Wuertz 2019). Due to the matrix secreted by the bacteria, biofilms bring the cells in close proximity (thus allowing for social interaction), shield them from environmental dangers and make different concentrated nutrients, signaling molecules and LGT agents available to the community (van Gestel, Vlamakis and Kolter 2015; Flemming and Wuertz 2019). In such multicellular organizations, death becomes a common regulatory mechanism that ends certain bacterial lineages (for example, to abort the spread of phages while in their premature stages) as well as further supplying the biofilm with extracellular polymeric substances (DNA, proteins and exopolysaccharides), nutrients and lipids (Allocati *et al.* 2015). The CD-based vesiculation is based on the established relationship between the bacteria and some of their temperate bacteriophages existing as prophages (Turnbull *et al.* 2016; Toyofuku *et al.* 2017). This type of vesiculation involves the activity of the prophage-encoded holin–endolysin dyad (Turnbull *et al.* 2016; Toyofuku *et al.* 2017).

Holins are small hydrophobic pore-forming peptides that oligomerize within the CM and, upon pore formation, allow the endolysins (PG hydrolases) to escape and cleave the PG, followed by cell lysis (Catalao *et al.* 2013). This cell lysis delivers exogenous DNA and membrane fragments that spontaneously form BMVs used for the mechanical maintenance of the biofilm structure (Turnbull *et al.* 2016). The mechanism underlying this type of vesiculation is RecA-dependent and can be induced either spontaneously or by agents that trigger the SOS response (Turnbull *et al.* 2016). This kind of vesiculation is described as 'explosive cell lysis' (schematically present in Fig. 1D) in the case of *Pseudomonas aeruginosa* the genome of which carries P2- and λ-like prophages coding for the R2 and F2 bacteriocins (pyocins), respectively (Nakayama *et al.* 2000; Turnbull *et al.* 2016). Such kind of vesiculation involves the expression of the *lys* gene (which codes for an endolysin) from the prophage gene cluster (Fig. 1D, panel II), which in turn cleaves the PG, thus causing cell rounding that then leads to cell lysis in a subpopulation of bacteria (Fig. 1D, panel III) in interstitial biofilms of *P. aeruginosa* (Turnbull *et al.* 2016).

The other type of prophage-triggered vesiculation has been described in the case of *B. subtilis* (Toyofuku *et al.* 2017). It is represented schematically in Fig. 1E. It involves the expression of the XhlAB–XlyA (holin–endolysin) dyad encoded by the PBSX prophage (Toyofuku *et al.* 2017). Unlike the explosive cell lysis during which bacteria become spherical, the prophage-triggered vesiculation in *B. subtilis* does not lead to morphological changes of the bacteria, and the BMVs are released via vesiculation of the CM, the vesicles of which are released through the pores introduced in the PG by the XlyA activity (Turnbull *et al.* 2016; Toyofuku *et al.* 2017); for clarity, see Fig. 1D and E. The death of the cell is presumably caused by the disrupted membrane potential due to pores formed by the holin, which then leads to the loss of membrane integrity (Catalao *et al.* 2013; Toyofuku *et al.* 2017).

THE ROLE OF BMVs IN BACTERIAL ECOLOGY

In the previous section, we outlined the bacterial diversity—generated and preserved by various ecological, evolutionary and geological processes—that has resulted from successive selections in different extinct and extant environments. While we also introduced the dynamics of BMVs, in this one we will outline some of the specific roles of BMVs in bacterial ecology. We will also propose eco-evo feedback mechanisms in which BMVs may be active players.

As a general rule, the populations of every species have defined tolerances to, and requirements for, certain environmental parameters that together represent the ecological niche

of the species (Begon, Townsend and Harper 2005). Being a resident in a particular habitat requires several factors: (i) a specific metabolism for a place in the food web, (ii) adaptation to the environmental physicochemical conditions and (iii) the means for competition or co-existence with other organisms (Kassen and Rainey 2004; Schlegel and Jannasch 2006; Zavarzin 2006). To achieve all of the above, bacterial populations, as is the case with individual bacteria per se, may develop a certain degree of individuality shaped by the expression of distinct systems for substrate utilization, by the production of extracellular organelles for attachment or motility and by the development of defensive mechanisms against environmental stress, predators and competitors (Kassen and Rainey 2004; Schlegel and Jannasch 2006). Ecosystems of strongly restrictive environmental characteristics, such as the Atacama lithic habitats, acid mines, black smokers and the Deccan traps, thermal springs, soda lakes and aquatic cold environments, are inhabited by a few bacterial species present in relatively large numbers, while ecosystems of fluctuating physicochemical and nutrient conditions, such as certain plant and animal hosts, soils and water ecosystems, among others, may be inhabited by a variety of bacterial species with individuals present in different numbers (Johnson and Hallberg 2003; Schlegel and Jannasch 2006; Ward 2006; Lozupone and Knight 2007; Mouchka, Hewson and Harvell 2010; Margesin and Miteva 2011; Johnson 2012; Huang *et al.* 2013; Sorokin *et al.* 2014; Jebbar *et al.* 2015; Gomez-Silva 2018; Cordovez *et al.* 2019; Dutta *et al.* 2019). The trend may be exemplified by a study that demonstrated the presence of 3560 operational taxonomic units (OTUs) in soil samples isolated from favorable conditions compared with the OTUs detected in sea ice isolates or acidic environments, which were found to range from 239 to 78 OTUs (Lozupone and Knight 2007). Other ecosystems exist with favorable environmental conditions and a constant flux of nutrients, such as the gastrointestinal ecosystems of different homeothermic animal hosts (Lozupone and Knight 2007; Oakley *et al.* 2014; Nishida and Ochman 2019).

'Everything is everywhere, [but] the environment selects' is also a good start for this part of the review. The idea can be interpreted from the perspective of the BMV release: every bacterium releases BMVs, but the environment may select the type, content and number of the BMVs (Mayrand and Grenier 1989; Beveridge *et al.* 1997; Kulp and Kuehn 2010; Manning and Kuehn 2013; Kulkarni and Jagannadham 2014; Schwechheimer and Kuehn 2015; Orench-Rivera and Kuehn 2016; Eberlein *et al.* 2018).

Figure 2 presents the relationships of the main bacterial species considered in this review, combined with their physiological properties and niche preferences. These species have a combination of adaptive phenotypes that provide a certain degree of individuality that stands out when we compare a species with the closely related species shown in Fig. 2 (for example, *Vibrio cholerae* bacteria are mesophilic, while the *V. rumoiensis* cells are psychrophilic). We can reason that this degree of individuality can be transferred to the BMVs, the different components of which may contribute to adaptations to different environmental factors, such as by a combinatorial result of the membrane structure and the luminal content. Thus, the secreted BMVs can serve as a multifunctional delivery system through which bacteria can respond efficiently and readily to different environmental changes (Mayrand and Grenier 1989; Beveridge *et al.* 1997; Kulp and Kuehn 2010; Manning and Kuehn 2013; Kulkarni and Jagannadham 2014; Schwechheimer and Kuehn 2015; Orench-Rivera and Kuehn 2016; Eberlein *et al.* 2018).

The production of BMVs also provides some case-study evidence that can lead to a better understanding of ecosystem engineering: a process that contributes to the modification of the physical environment (Wright and Jones 2006). Ecosystem/ecological 'engineers' are what Jones, Lawton and Shachak (1994) define as 'organisms that directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. In so doing, they modify, maintain or create habitats through a process known as ecosystem engineering (Jones, Lawton and Shachak 1994; Wright and Jones 2006). The BMVs are involved in the structure and maintenance of different biofilms, as reviewed in several previous works (Beveridge *et al.* 1997; Schooling and Beveridge 2006; Kulp and Kuehn 2010; Tashiro, Uchiyama and Nomura 2012; Manning and Kuehn 2013; Brown *et al.* 2015; Wang, Chanda and Zhong 2015), but it is the ecological context that will be considered in the following section that defines their dynamics in different biofilms.

In the following section, we will outline how BMVs stabilize different communities; we will also provide examples of how BMVs can serve as ecosystem engineers that can act as 'detergents' for hydrophobic environments, be mediators in biochemical cycles, sustain microenvironments and serve as co-aggregation bridges, among other functions.

Strongly restrictive environments

To accommodate the purpose of this review, any environment that offers conditions that vary from any of those found in environments that support relatively high biodiversity (i.e. habitats with pH 5–9, 1 atm pressure, salinity lower than 2 M NaCl, temperature between 15 and 60°C and abundant nutrients present in higher than 1–15 mg of C per liter) is considered 'extreme', and the inhabitants of such environments are called extremophiles (Morita 1975; Kuznetsov, Dubinina and Lapteva 1979; Rothschild and Mancinelli 2001; Schlegel and Jannasch 2006). Due to their physicochemical parameters, the extreme environments contain substrates either of low energy or of very low concentrations and such situation reflects on the generation time of the extremophiles that can range from several hours (as in some psychrophilic, acidophilic and halophilic bacteria) to a theoretically estimated thousands of years as in the bacteria inhabiting the deep-sea bed (Morita 1975; Kuznetsov, Dubinina and Lapteva 1979; Jorgensen and Boetius 2007; Johnson 2012; Mesbah and Wiegel 2012). The relatively long generation time compared with that of some of the better-studied non-extremophiles is one of many reasons that the biology of these exotic bacteria remains understudied—a lack that also includes the role of their BMVs. The BMVs of extremophiles are an extension of the key aspects of their adaptive biology, which creates a microenvironment that increases the chances for the bacterial population to exist. Learning the mechanism and function of the vesiculation of extremophiles compared with that of their mesophilic relatives should help to illuminate the organisms' adaptive potential to challenging environmental factors.

Acidic environments

The biomineralization of the compounds of biologically significant chemical elements such as S and Fe, as well as the precipitation of toxic minerals containing chemical species of U and As, is often mediated by bacterial groups that are capable of either the oxidation or reduction of the ions of these aforementioned elements. The majority of these bacteria live in defined

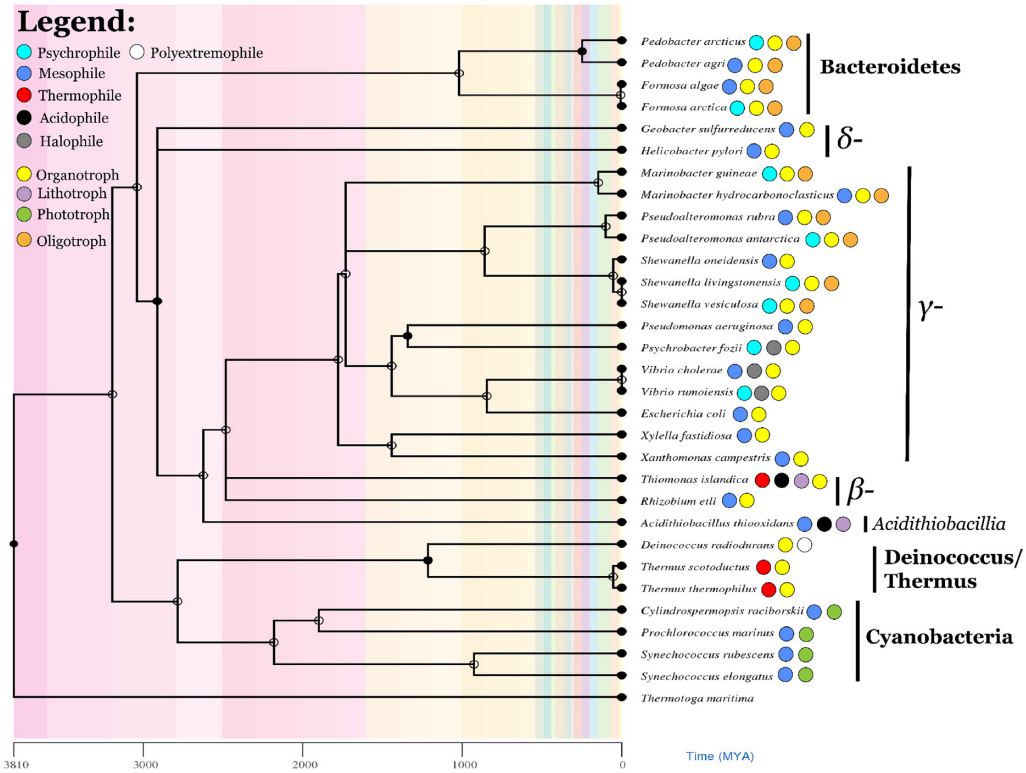

Figure 2. Time tree, built with MEGA TimeTree (Kumar *et al.* 2017), representing the hypothetical relationships and evolutionary time scale of the main bacterial taxa present in the review (MYA = million years). The proteobacterial classes are shown in italics (*Beta-*, *Gamma- Delta-proteobacteria* and *Acidithiobacillia*); the phyla are shown in **bold**. The ecological representation of each of the bacterial species is shown as a combination of circles, as explained in the legend. *Thermotoga maritima* is used as an outgroup.

environments in which the air-exposed minerals from ores produce iron- and sulfur-enriched water ecosystems with a pH that can range from 6.5 (e.g. the Storwartz mine in Norway) to 0.5 (the Iron Mountain in California) (Johnson and Hallberg 2003; Johnson 2012). Some mines may also contain, associated with the ore's minerals, metalloids and actinides such as As and U present in different oxidized states (Johnson and Hallberg 2003; Mkandawire 2013).

Acidithiobacillus thiooxidans is a Gram-negative aerobic mesophilic chemolithoautotroph that gains energy from the oxidation of sulfides (S^{2-}), elemental sulfur (S^0), thiosulfates ($S_2O_3^{2-}$) and sulfites (SO_3^{2-}) for CO_2 fixation (Kelly and Wood 2004). It is a true acidophile that inhabits sulfur-enriched acid mine drainages with a pH of 2–3 (Kelly and Wood 2004). In order for these bacteria to gain energy from elemental sulfur, they first have to overcome the solid's hydrophobicity (Yang *et al.* 2019). *Acidithiobacillus thiooxidans* has been shown to overcome this hydrophobic barrier via BMVs, which, due to their amphipathic nature, allow the bacteria to colonize and then dissolve the sulfur (Knickerbocker, Nordstrom and Southam 2000). Interestingly, the vesicle release has not been observed in sulfite-enriched cultures, which further supports the hypothesis that BMVs may shape a microenvironment that promotes colonization, but BMVs may also serve as a detergent that decreases the hydrophobicity of the elemental sulfur and solubilizes the sulfur so that bacteria can transport it to their periplasm and oxidize it to sulfuric acid with the help of oxygen (Knickerbocker, Nordstrom and Southam 2000).

Garcia-Meza *et al.* (2013) have shown that the electrooxidized chalcopyrite produces $S_n^{2-}/S^0/CuS$ phases on which *A. thiooxidans* bacteria form a mat-like biofilm composed of different layers with a zonal hydrophobicity in the biofilm due to the synthesis of lipids and hydrophobic extracellular polymers (Garcia-Meza *et al.* 2013). One intriguing area of research would be to explore the role of *A. thiooxidans* BMVs in the formation of these hydrophobic zones and to determine whether the sulfur hydrophobicity serves as a trigger for their formation. The secretome of exponentially grown *A. thiooxidans* bacteria in media supplemented with 1% sulfur has been found to contain mainly Omp40-like proteins and a trimeric coiled-coil lipoprotein licanantase that increases the sulfur oxidation (Bobadilla Fazzini, Levican and Parada 2011; Abarca *et al.* 2014). As Bobadilla Fazzini, Levican and Parada (2011) briefly speculated, the sulfur-induced secretome of *A. thiooxidans* may partially be delivered via BMVs.

A further hypothesis is that the BMVs released by these bacteria may play an important role in biomineralization processes in extreme environments where heavy metals are present and where the released BMVs can serve as a nucleation center for the formation of novel biominerals (Benzerara *et al.* 2008). This hypothesis is further supported by the finding that microbial polysaccharides can serve as a template for the production of unusual crystal structures (Chan *et al.* 2004), which adds a novel role of BMVs as mediators in the biogeochemical cycle of metals in nature. Bacteria, as mentioned above, most probably release these mineralized vesicles to let go of the precipitates encrusted on their cells (Fig. 3A).

Studies on the acid-mine-drainage microbial communities, found in Carnoules, France—the representatives of which belong to *A. ferrooxidans*, *Gallionella ferruginea* (both species capable of ferrous oxidation) and *Thiomonas* spp. [capable of oxidizing

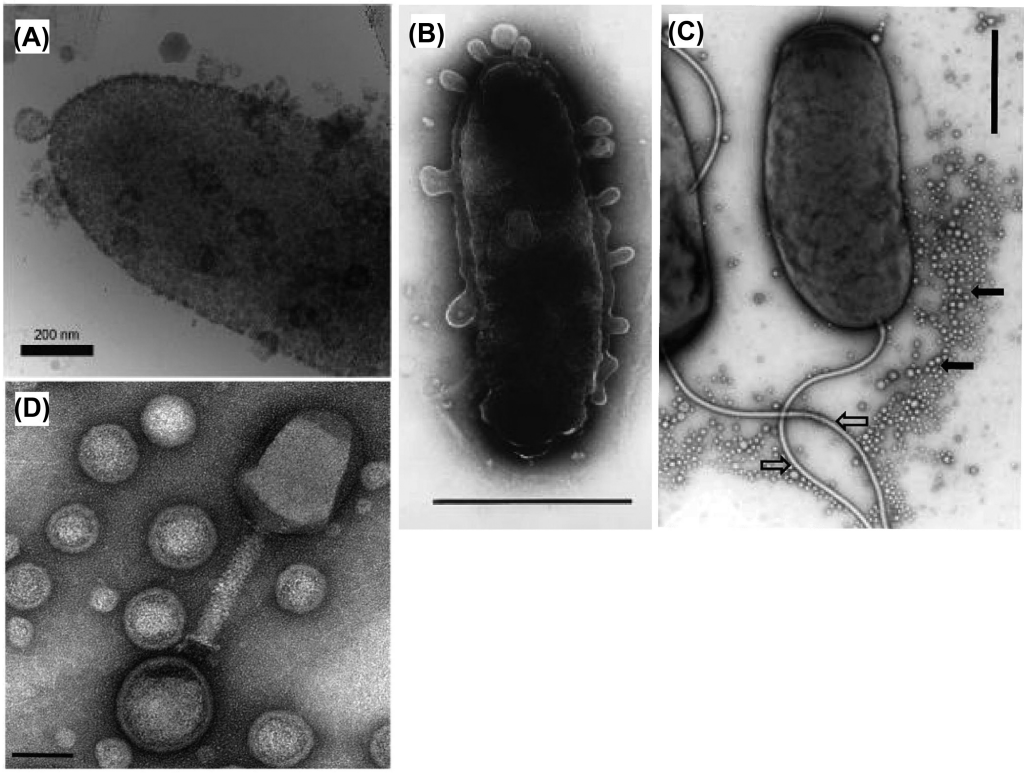

Figure 3. Eco-evolutionary dynamics mediated via BMVs. For example: **(A)** biofilm-deficient strain releases BMVs to shed metal compounds; differences between the type and degree of vesiculation between closely related species, belonging to the same genus, exemplified with *Vibrio rumoiensis* **(B)** and *V. cholerae* **(C)**; and **(D)** the BMVs can provide shielding from phage attacks, which helps bacteria avoid evolutionary trade-offs. (A) Cryo-TEM images of intact *S. oneidensis* Δ*mxdA* biofilm-deficient mutant producing extensive BMVs coated with electron-dense U. (Shao, Comolli and Bernier-Latmani 2014; reproduced under the terms of the Creative Commons CC BY license—https://creativecommons.org/licenses/by/4.0/legalcode) (B) Electron micrograph of a negatively stained cell of *V. rumoiensis* S-1^T, showing blebs on the cell surface. Bar, 1 μm (Yumoto *et al.* 1999; **Applied and Environmental Microbiology**, 1999, Vol. 65, page 68, PMID: 9872761; PMCID: PMC90984, reproduced with permission from American Society for Microbiology). This image/content is not covered by the terms of the Creative Commons license of this publication. For permission to reuse, please contact the rights holder. (C) Ultrastructural analysis of *V. cholerae* by electron microscopy. An electron micrograph showing the flagella (open arrows) and OMVs (closed arrows) (size bar = 500 nm) (Manning and Kuehn 2011; reproduced under the terms of the Creative Commons by license; https://creativecommons.org/licenses/by/4.0/legalcode). (D) Negative stain electron micrograph of a complex of T4 bacteriophage and BMV released by *E. coli* (size bar = 50 nm) (Manning and Kuehn 2011; reproduced under the terms of the Creative Commons by license; https://creativecommons.org/licenses/by/4.0/legalcode).

Fe(II) and As(IV)]—have revealed two different mineral precipitation patterns (Benzerara *et al.* 2008). Besides being associated with the bacterial cells, most of the mineral precipitates were found to be biomineralized vesicles (Benzerara *et al.* 2008). In addition to examining BMVs, another study on arsenic immobilization by a microbial community mainly composed of *G. ferruginea* showed that As(V) accumulated in the biomat mainly on sheath-like and stalk-like structures as well as co-precipitated as extracellular granules under the form of Fe-S-As-bearing mineral (Ohnuki *et al.* 2004).

Researchers who have examined other bacteria capable of reducing U(VI) to U(IV), and thus accumulating uraninite crystals, have also proposed mineralized BMV release as a novel option for the secretion of insoluble compounds. For example, *Shewanella oneidensis* is a facultative anaerobic alteromonade capable of metal respiration. To prevent the accumulation of insoluble U(VI) on the cell wall, *S. oneidensis* bacteria produce exopolysaccharide (EPS), which moves the precipitate away from the cells; as a second strategy, they also secrete the encrusted uranates as part of their BMVs; see Fig. 3A (Shao, Comolli and Bernier-Latmani 2014).

BMV release as a metal-detox strategy is also exploited by another U(VI) reducer: *Geobacter*. Cologgi *et al.* (2011) have demonstrated that the reduction of U(VI) and the subsequent immobilization of U(IV) both take place on the pili of the bacteria, which they found prevented the precipitation of U(IV) in the periplasm. Furthermore, as Shao, Comolli and Bernier-Latmani (2014) have pointed out, the pili-deficient mutant exhibited increased vesiculation compared with the wild-type strain when the medium was supplemented with U(VI); in this way, the vesiculation is thought to serve as a back-up electron transfer mechanism (Cologgi *et al.* 2011; Shao, Comolli and Bernier-Latmani 2014).

Low-temperature environments

Membrane vesicles have been described as a common feature of some psychrophiles—bacteria whose optimal growth temperature is 15°C or lower, while their maximal temperature for growth is ∼20°C (Ingraham and Stokes 1959; Morita 1975; Schlegel and Jannasch 2006). The ecological distribution of the psychrophiles is quite vast from Arctic and Antarctic ecosystems to human-generated cooling-system environments to the ocean waters, 90% of the volume of which is 5°C or lower (Ingraham and Stokes 1959; Morita 1975; Schlegel and Jannasch 2006). A certain group of bacteria that grows at temperatures above 20°C, but can also grow at lower temperatures, is referred to as being 'psychrotolerant'; these bacteria are also known as facultative psychrophiles or psychrotrophs (Ingraham and

Stokes 1959; Morita 1975; Schlegel and Jannasch 2006). Also, very few studies on obtaining psychrophilic mutants from their mesophilic wild types have been recorded in the chronicles of microbiology—it has only been achieved with UV-irradiated mesophilic *P. aeruginosa* bacteria (Azuma, Newton and Witter 1962; Olsen and Metcalf 1968). In this review, we will collectively call the cold-loving bacteria 'psychrophiles'. To survive in such environments, these bacteria have evolved capabilities that help them adapt to oxidative stress, low water-activity levels and physical disruption due to ice formation, among other adaptations (Scherer and Neuhaus 2006).

A common trend among some psychrophilic bacteria is to form biofilm composed of diverse EPS polymers and BMVs (Nevot *et al.* 2006b; Frias *et al.* 2010; Perez-Cruz *et al.* 2013; Yokoyama *et al.* 2017). Studies on *Shewanella livingstonensis* NF22, *Shewanella vesiculosa* M7, *Pseudoalteromonas* sp. M4.2, *Pseudoalteromonas antarctica* NF3, *Psychrobacter fozi* NF23 and *Marinobacter guineae* M3B have confirmed that an extracellular matrix composed of EPS and BMVs may be a common feature among these cold-loving bacteria (Nevot *et al.* 2006a,b; Frias *et al.* 2010). The BMVs stability in the psychrophilic biofilm may be reinforced by the secreted EPS. In addition to its role in cryoprotection, osmoprotection, adhesion and nutrient scavenging (Nichols, Guezennec and Bowman 2005; Deming and Young 2017; Casillo *et al.* 2018; Lo Giudice *et al.* 2020), the EPS could also be involved in the stabilization of the released BMVs. Evidence for such kind of implication comes from studies on *P. antarctica*'s EPS that is predominantly glycoproteinaceous (de La Maza *et al.* 1999; Cocera *et al.* 2000; Cocera *et al.* 2001). The studies have demonstrated that the secreted EPS is able to protect PC-liposomes from the activity of different detergents such as SDS, dodecyl maltoside and octyl glucoside (de La Maza *et al.* 1999; Cocera *et al.* 2000; Cocera *et al.* 2001). The psychrophilic BMV componentry, represented by putative TonB-dependent receptors, bifunctional UDP-sugar hydrolase, P-binding protein and phosphate-selective porins O and P, hints to involvement of the BMVs in nutrient acquisition (Nevot *et al.* 2006a,b; Frias *et al.* 2010) that could act in synergy with the produced EPS.

In a nutshell, the BMVs of the studied psychrophiles are part of a microenvironment in which the cold-loving bacteria can thrive under harsh conditions. Yet, the role(s) of the BMVs in cold adaptation remains per se unknown.

Be that as it may, we will also consider indirect studies that may hint to cryoprotection exerted by the psychrophilic BMVs. The first group of findings indicates that some bacteria may use membrane vesicles to deliver ice crystallization proteins (Phelps *et al.* 1986; Muryoi *et al.* 2003). Among other features, an important adaptation for life in thermal equilibrium with its environment of low temperatures is the regulation of different phase transitions (Lorv, Rose and Glick 2014; Collins and Margesin 2019). Studies on *Pantoea agglomerans* (*Erwinia herbivora*) and *Pantoea ananatis* have illustrated that these bacteria (even though not psychrophilic) can secrete ice-nucleating proteins (membrane proteins that trigger extracellular ice-formation; INPs) via BMVs under the form of extracellular ice-nucleating matter (EIM) (Phelps *et al.* 1986; Muryoi *et al.* 2003). Moreover, incorporation of INPs into PC-PE liposomes has increased the ice-nucleating activity of *P. ananatis* INPs (Muryoi *et al.* 2003). It is proposed that the secretion of INPs may prevent or reduce the cryodamage due to intracellular ice crystals (Lorv, Rose and Glick 2014; Collins and Margesin 2019). The second group of studies suggest that psychrophiles may use their vesicles for the delivery of PLs (such as PC, PE and others) as a part of the vesicular componentry that may act as biosurfactants. The biosurfactants have been proposed by Collins and Margesin (2019) and Perfumo, Banat and Marchant (2018) to contribute to ice growth inhibition, osmoprotection and decrease in surface tension. Finally, studies on cryopreservation with artificial vesicles,

which showed increased β-glucuronidase stability at low temperatures (4°C and –80°C) when the enzyme was encapsulated into unsaturated fatty acid-esterified PC liposomes (Frank *et al.* 2018), hint to a putative cryoprotective role, i.e. the BMVs may also stabilize the proteins delivered by them.

The psychrophiles have also developed capabilities for homeoviscous maintenance of their membranes. To achieve the right membrane fluidity, they can increase the number of their unsaturated, branched or short fatty acids of the PLs (Scherer and Neuhaus 2006). Yokoyama *et al.* (2017) reported that membrane stress in *S. livingstonensis* Ac10 due to mutational deficiencies in eicosapentaenoic acid (EPA) and branched-chain fatty acids causing altered lipid composition increased the vesicle production by up to 5-fold (Yokoyama *et al.* 2017). Based on previous studies on the importance of EPA for cellular morphogenesis and proper membrane protein folding (Kawamoto *et al.* 2009; Dai *et al.* 2012), Yokoyama *et al.* (2017) have proposed that the observed increase in the BMV release is a stress response of the bacteria resulting in the release of misfolded proteins similar to the BMV response to unfolded or misfolded periplasmic proteins (McBroom and Kuehn 2007). Interestingly, while the addition of unsaturated fatty acids in the growth medium could compensate for the cryosensitivity of some mesophilic mutant strains (for example, *E. coli* Δ*fabA*), the presence of EPA could not restore the normal growth of EPA-deficient *S. livingstonensis* at 4°C (Calcott 1986; Kawamoto *et al.* 2009). The extracellularly present EPA-PLs could do that (Kawamoto *et al.* 2009). This finding hints that the BMV-delivered PLs may also contribute to the maintenance of the homeoviscous adaptation.

Among the psychrophilic BMV producers studied, one species stands out: *Shewanella vesiculosa*, which is so named for its ability to release BMVs (Bozal *et al.* 2009). Indeed studies on *S. vesiculosa* M7^T were what led to the discovery of the O-IMVs representing 0.1% of the total BMVs produced by these bacteria (Perez-Cruz *et al.* 2013). Pérez-Cruz and her colleagues also found that the O-IMVs carry short DNA molecules of ~600 nucleotides. Based on their EM experiments, the researchers proposed a mechanism for O-IMV biogenesis (illustrated in Fig. 1C) in which the cytoplasm is included in the vesicle biogenesis in a budding process that involves the CM and OM (Perez-Cruz *et al.* 2013). Their model has also confirmed the Kadurugamuwa/Beveridge double-step hypothesis proposed to explain the presence of cytosolic components and DNA in *P. aeruginosa*'s BMVs (Kadurugamuwa and Beveridge 1995). The BMVs of another isolate of *S. vesiculosa*, strain HM13, have been shown to contain a single major transmembrane protein of unknown function called P49 (Chen *et al.* 2019). The authors demonstrated that the disruption of the P49 gene does not affect the BMV production and it was concluded that the loading of this protein takes place independently from the BMV biogenesis (Chen *et al.* 2019). From follow-up work, it has been reported that the secretion of P49 depends on the type 2 secretion system (for further information about the different secretion systems, see Fig. 5) and that the P49 association with BMVs requires the presence of a glycolipid, which, if not produced, results in BMV-independent P49 liberation in the extracellular environment (Kamasaka *et al.* 2020). Overall, further studies, on BMVs produced by *S. vesiculosa* HM13 may offer new possibilities to study the processes of the sorting and enrichment of the vesicle cargo, as suggested by Kamasaka *et al.* (2020).

High-temperature environments

Thermophiles, in contrast to the psychrophiles, are a group of bacteria adapted for life at high temperatures. *Thermus* spp., which are aerobic heterotrophic bacteria typically found in hydrothermal areas, have an optimal growth temperature of 70°C (da Costa Allgayer, Nobre and Rainey 2001). Researchers have suggested that the LGT between the hyperthermophilic archaea and the representatives of *Thermales* played a significant role in the adaptation of the latter to high temperature (Averhoff 2009). Blesa and Berenguer (2015) have shown that different species of *Thermus* (e.g. *T. aquaticus* subsp. *thermophilus* and *T. scotoductus*) release a variety of vesicles in which extracellular DNA (eDNA) can be present and protected from the harsh environment (Blesa and Berenguer 2015). These thermophiles can be transformed at 100-fold higher efficiency with the eDNA present in the vesicles than with the free genomic DNA (Blesa and Berenguer 2015). In the case of *Thermus* spp., the BMVs not only serve as a possible source for genetic material but also play a role in preserving the DNA, and they function as a vehicle for an effective alternative route of transformation (Blesa and Berenguer 2015).

These findings should prompt further studies. One avenue of interest would be to conduct genetic experiments to test directly if exchange occurs between representatives of *Thermus* and hyperthermophilic archaea that involve BMV-mediated LGT. Another suggested direction for future study could be based on the comparison between the BMVs released by representatives of *Thermus* compared with those secreted by the group of psychrophilic bacteria mentioned above. The common theme for these future studies may be the content of DNA, the thermophiles can seemingly use their BMVs as a form of LGT (Blesa and Berenguer 2015), while the role of the short DNA pieces within the BMVs of psychrophiles in LGT has yet to be clarified (Perez-Cruz *et al.* 2013). The DNA within the psychrophiles' BMVs, in combination with the EPS, can also be studied regarding the maintenance of water activity and decreased freezing temperatures due to the presence of these components in biofilm.

Aquatic ecosystems

The aquatic ecosystem, the largest ecosystem on earth, includes both marine and freshwater environments. Based on the nutritional availability, the aquatic ecosystem incorporates two types of habitats: coastal and oligotrophic (Kuznetsov, Dubinina and Lapteva 1979; Poindexter 1981; Koch 2001). The first, occupied by copiotrophs, provides favorable growth and nutritional conditions (for example, the carbon concentration of the available C sources is above 500 mg L^{-1}), while the second is characterized by a scarcity of nutrients where the available C sources range between 1 and 15 mg L^{-1} (Kuznetsov, Dubinina and Lapteva 1979; Poindexter 1981). Examples of such oligotrophic environments (in addition to the cold ecosystems, and some soil-embedded and soil-embedding environments) include the open seas and oceans, which are typically inhabited by bacteria, called oligotrophs (Kuznetsov, Dubinina and Lapteva 1979; Poindexter 1981; Koch 2001; Torsvik and Øvreås 2008). (Also of note is that copiotrophs can still be found in oligotrophic systems, and oligotrophs can be present in coastal waters.) To survive in environments with depleted energy sources, the oligotrophs have diverse nutrient uptake systems with low substrate specificity and multiple substrate-biding sites (Kuznetsov, Dubinina and Lapteva 1979; Poindexter 1981; Koch 2001). The nutrient flux is further facilitated by the increased surface-to-volume (S/V) ratio that oligotrophs achieve either by having the morphology of small spheres or thin rods, or by producing membrane projections such as the stalk of caulobacteria and hyphomicrobia

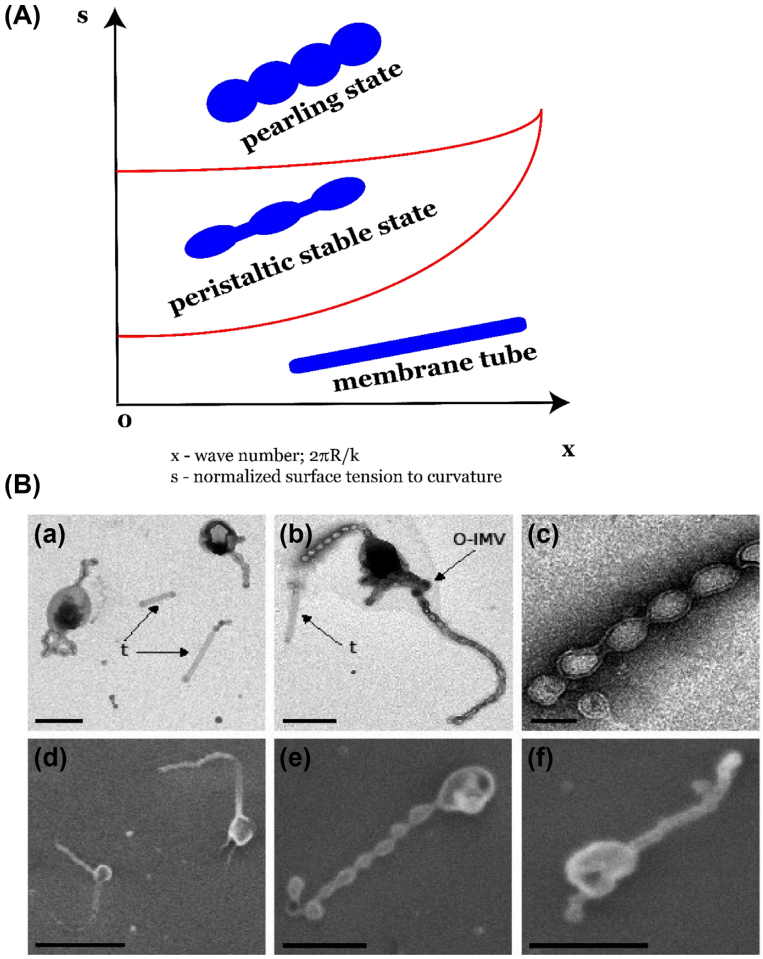

Figure 4. Comparison of the physically induced pearling with the 'biopearling' produced by oligotrophs. **(A)** Bar-Ziv–Moses' diagram describing the pearling state of the cylindrical membrane due to competition between curvature and tension. The bottom line represents the instability line; above the second line, the pearling state of the membrane is expected to appear (modified from Bar-Ziv and Moses 1994). **(B)** Micrographs of strain Hel3_A1_48 cells in stationary growth phase. Membrane tubes (t) and thicker vesicles (assigned as O-IMVs) are indicated. Cells grown in HaHa_100V medium at 21°C to stationary phase were negatively stained with 1% uranyl acetate for transmission electron microscopy (TEM) **(a–c)**. The cells were passively settled on a silica wafer, dehydrated by an ethanol series and preserved using critical point drying for scanning electron microscopy (SEM) **(d–f)**. Bar corresponds to 100 nm (c), 500 nm (a, b, e and f) or 1 μm. (d) (**Applied and Environmental Microbiology**, 2019, Vol. 84, page 3, DOI: 10.1128/AEM.00829-19, reproduced with permission from American Society for Microbiology). This image/content is not covered by the terms of the Creative Commons license of this publication. For permission to reuse, please contact the rights holder.

(Kuznetsov, Dubinina and Lapteva 1979; Poindexter 1981; Koch 2001).

The marine members of **Bacteroidetes** are among the main representatives of the bacterioplankton (Pinhassi, Zweifel and Hagström 1997; Pinhassi and Hagström 2000; Giovannoni and Stingl 2005; Anne-Carlijn, Eva and Gerhard 2006; Pommier *et al.* 2007; Lindh *et al.* 2015; Bunse and Pinhassi 2017). One recently discovered member of *Flavobacteriaceae*, *Formosa* strain Hel3_A148, revealed another strategy for the optimization of nutrient uptake that involves BMV release (Fischer *et al.* 2019). As a result of a process called 'biopearling'—the membranes of these bacteria protrude and form up to 2-μm-long tubular appendages and start to oscillate due to abiogenic physical forces resulting from the competition between membrane curvature and tension that together introduce instability in the tubular structure and produce a chain of linked with short neck vesicles or 'pearls'; see Box 2 and Fig. 4B (Fischer *et al.* 2019). Proteomics studies of the BMVs of this strain have shown that the BMVs are enriched in porins (the OmpC-like type), hydrolytic enzymes (including endonucleases, peptidases and glycoside hydrolases), TonB-dependent proteins and gliding motility-associated proteins, while BMVs have also been found to exhibit laminarin-binding properties, which suggests a role in carbohydrate binding and utilization (Fischer *et al.* 2019).

Box 2.
Physical factors that induce pearling

Physics can also help to explain the dynamics of vesiculation. For example, Bar-Ziv and Moses (1994) have shown that optical tweezers can be used to induce sinusoidal perturbations in a cylindrical membrane vesicle (Bar-Ziv and Moses 1994) (Fig. 4A). In their experiment, this excitation transformed the membrane tube into vesicles linked by small necks in a way that the overall structure resembled 'pearls on a string'—the same structure observed as a result of the biopearling described by Fischer *et al.* (2019) in the oligotrophic bacteria (Bar-Ziv and Moses 1994; Fischer *et al.* 2019); see Fig. 4A and B. Bar-Ziv and Moses have found that the laser-induced membrane tension competes with the membrane curvature, for example with E_b, which forms the peristaltic mode of the system (Bar-Ziv and Moses 1994).

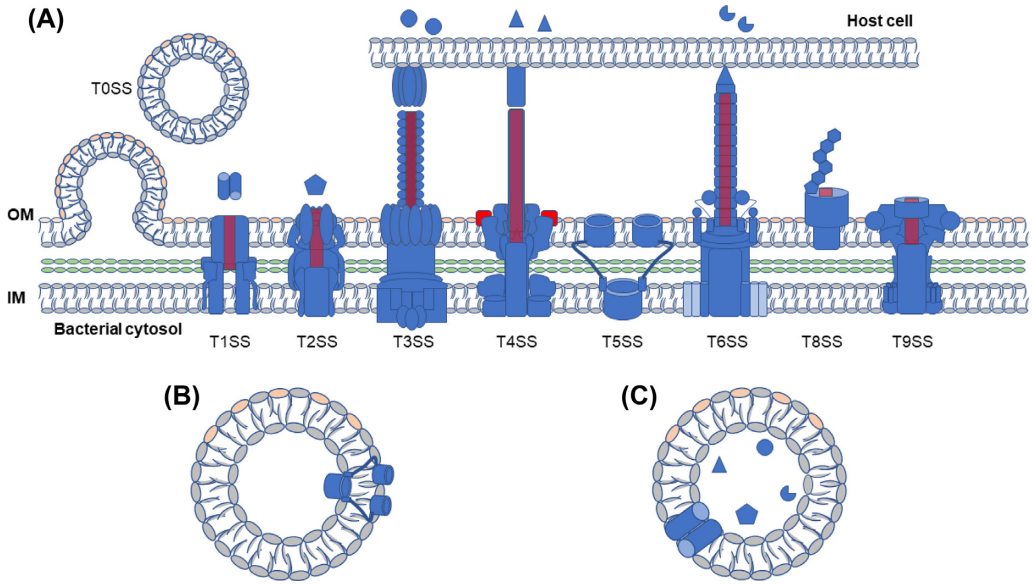

Figure 5. Illustration of BMVs and other bacterial secretion systems of Gram-negative bacteria. The figure is in relation to studies discussed in this review concerning substrates of different secretion systems that can be delivered via BMVs. **(A)** Schematic overview (adapted from Bocian-Ostrzycka *et al.* 2017) of different secretion systems of Gram-negative bacteria illustrating different modes of interaction of bacteria with their environment; abbreviated as T1SS (Type 1 Secretion System) to T9SS (the T7SS, i.e. the chaperone-usher pathway is not included in the figure). The BMV release is annotated as T0SS, type 'Zero' secretion system, as proposed earlier for secretion of the ClyA protein from *E. coli* by Uhlin, Oscarsson and Wai (2014). While the T1SS, T2SS and T5SS to T9SS deliver effectors directly to the extracellular environment or to the extracellular surface of the OM, the T3SS, T4SS and T6SS deliver their effectors in a manner that involves a contact to the either cell envelope of other bacteria or membranes of their hosts. **(B)** Bacterial membrane vesicles may also carry another secretion system, such as T5SS. **(C)** Bacterial membrane vesicles may combine the effectors of different secretion systems.

Mechanical stress and other factors, such as heating, electricity and van der Waals forces, can also destabilize membrane cylinders (summarized by Bar-Ziv and Moses 1994). These kinds of oscillating systems, as exemplified by the cylindrical membrane vesicle with induced perturbations, increase the surface area while simultaneously keeping the volume unchanged, which may provide the oligotrophs (such as flavobacteria) with the ability to interact with their nutrient-poor environment in the least energetically costly manner (Fig. 4B).

This type of vesiculation has also been observed in not only other bacterial species that exhibit oligotrophic growth, such as *S. livingstonensis*, *S. vesiculosa*, *S. oneidensis* and *Flavobacterium psychrophilum*, but also some non-oligotrophic bacteria such as *Neisseria meningitidis*, *Myxococcus xanthus* and *Francisella novicida* (Devoe and Gilchrist 1973; Moller *et al.* 2005; McCaig, Koller and Thanassi 2013; Perez-Cruz *et al.* 2013; Remis *et al.* 2014; Yokoyama *et al.* 2017; Subramanian *et al.* 2018). Nevertheless, the BMV-mediated biopearling may be an adaptive mechanism used by oligotrophs because it leads to an increased S/V ratio in the least energetically costly manner (Box 2 and Fig. 4), as well as providing an adhesive mechanism coupled with the delivery of the hydrolytic enzymes (Fischer *et al.* 2019).

Notably, the physiological nature of bacteria sometimes blurs the borders between different trophic modes: some copiotrophs may thrive or persist in oligotrophic environments, while some oligotrophs can grow in nutrient-rich places (Kuznetsov, Dubinina and Lapteva 1979; Koch 2001; Fierer, Bradford and Jackson 2007; Torsvik and Øvreås 2008). Once they experience nutrient depletion, some copiotrophs trigger a program, similar to that of the oligotrophs, which involves upregulation of nutrient uptake systems and/or an increase of their S/V ratio (Koch 2001; Fierer, Bradford and Jackson 2007; Torsvik and Øvreås 2008). A part of this management can also result in the formation of membrane structures that may be a product of biopearling (McCaig, Koller and Thanassi 2013; Remis *et al.* 2014). In such cosmopolitan bacteria, the biopearling may have other functions than adaptation to oligotrophic conditions.

Myxococcus xanthus is a soil deltaproteobacterial species with a 'biphasic' life cycle defined by the presence or absence of available food sources (Dworkin and Bonner 1972; Zusman *et al.* 2007; Zhang *et al.* 2012). The bacteria of this species can also be isolated from different aquatic environments (Hook 1977; Reichenbach 1999; Li *et al.* 2002). If nutrients exist in sufficient quantities, then the bacteria can form 'wolf packs' to either prey on other bacteria or to acquire the amino acids and protein sources present in the environment. Using this lifestyle, also called vegetative, *M. xanthus* can target other bacteria via their BMVs the cargo of which is enriched with peptidases/proteases, chitinases, antibiotics and chelating agents (Kahnt *et al.* 2010; Evans *et al.* 2012; Berleman *et al.* 2014). In oligotrophic situations (i.e. any environment with unavailable or lacking amino acids and peptides), *M. xanthus* bacteria aggregate and initiate a developmental program that results in the production of fruiting bodies filled with myxospores (Dworkin and Bonner 1972; Zusman *et al.* 2007; Zhang *et al.* 2012). In the initial stage of development, the cellular aggregation (biofilm) is achieved by cell-to-cell and cell-to-surface interactions mediated via intercellular appendages, slime and BMVs, some of which are arranged in chains (Arnold and Shimkets 1988; Palsdottir *et al.* 2009; Remis *et al.* 2014). The fact that the vesicle chains have rarely been observed in the planktonic phase implies that these formations are typical for the biofilm stage of *M. xanthus*, during which the BMVs produced by the developmental cells (Remis *et al.* 2014). This may promote adhesion and nutrient scavenging, coupled with other

functions, due to the lifestyle of these bacteria, such as cell-to-cell bridging, and intercellular signaling (Palsdottir *et al.* 2009; Kahnt *et al.* 2010; Remis *et al.* 2014). A future avenue of study that piques our curiosity is to check whether the BMVs extracted from the two different lifestyles of *M. xanthus* can perform the extracellular complementation of the different mutant groups, each deficient in a certain stage of fruiting-body development (Shimkets 1999).

Another bacterial species that occurs in oligotrophic situations is *Francisella novicida* (Margolis *et al.* 2009). The species was initially isolated from freshwater environments and it is considered as a waterborne intracellular human pathogen that can also be transmitted via arthropod vectors (Larson, Wicht and Jellison 1955; Hennebique, Boisset and Maurin 2019). When challenged in oligotrophic environments, *F. novicida* forms biofilms on abiotic surfaces and chitin and with the help of chitinases (ChiA and ChiB), the bacteria use *N*-acetyl-D-glucosamine as a carbon source (Margolis *et al.* 2009). McCaig, Koller and Thanassi (2013) have reported the production of OMVs and OM tubes (OMV/Ts) by *F. novicida*, as the tubes most probably are the released 'horn-like' (or stalk-like) structures previously described by Gil, Benach and Thanassi (2004) observed when the bacteria were incubated on solid Mueller–Hinton chocolate agar. The isolated OMV/Ts have been shown to be enriched with porins, to exhibit low cytotoxicity to the host cells (murine macrophages) and to trigger a proinflammatory response in a dose-dependent manner (McCaig, Koller and Thanassi 2013). McCaig, Koller and Thanassi (2013) have also noticed that the stalk-like structures are predominantly formed by the intracellular bacteria or by the exponentially growing bacteria in brain-heart infusion broth. One may speculate that the bacteria use biopearling to regulate their adhesion and host colonization by producing and releasing their stalks in the form of OMV/Ts depending on the environmental conditions defined by the habitat.

Various marine habitats, salt lakes, brines and other habitats are also inhabited by halophilic eubacteria that can thrive in high-salt concentrations. Although the actual contribution of BMVs in the bacterial adaptation to high-salt concentrations has yet to be elucidated, EM studies on the cellular morphology of certain halophilic bacteria and BMVs have revealed the potential role of BMV involvement (Vreeland, Anderson and Murray 1984; Gauthier *et al.* 1992; Yun *et al.* 2017). The gammaproteobacterial heterotrophic species that belong to genus *Halomonas* are typically isolated from a variety of saline ecosystems, ranging from estuarine and open-ocean ecosystems to hypersaline lakes. *Halomonas elongata* can grow in environments with salt concentrations ranging from 0.05 to 3.4 M NaCl (Vreeland *et al.* 1983). In their studies on the adaptive physiology of this organism, Vreeland, Anderson and Murray (1984) showed that upon increased NaCl, the cell wall of *H. elongata* became more compact, with an increased quantity of negatively charged lipids, which overall resulted in structural maintenance and an increase in less-mobile 'structured cell' water that prevented the cell from dehydrating. Following this line of logic, one should expect decreased vesicle release by the halophilic bacteria: because of their structural integrity, their cell walls become more rigid. This scenario is also what Vreeland, Anderson and Murray (1984) observed in their study: *H. elongata* cells incubated in 0.05 M NaCl produced blebs (released as BMVs), while the bacteria grown in 3.4 M NaCl did not. Further studies on the BMVs released by halophiles will be required to clarify how the observed decrease of BMV release might function as an adaptive strategy to high salt concentration.

Among primary inhabitants of different aquatic ecosystems are the oxygenic photosynthetic cyanobacteria. By combining data from culture and field studies, Biller *et al.* (2014) have shown that bacterial vesicles released by diverse bacterial species are abundant in marine ecosystems. The number of vesicles in coastal waters can reach $\sim 6 \times 10^6$ ml^{-1}, and in the Sargasso Sea can reach $\sim 3 \times 10^5$ ml^{-1}; both numbers are similar to the bacterial numbers found in these habitats (Biller *et al.* 2014). The bacterial vesicles also seem to be present in freshwater ecosystems produced by autotrophs (Silva *et al.* 2014; Gamalier *et al.* 2017).

Two interesting parallels may be suggested if we compare BMV distribution in aquatic ecosystems with their distribution in acid mines. For the first parallel, let us assume that vesicles released in the oceans can also serve as sites for nucleation, similar to the hypothesized role of acid-mine BMVs in biomineralization. If that is the case, then aquatic BMVs may have played an essential role in the formation of the stromatolites that are stratified rocks presumed to be lithified bacterial mats (Stal 2012). The bacterial mats are biofilms of vertically stratified layers of different physiological groups of bacteria that usually form on the bottom of different shallow aquatic environments (Stal 2012). These mats can precipitate insoluble inorganic salts, resulting in their mineralization (Stal 2012). As a result, the bacterial mats have been found to be calcified, silicified and sulfidized among other processes, which has presumably allowed the preservation of these biofilms in the form of laminated rocks (stromatolites) during different geological times (Wacey *et al.* 2011; Stal 2012; Baumgartner *et al.* 2019). One interesting line of speculation is whether the BMVs released by the different bacterial guilds forming each layer of the mat—the top layer is represented by the cyanobacteria, the middle layer is composed of the representatives of the purple sulfur bacteria, followed by the green sulfur bacteria (Stal 2012)—may have played a role in this mineralization process, for example, by either serving as nucleation centers of pseudo-single crystals or bridging Ca^{2+}, SO_4^{2-}, S^{2-} or other ions that can be precipitated with the EPS to play a role as a matrix.

The second parallel is as follows: even though they are produced by very different bacterial groups, acid-mine BMVs may exhibit some of the same ecological trends of aquatic BMVs.

Among the primary BMV producers is *Prochlorococcus*, which is the dominant cyanobacterial species of the open ocean, an oligotrophic zone to which the bacterium has adapted via the reduction of its genome and cell size (Partensky and Garczarek 2010; Flombaum *et al.* 2013). In addition to *Prochlorococcus*, other cyanobacteria such as *Synechococcus* and *Synechocystis* also produce BMVs (Biller *et al.* 2014; Pardo *et al.* 2015). *Prochlorococcus* has been further demonstrated to produce large quantities of vesicles, in numbers ten times higher than those of the bacterial cells (Biller *et al.* 2014). The BMVs of *Prochlorococcus* are isolated from the environment with a rigid membrane made of the non-bilayer lipids monoglycosyldiacylglycerol and sulfoquinovosyldiacylglycerol esterified with saturated fatty acids, as well as certain unidentified glycolipids (Biller *et al.* 2014). The vesicles contain proteins, belonging to different functional groups (such as nutrient transporters, porins, hydrolases as well as many of unknown function), DNA ($\sim$3 kb) and RNA (Biller *et al.* 2014).

Based on calculations that *Prochlorococcus*-derived dissolved organic carbon delivered via BMVs represents 10^4 to 10^5 tonnes of organic carbon exported into the ocean per day, Biller *et al.* (2014) have demonstrated that these BMVs could serve as a carbon source for heterotrophs, especially for oligotrophs such as *Alteromonas* and *Halomonas*. To extend this concept, an examination of the extent to which BMVs can participate in the cross-feeding of substrates (i.e. syntrophy) among different

oligotrophic bacterial species would be an intriguing line of research.

Due to the natural competence of the unicellular cyanobacteria and the length of the DNA present in the BMVs (which covers half the bacterial chromosome, with sequence reads overrepresented at the terminus region) in the BMVs, a role can be suggested for BMVs in the LGT (Biller *et al.* 2014). This potential role of the cyanobacterial vesicles in LGT is even more interesting for several reasons. First, while it has been recently shown for the model cyanobacteria, such as *S. elongatus*, that the natural competence is determined by type 4 transformation machinery (Taton *et al.* 2020), many of the naturally competent cyanobacterial species do not code for the full set of genes of the transformation pilus (Wendt and Pakrasi 2019). An alternative mechanism such as the BMV-mediated transformation hence may be a good substitute for the lack of DNA-uptake machinery. The BMVs are also potentially useful LGT vehicles because of the protective role of the BMV envelope against certain DNA-degrading agents and because of the form in which the DNA is present in the vesicles. Previous studies have demonstrated that the DNA for transformation can be DNA/DNA or DNA/RNA complexes of homologous or heterologous DNA the uptake of which is dose-dependent and severely disrupted in the presence of DNases (Trehan and Sinha 1981; Porter 1986; Essich, Stevens and Porter 1990; Barten and Lill 2006; Almeida *et al.* 2017).

The occasional observations of bacteriophages attached to BMVs from environmental samples suggest that BMVs can also serve as a broad phage-decoy mechanism (i.e. the bacteria titer away the bacteriophages via BMVs, which may carry the receptors for the adhesion of different phages) (Biller *et al.* 2014). This idea is further supported by two studies. First, Manning and Kuehn have aptly demonstrated that BMVs produced by *E. coli* can irreversibly bind T4 bacteriophages, which reduces the infection of *E. coli* by 90% (Fig. 3D) (Manning and Kuehn 2011). The second study, by Reyes-Robles *et al.* (2018), has demonstrated that BMVs released by *V. cholerae* can neutralize T4- and T7-like bacteriophages. The BMVs released by planktonic bacteria are a good example of an alternative eco-evo feedback. Eco-evo studies on *E. coli*–bacteriophage communities have demonstrated that if an ecological niche is structured, then the niche can offer a spatial escape to refugees that can hide from phages (Bohannan and Lenski 2000). Since the planktonic lifestyle cannot offer this kind of structured ecosystem per se, one can speculate that the bacteria may obtain a relatively phage-free environments with the help of the BMVs. One hypothetical scenario envisions the formation of spatial environments created by the bacteria and their BMVs due to the differences in their diffusion coefficient (D), which expresses how fast the particles of one species (atoms, ions, molecules and so on) can diffuse through another environment (higher values of D define faster diffusion). Based on experiments with artificial membrane vesicles, the D of the BMVs of 50 nm in diameter can be approximated to fall in the range of 10^{-8} cm^2 s^{-1} and it can be spatially restricted by the ionic strength and the pH of the environment, as well as by the size and composition of the BMVs (Kyoung and Sheets 2008). Yet, the range of D for a bacterial population (*E. coli*) in liquid medium is between 10^{-6} and 10^{-5} cm^2 s^{-1} suggesting that bacteria may diffuse faster than the BMVs (Licata *et al.* 2016). One fascinating finding is that the bacteria can regulate their D coefficient with the help of motility (Licata *et al.* 2016). In their particular study, Licata *et al.* (2016) showed that *E. coli* can decrease D via tumbling to such a degree that 'hypertumbly' cells are theoretically calculated to have $D = 0$. Based on these two approximations, one can envision that bacteria can regulate their D coefficient so they can diffuse faster, slower or together with the BMVs, which can bind and clear away the bacteriophages before the phages have the chance to encounter the cells. In addition, BMV release helps bacteria, avoid or temporarily delay, certain evolutionary trade-offs (at least until these trade-offs become a necessity) that can provide them with genetic resistance to phages but can also make them less competitive over phage-sensitive bacteria, for example via mutations in the *lpcA* and *lpcB* genes, whose products are important for LPS core formation and provide resistance against T4 phages but can also reduce the fitness of the resistant bacteria (Bohannan and Lenski 2000).

UV radiation, which is likely among the most ancient of dangers, existed before the Great Oxygenation Event (Bekker *et al.* 2004). Bacteria have developed different defense mechanisms to endure such radiation. Studies on a toxin-producing cyanobacterium, *Cylindrospermopsis raciborskii*, have demonstrated that this bacterium responds to UV treatment by increased vesiculation (Zarantonello *et al.* 2018). A triggering signal for the bacteria's BMV release may be the exposure of phosphatidylserine (a very atypical bacterial phospholipid) on the OM of its cells (Zarantonello *et al.* 2018). Increased vesiculation due to UV irradiation has also been observed in other aquatic bacteria (Gamalier *et al.* 2017). We should point out that together with the increased vesiculation triggered by UV irradiation, Zarantonello *et al.* (2018) and Gamalier *et al.* (2017) have also observed a decrease in cell viability, which suggests that this type of vesiculation is due to the vesicle release accompanied by cell death. Whether the increased vesicle formation under UV stress (which is accompanied with cell death) is at the end protecting the population remains to be shown. In this line, we have previously shown that the occasional human marine pathogen *V. cholerae* increases BMV release upon UV exposure, and that the released BMVs do indeed protect the bacteria from UV irradiation (Song and Wai 2009). The molecular mechanism behind the defense mechanism of *V. cholerae* is σ^E mediated, which signals activation of the expression of the sRNA VrrA, which in turn downregulates the OmpA transcript (Song and Wai 2009). Thus, due to decreased OmpA protein in the OM, the process leads to increased vesicle production (Song and Wai 2009).

Host-provided ecosystems

Plant hosts

Compared with what appears to have been the first report on BMVs released by *E. coli* in 1966, the first analysis of BMV release by phytopathogenic bacteria (*Xanthomonas campestris*) was published only in 2008, and thus the exploration of the role of BMVs in the development of pathogenic or symbiotic relationships with plants has begun more recently (Knox, Vesk and Work 1966; Sidhu *et al.* 2008). Although the human-associated bacteria have received the most attention, recent devastating outbreaks caused by certain bacterial phytopathogens in different agroecosystems have revealed the need for more in-depth studies on plant–bacteria interactions. The importance of a better understanding of the ecology of bacteria–plant associations has re-emerged as an urgent topic in the prevention of such outbreaks from happening again (Saponari *et al.* 2013; Sicard *et al.* 2018). In addition, understanding the symbiotic associations between plants and N_2-fixing bacteria can further improve the primary production in various agroecosystems. In the following section, we summarize recent studies that have been conducted on the role of BMVs released by plant-associated bacteria.

Members of genus *Xanthomonas* are chemoorganotrophic, motile, aerobic gammaproteobacteria that cause diseases in 124

monocots and 268 dicots (Saddler and Bradbury 2005a; Jacques *et al.* 2016). Among the main characteristics to contribute to the pathogenicity of these bacteria is their ability to secrete enzymes that degrade cellulose, hemicellulose, pectin and proteins: the various components of the plant cell wall (Saddler and Bradbury 2005a; Jacques *et al.* 2016). A comparison between the BMVs produced by *Xanthomonas campestris pv. campestris* and those produced by *pv. vesicatoria* reveals several interesting features. In both pathovars, the BMVs are part of the secretome of these bacteria, in which different cellulolytic enzymes are sorted and concentrated in such a way that, when secreted, the BMVs can cause sequential degradation to the plant cell wall from a distance before the bacteria come in close proximity to the plant cells (Sidhu *et al.* 2008; Sole *et al.* 2015). The presence of different cellulolytic enzymes in the BMVs, produced by *X. campestris pv. vesicatoria*, has been demonstrated via immunogold EM, but enzymatic activity has yet to be shown *in vitro* (Sole *et al.* 2015). The BMVs produced by *X. campestris pv. campestris* contain cellulase (Egl), xylan 1,4-beta-xylosidase (XynB) and beta-glucosidase (Bgl) (Sidhu *et al.* 2008). Sole *et al.* (2015) also demonstrated in *X. campestris pv. vesicatoria* that a protease and a lipase, in addition to the xylanases, could be exported either via the type 2 secretion system (T2SS) or via the BMVs (Fig. 5A and C). These studies suggest that BMVs can serve as an alternative secretion mechanism to the T2SS of *X. campestris*. The fact that Sidhu *et al.* (2008) also detected substrates of the type 3 secretion system (T3SS), such as AvrBsI, suggests that the BMV release can deliver a combination of T2SS and T3SS substrates that may cause greater damage to the plant cell in a contact-independent manner; see Fig. 5C (Sidhu *et al.* 2008; Sole *et al.* 2015). Finally, infection experiments conducted on the EPS-deficient *X. campestris pv. vesicatoria* bacteria have demonstrated that BMVs are also released *in planta* and have highlighted their presence *in vivo* (Sole *et al.* 2015).

Xylella fastidiosa is closely related to *Xanthomonas*. The current era is the second time this phytopathogen has been in the spotlight in the history of microbiology. For >80 years before the pathogen was recognized as an important and globally emerging bacterial agent that causes necrosis of the vascular tissue in broad-leaved trees and vines due to water stress, it was thought to be a virus until Davis, Purcell and Thomson (1978) successfully showed that the pathogen is a bacterium (Gardner and Hewitt 1974; Davis, Purcell and Thomson 1978; Hopkins and Purcell 2002; Chatterjee, Almeida and Lindow 2008a; Purcell 2013; Saponari *et al.* 2013; Martelli *et al.* 2016). *Xylella fastidiosa* can also be isolated as commensal from different plant species such as weeds (*Taraxacum officinale*, *Vernonia* sp. and *Digitaria* sp., among other plant species) or bushes (e.g. *Rubus* sp.), which can serve as natural reservoirs (Saddler and Bradbury 2005b). From there, they can be transmitted into susceptible hosts where the bacteria are unwelcome 'guests' (Saddler and Bradbury 2005b). This species circumscribes xylem-inhabiting, non-flagellated gammaproteobacteria (fam. *Xanthomonadaceae*) with *de rigueur* vector transmission by sap-feeding hemipters of the *Cicadellidae* family (Chatterjee, Almeida and Lindow 2008a; Sicard *et al.* 2018). Interestingly, in a competition with other phytopathogens, only *X. fastidiosa* bacteria managed to adhere to insect wings that mimicked the foregut cuticle, thus highlighting how closely associated this bacterium has evolved to its vector (Killiny and Almeida 2009).

The biology of *X. fastidiosa* allows the bacteria to exist in two different habitats: in the xylem of its host and in the foregut of its vectors (Chatterjee, Almeida and Lindow 2008a; Sicard *et al.* 2018). In both habitats, the bacteria form structurally different biofilms on the dead tissue of the host and the vector; *in planta*, they co-aggregate and adhere to the xylem wall and secrete EPS, in which the bacteria remain embedded either tightly or loosely associated with each other (Purcell, Finlay and McLean 1979; Newman *et al.* 2004; Chatterjee, Wistrom and Lindow 2008b; Sun *et al.* 2013). In its vector, the bacteria colonize the oesophagus and the cibarium of the foregut in a polar manner, forming a mat-like biofilm (Purcell, Finlay and McLean 1979; Newman *et al.* 2004; Chatterjee, Wistrom and Lindow 2008b; Sun *et al.* 2013). Both biofilms must be strong enough to allow the bacteria to remain in the particular environment; at the same time, they must not be so strong that the bacteria can be disseminated or transmitted, which happens when a subpopulation of the bacteria in the biofilm enters its 'exploratory phase', as shown in Fig. 6 (Chatterjee, Almeida and Lindow 2008a).

This sophisticated lifestyle is tightly regulated by the RpfF–RpfC system, which is involved in quorum sensing via α,β-unsaturated fatty acid diffusible signal factor (DSF), in such a way that *rpfF* codes for an enoyl-CoA hydratase, and *rpfC* codes for a two-component hybrid system (Newman *et al.* 2004; Chatterjee, Wistrom and Lindow 2008b). The Δ*rpfF* mutants have been shown to be more virulent, but they fail to colonize and form biofilms in their vector, which determines their meagre transmission rate (Newman *et al.* 2004). But, Δ*rpfC* mutants overexpress *rpfF*, leading to DSF overproduction and the formation of robust biofilm due to hyper-attachment, which overall results in reduced virulence and spreading in plants, as well as reduced vector transmission (Chatterjee, Wistrom and Lindow 2008b).

Xylella fastidiosa's biology also provides its bacteria with the adaptive traits required for a host jump (i.e. infecting new plant species) and for persistence, both of which lead to recurrent infection (Hopkins and Purcell 2002; Chatterjee, Almeida and Lindow 2008a; Sicard *et al.* 2018). Genetic and experimental data have shown that *X. fastidiosa* does not code for T3SS, as shown in Fig. 5 (since its lifestyle does not involve direct contact with living host or vector cells), or its effector substrates, but *X. fastidiosa* secretes the extracellular enzymes through T2SS (Fig. 5), produces the typical EPS xanthan (with slight modifications) and can perform twitching motility with the help of type IV pili (Simpson *et al.* 2000; Chatterjee, Almeida and Lindow 2008a; Sicard *et al.* 2018). The adhesion and colonization of the host and the vector are achieved via the differential expression and localization of fimbrial and afimbrial adhesins (Simpson *et al.* 2000; Chatterjee, Almeida and Lindow 2008a; Sicard *et al.* 2018).

Xylella fastidiosa may regulate the different states of its lifestyles via BMV release (Fig. 6). Besides demonstrating the delivery of virulence factors (such as lipases/esterases, proteases and pectinases among others), proteomic and metabolomic experiments have also demonstrated that the BMVs produced by *X. fastidiosa* are rich in DSF, which suggests their involvement in the intercellular communication of these bacteria (Nascimento *et al.* 2016; Feitosa-Junior *et al.* 2019). Reports on different afimbrial adhesins, such as the hemagglutinins (HAs) HxfA and HxfB, the AT-1 autotransporters XadA1 and XatA, and the Omp PD1063, have also shown that the adhesins can be exported within BMVs and, together with the type 1 fimbriae, these adhesins contribute to the different stages of biofilm formation and cell-to-cell aggregation (Guilhabert and Kirkpatrick 2005; Feil, Feil and Lindow 2007; Voegel *et al.* 2010; Matsumoto *et al.* 2012; Ionescu *et al.* 2014; Pierce, Voegel and Kirkpatrick 2014). Based on mutational and phenotypic experiments, afimbrial PD1063 has been found to predominantly facilitate cell-to-cell contact, HxfA and HxfB to initiate host colonization and XatA1 to participate in both

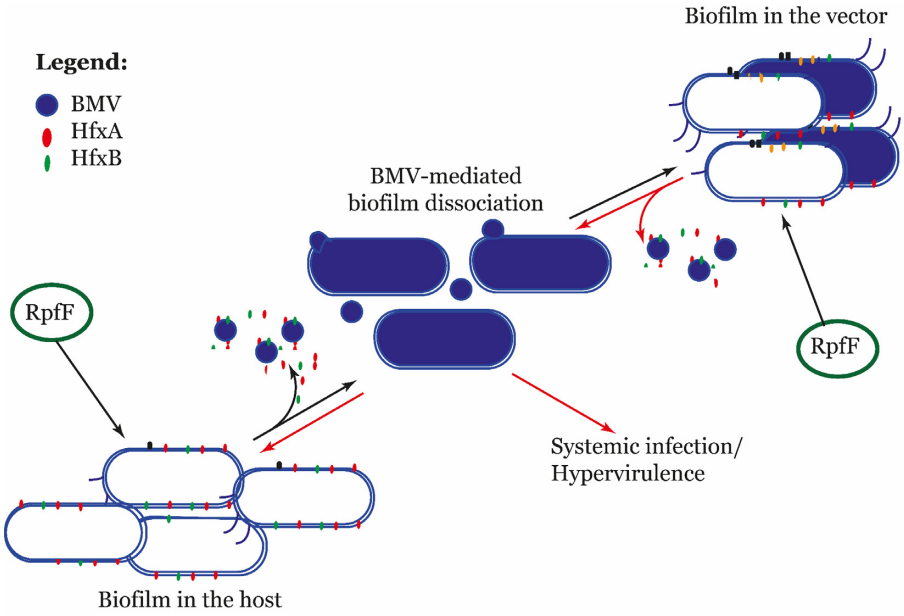

Figure 6. The life cycle of *Xylella fastidiosa*. Under the regulation of RpfF, the bacterium forms a biofilm in plants and in the bacterium's vector. Both transmissions (from the vector into the plant and from the plant into the vector) require a loosening of the biofilm, which is mediated via BMVs, by serving as either an anti-adhesion factor or a secretion system of the afimbrial adhesins HfxA and HfxB. The black arrows represent the transmission to its vector, while the red arrows represent the processes that take place upon infection.

intercellular contact and initial adhesion (Feil, Feil and Lindow 2007; Matsumoto *et al.* 2012).

Studies on the BMV released by the Pierce's disease-causing *X. fastidiosa* strain Temecula 1 have demonstrated that the final destination of HxfA and HxfB depends on the way they are secreted: they can be located on the bacterial OM, secreted independently in the extracellular milieu or released within BMVs; see Fig. 6 (Voegel *et al.* 2010). By random mutagenesis and infection experiments with Chardonnay grapevines, Guilhabert and Kirkpatrick (2005) demonstrated that Δ*hfxA* and Δ*hfxB* deletion mutants exhibit hypervirulent phenotypes. They proposed that HfxA and HfxB can serve as anti-virulence factors involved in the intercellular aggregation and attenuation of bacterial colonization (Guilhabert and Kirkpatrick 2005). If we combine their findings with those from the work of Voegel *et al.* (2010), we can propose that the BMV release of HAs can serve as a regulatory mechanism for the virulence of *X. fastidiosa*. When required, HfxA and HfxB are present on the cell envelope, but when the bacteria need to decrease their adhesiveness to be taken in by the vector, they secrete the HAs packed in BMVs, as shown in Fig. 6 (Voegel *et al.* 2010).

Killiny and Almeida's (2009) experiments, however, demonstrated that HfxA and HfxB are upregulated by RpfF and that their importance for the early attachment and biofilm formation of *X. fastidiosa* bacteria in the vector also reflects their later transmission to plants. The transmission rates were found to be lower when the bacterial strains were inoculated separately in the vectors, most probably due to the reduced ability to remain in the vector: the bacteria showed a 36% transmission rate for the Δ*hfxA* bacteria, 46% for the Δ*hfxB* bacteria and 88% for the wild-type cells (Killiny and Almeida 2009). The presence of XatA further shows evidence of the involvement of the *X. fastidosa*'s BMVs in biofilm formation (Matsumoto *et al.* 2012). XatA is an AT-1 autotransporter, previously shown to be involved in autoaggregation and biofilm formation; the symptoms caused by bacteria deficient in XatA are severely attenuated (Matsumoto *et al.* 2012). The fact that Matsumoto *et al.* detected XatA together with its passenger domain in the BMVs provides another layer of complexity: a secretion system within a secretion system that can be activated under the right conditions; see Fig. 5B.

In addition to the sessile and motile bacteria, a portion of *X. fastidiosa* cells (0.005% of the bacterial population) may endure extreme environmental hardships, such as the presence of copper and tetracycline, via entering growth arrest, associated with reduced metabolic activity: *X. fastidiosa* can form persisters (Muranaka *et al.* 2012). So far, studies have shown that MqsR is the main effector to not only positively regulate the biofilm formation but also increase the formation of persisters upon Cu stress (Lee *et al.* 2014; Merfa *et al.* 2016). Interestingly, *mqsR* expression also occurs under the regulation of the RpfF–RpfC system, since its gene expression is increased in the Δ*rpfF* mutant background (Wang, Li and Lindow 2012). Santiago *et al.* (2016) have provided evidence that the MqsR anti-toxin (YgiT) is secreted via BMVs. Although the mechanism of cytosolic YgiT anti-toxin secretion has yet to be discovered, one can envision that these bacteria might secrete the anti-toxin to shift the balance toward increased toxin activity, which eventually upregulates biofilm formation or activates the persister state (Santiago *et al.* 2016).

Xylella fastidiosa's BMVs offer an interesting case for consideration—can they be regarded as bona fide virulence-associated factors, or are they present due to general mechanisms such as envelope stress, which has been shown to lead to increased vesiculation? Studies by Ionescu *et al.* (2014) offer interesting insights into the answer. The researchers have shown that *X. fastidiosa* uses the BMVs to control adhesion

to different surfaces, thus contributing to the transition from biofilm to the 'exploratory', planktonic phase (Fig. 6). *In vivo* and *in vitro* experiments demonstrated that the BMVs' pre-treatment of insect wings led to a 3-fold decrease in bacterial attachment, and the presence of BMVs led to a 20-fold reduction in the bacterial adhesion to xylem 20-fold. Ionescu *et al.*'s study was one of the few to provide a direct link between BMV production and regulation by a dedicated virulence-regulatory system: the *X. fastidiosa* Δ*rpfF* hypervirulent mutant produced three times the number of BMVs, which, together with the other findings, suggests that these BMVs might have evolved into a mechanism to block bacterial adhesion.

Rhizobium etli is a soil-based, N_2-fixing alphaproteobacterium that enters symbiotic associations predominantly with the legumes *Phaseolus vulgaris* and *Mimosa affinis* (Poole, Ramachandran and Terpolilli 2018). Two recent studies on the BMVs produced by the plant endosymbiont *R. etli* demonstrated that these bacteria produce different BMV populations in a growth-phase-dependent manner and upon stimulation with naringenin (Taboada *et al.* 2019a,b). Taboada *et al.* (2019b) found that vesicles isolated from non-naringenin induced bacteria, mimicking the life of the bacteria in soil, contained different classes of proteins. The presence of the Rap and curlin autoaggregation/adherence proteins, and the PlyB-orthologous, PlyC and polysaccharidases in the vesicular content suggested involvement in biofilm formation and the presence of different ABC substrate binding transporters for substrate scavenging: a strategy used by oligotrophs. The BMVs were also shown to contain iron(III), hemin and hemichrome acquisition proteins, as well as Cu oxidase and KatG (catalase), which provide a cross-resistant adaptive mechanism linked to iron supply and defense against Cu- and oxidative stress (Taboada *et al.* 2019a,b). The authors also directly demonstrated that the rhizobial BMVs inhibited the growth of another bacterial soil competitor, *B. subtilis* (Taboada *et al.* 2019b). In contrast, the BMVs isolated from bacteria treated with naringenin contained increased levels of proteins that were found to participate in carbohydrate and nitrogen utilization (Taboada *et al.* 2019a). Ultimately, the authors showed that the BMVs did indeed carry the Nod factors (chitooligosaccharides, which serve as a signal for nodulation), and they demonstrated that the BMVs from naringenin-treated bacteria could induce root-hair curling, which is a physiological response for establishing a symbiotic relationship (Taboada *et al.* 2019a).

Animal hosts

The majority of previous research on BMVs has focused on their mechanism and release by certain human pathogens and their interactions with hosts. Bacterial BMVs secreted by pathogenic bacteria can deliver a broad range of virulence factors to the host cells, including small molecules, endotoxins, exotoxins, lipids, DNA, RNA and microRNAs. BMVs thus enable both intracellular and extracellular bacteria to communicate with the host cell and to deliver their virulence factors to the intracellular compartments important for the modulation of various signaling pathways.

We have earlier demonstrated the role of the BMV-mediated transport of *E. coli* pore-forming toxin, cytolysin A (ClyA) (Wai *et al.* 2003). Based on our findings, we proposed that the BMV release can serve as a secretion mechanism for proteins that do not have a dedicated signal sequence for one of the secretion machineries. Furthermore, the BMV-associated ClyA has found to have considerably higher cytotoxicity toward mammalian cells compared with purified toxin due to the favorable redox environment offered by the vesicles (Wai *et al.* 2003). Several other studies have shown the importance of BMVs in the delivery of other virulence factors during host infection, including the

delivery of type I-secreted α-hemolysin (Balsalobre *et al.* 2006) and cytotoxic necrotizing factor-1 (CNF1) (Kouokam *et al.* 2006). Similarly, Bielaszewska *et al.* (2013) have shown that Enterohemorrhagic *E. coli* (EHEC) delivers pore-forming toxins via BMVs that induce apoptosis in the host cells. In addition to delivering pore-forming toxin hemolysin, EHEC BMVs act as a potent delivery system for the transport of several other virulence factors to different cellular compartments of host cells. These factors include Shiga toxin 2a (Stx2a), flagellin and cytolethal distending toxin V (CdtV) (Bielaszewska *et al.* 2017).

Endotoxin (lipid A) is reportedly responsible for immune modulation during pathogenic invasions. Vanaja *et al.* (2016) revealed that BMVs produced by Gram-negative bacteria may act as vehicles for the delivery of LPS into the host cytosol and therefore lead to atypical inflammasome activation *in vitro* and *in vivo*. Several toxins bound to BMVs have been reported among *Vibrio* species, including the cholera toxin and other secreted toxins and enzymes that are important to *V. cholerae* pathogenesis, such as the metalloprotease of *Vibrio* (PrtV) (Vaitkevicius *et al.* 2006; Rompikuntal *et al.* 2015), Zn-dependent HA protease (HAP) (Hase and Finkelstein 1991; Ghosh *et al.* 2006), *Vibrio* cytolysin (VCC) (Olivier *et al.* 2007), trypsin-like serine protease (VesC) (Syngkon *et al.* 2010) and accessory cholera enterotoxin (Ace) (Kaper, Morris and Levine 1995).

BMVs produced by *Helicobacter pylori* play an important role in the bacteria's pathogenesis by inducing local and systemic host inflammatory and immune responses. BMVs isolated from *H. pylori* are enriched in several lipid species including PE, lyso-PE, PGl, CL, phosphatidylcholine (PC) and lyso-PC (Olofsson *et al.* 2010). LPS anchored by lipid A in the OM is another important virulence component of *H. pylori* BMVs. In addition to lipid species, several proteins essential to effective *H. pylori* virulence have been found to be associated with BMVs, including blood-group antigen-binding adhesin (BabA), outer inflammatory protein A (OipA), vacuolating cytotoxin (VacA), CagA, *H. pylori* neutrophil-activating protein (HP-NAP), adherence-associated lipoprotein (AlpA), urease and sialic acid-binding adhesin (SabA) (Olofsson *et al.* 2010; Lekmeechai *et al.* 2018). The free-soluble toxin VacA secreted by *H. pylori* is highly toxic to the epithelial cells, as it induces vacuolization in the host cells; BMV-bound VacA, however, mostly remains inactive (Ricci *et al.* 2005). The shedding of *H. pylori* OM has been detected by EM not only during the late stationary growth phase but also in gastric biopsies of infected individuals (Keenan *et al.* 2000; Yonezawa *et al.* 2011; Grande *et al.* 2015). BMVs are generally considered to be an effective way for extracellular bacterial pathogens to translocate the bacterial effector molecules through the mucosa to immune cells in tissues or even to the blood-circulation system.

Ecospeciation

At the end of this section on the role of BMVs in bacterial ecology, we would like to speculate how the development of a new vesiculation pattern, defined by the size, amount and content of the released BMVs, can contribute to certain ecospeciation, i.e. adaptive divergence of bacterial populations inhabiting different ecological environments (Begon, Townsend and Harper 2005; Nosil 2012; Lassalle, Muller and Nesme 2015). For ecological speciation to come into play, a bacterial subpopulation has to respond to a new environmental factor with a change in the pattern of their BMVs—a change that is considered adaptive. Consequently, this adaptive change should have a genetic basis that allows divergence between the given subpopulation and the rest of the individuals of the species. But if a pattern for bacterial vesiculation can develop, and be selected for due to external environmental stimuli, could it become genetically maintained? Even though the question regarding genetic control mechanism(s) of the degree of vesiculation has yet to be settled, we may consider findings pointing toward the environments where a hypothetical vesiculation pattern could be selected for and upon which genes natural selection could presumably act.

A body of evidence illustrates that different bacterial species can respond to challenging environments or the presence of nutritional substrate(s) with increased vesiculation (Dutson *et al.* 1971; Marvin, ter Beest and Witholt 1989; McBroom and Kuehn 2007; Manning and Kuehn 2011; Baumgarten *et al.* 2012; Maredia *et al.* 2012; Bager *et al.* 2013; Macdonald and Kuehn 2013; Yamaoka *et al.* 2014; Orench-Rivera and Kuehn 2016; Eberlein *et al.* 2018; Gerritzen *et al.* 2018). For example, *Mycobacterium tuberculosis*, *H. pylori*, *Haemophilus influenzae*, *V. cholerae* and *E. coli* increase their formation of BMVs upon iron limitation as summarized in Orench-Rivera and Kuehn (2016). Besides iron limitation, stresses due to oxygen radicals, temperature and osmotic changes, detergents and antibiotics have also been shown to enhance BMV production (McBroom and Kuehn 2007; Song *et al.* 2008; Manning and Kuehn 2011; Baumgarten *et al.* 2012; Maredia *et al.* 2012; Macdonald and Kuehn 2013; Orench-Rivera and Kuehn 2016; Gerritzen *et al.* 2018). In addition to the changes in different conditions, the presence of certain nutrients can also trigger BMV release as is the case with *Pseudomonas fragi*, the proteases of which are exported via BMVs when its bacteria are incubated on muscle tissue, or with *Meiothermus ruber* whose bacteria increase their vesiculation in the presence of feather keratin (Dutson *et al.* 1971; Tarrant *et al.* 1973; Yamaoka *et al.* 2014).

Furthermore, an increase in the BMV production, due to environmental stimuli, should also contribute to the bacterial fitness, i.e. the BMVs would be an adaptive trait. Such contributions may be part of a compensatory mechanism for the loss of envelope stress regulation, as exemplified with the BMV production by hypervesiculating *E. coli* strains, for example with a deletion in the *yieM* gene, when incubated in the presence of ethanol, polymixin B or colistin (McBroom and Kuehn 2007; Manning and Kuehn 2011). The BMV-mediated adaptive traits may also be linked to the increase in hydrophobicity resulting in biofilm formation by *Pseudomonas putida* when subjected to osmotic stress and heat shock (Baumgarten *et al.* 2012).

While the aforementioned examples introduce the putative selective conditions for vesiculation, other evidence resulting from genetic screens hints to the potential genetic pool of candidate genes for such natural selection (McBroom and Kuehn 2007; Song *et al.* 2008; Macdonald and Kuehn 2013; Kulp *et al.* 2015). A genome-wide screen revealed that mutations that result in defects in the enterobacterial common antigen and in changes of the LPS chain are leading to increased BMV release by *E. coli* (Kulp *et al.* 2015). Other evidence from genetic studies with *E. coli*, *Salmonella*, *P. aeruginosa* and *V. cholerae* suggests that mutations in genes that code for the Tol-Pal and OmpA proteins can change the degree of vesiculation and that is also an outcome of the overexpression of σ^E, the alternative sigma factor for envelope stress response (McBroom and Kuehn 2007; Song *et al.* 2008; Macdonald and Kuehn 2013; Kulp *et al.* 2015).

Some of the genes in the pool subjected to adaptive mutagenesis may also be linked to certain trade-offs, as exemplified from earlier studies on cryoprotection (Bennett, Seaver and Calcott 1981; Calcott 1986). Experiments with *S.* Typhimurium strains that carry different defects in their LPS structure, such as Δ*rfaG* and Δ*rfaF*, demonstrated that these strains are more sensitive

to low temperatures, detergents and antibiotics (Bennett, Seaver and Calcott 1981; Calcott 1986). Even though their corresponding *E. coli* deletion mutants are shown to be hypervesiculating (Kulp *et al.* 2015), it is unlikely for such kind of bacterial mutants to be selected as prolific vesicle producers.

On the other hand, some genes may undergo selection that can improve bacterial fitness at low temperature correlating with enhanced vesicle production (Calcott 1986; McBroom and Kuehn 2007). In the context of cryopreservation, Calcott (1986) showed how viability of different *E. coli* mutants deficient in outer membrane proteins was altered when bacteria were subjected to freeze-thawing. One of the mutants lacking the EnvZ-OmpR two-component system exhibited 1.4-fold increased viability compared with the wild type (Calcott 1986). Interestingly, deficiency in the *ompR* gene has been shown by McBroom and Kuehn (2007) to also trigger hypervesiculation and to improve bacterial fitness. From the findings of these two different studies, we suggest that the *ompR* gene would be a right candidate in the search for natural selection that can trigger ecospeciation under osmotic and low-temperature stress. Furthermore, we note that the *ompR* gene in fact has been shown to be a subject of adaptive mutagenesis as demonstrated by *E. coli* mutants deficient in the EnvZ–OmpR system that have already been isolated from the mouse gut after the bacteria successfully had become adapted to the intestinal environment (Giraud *et al.* 2008).

Another interesting aspect concerns the BMV release on supraspecies level. Again, when exactly the pattern of BMVs released by bacterial species within a genus (for example, compare Fig. 3B to C), and the regulation of this BMV release can be considered a new adaptive capability is still a matter of discussion. Let us assume for now that if an innovation in the pattern of vesiculation appears, the species can adapt to particular environmental conditions such as low or high temperatures, thus leading to ecospeciation.

One notable example is the gammaproteobacterial genus *Vibrio*, the representatives of which (some pathogenic) inhabit marine and estuarine ecosystems and can be associated with the surface or intestinal content of marine animals. All the vibrios examined to date produce BMVs, including *V. anguillarum*, *V. cholerae*, *V. tasmaniensis*, *V. shilonii*, *V. vulnificus* and *Aliivibrio fischeri* (Kondo, Takade and Amako 1993; Song *et al.* 2008; Hong *et al.* 2009; Vanhove *et al.* 2015; Aschtgen *et al.* 2016; Li *et al.* 2016; Hampton *et al.* 2017) as some examples are shown in Fig. 3B and C. The logical question that follows is whether the natural vesiculation typical of vibrios can contribute to the adaptation of a *Vibrio* species to the different conditions under which BMV production appears to be adaptive.

Adaptation to low temperatures is one such condition, and Yumoto *et al.* (1999) isolated one such *Vibrio* species (*V. rumoiensis*) from an H_2O_2-treated drainage pool. *Vibrio rumoiensis* is a facultatively psychrophilic bacterium with a growth range between 2 and 34°C; for an illustration, compare *V. rumoiensis* with *V. cholerae*, shown in Fig. 2; and Fig 3B and C. Even before the more intensive research on *Vibrio*'s BMVs started, Yumoto *et al.* (1999) had noticed that *V. rumoiensis* cells produce many blebs, i.e. BMVs (Fig. 3B). Besides being psychrophilic, *V. rumoiensis* also has a high tolerance to oxidative stress due to the VktA catalase activity of its bacteria (Yumoto *et al.* 2000; Ichise *et al.* 2008). Since there is no N-terminal secretion signal revealing a likely secretion route, the development of the new BMV pattern may be considered as a potential mechanism for the extracellular delivery that contributes to the peroxide resistance (Yumoto *et al.* 2000; Ichise *et al.* 2008).

We can ask then whether the cold temperature *de facto* triggers the bacteria's vesicle production (as is evident from the explored BMV-producing cold-loving γ-proteobacteria shown in Fig. 2) that later becomes a genetic trait. If that is the case, then we may consider that there must be a facultative psychrophile, which is proficient in vesiculation within a mesophilic genus of a species that is not. A new species was described in a study about isolates from the Norwegian tundra soil and denoted as the 'sea ice' bacterium *Pedobacter arcticus* (Zhou *et al.* 2012). *Pedobacter arcticus* is the only species from the soil inhabitants of *Pedobacter* (*Sphingobacteriaceae*, **Bacteroidetes**; compare *P. arcticus* to *P. agri* in Fig. 2) the bacteria of which were reported to both be psychrophilic and exhibit budding (Zhou *et al.* 2012).

CONCLUSIONS AND FUTURE DIRECTIONS

The guiding theme of this review has been the involvement of bacterial membrane vesicles in cyclic processes, collectively called eco-evolutionary (or 'eco-evo') dynamics, in which an environmental change induces an evolutionary change, and vice versa. While most of the studies from the literature examined for this review have considered the BMVs' role in ecosystem engineering, their role in influencing evolutionary feedbacks—for example as feedbacks that result in speciation—has yet to be discovered.

To conclude this review, we would like to propose possible approaches and examples for future study. The time might be right to consider that the more we understand BMVs, the more feasible they can become as real, naturally occurring models in combination with artificial models for studies of how vesicles might play a role in the origin of life. The use of the tools of physics for direct manipulation, as well as the use of force spectroscopy techniques such as optical tweezers, may be beneficial for the field of BMVs, because such tools can provide answers to questions that are linked to not only vesicle dynamics and related shape transformations but also which forces may trigger these processes and which changes in a particular habitat can lead to their induction. In general, a comparison of the pattern of vesiculation of extremophiles with that of their closely related non-extremophiles may help to illustrate the adaptive potential provided by vesicles, because we may assume that the results of extremophile homeostasis could likely be translated to a non-extremophile lifestyle. Another potentially intriguing avenue of research would be to examine the role of the vesiculation of the alkaliphilic bacteria (optimum pH 10–11.5) because of the fact that their membranes may function well at high pH, but around neutral pH, they may become too fluid.

As is often the case with scientific quests, searches lead to new searches, and research on bacterial membrane vesicles in particular can offer an abundance of research avenues.

ACKNOWLEDGMENTS

We would like to thank Professors Glenn Björk and Søren Molin for their critical reading of this review. We are also grateful to Ivan Tsonev for his feedback on the review's physics-related sections. We wish to thank also the anonymous reviewers for their thoughtful comments and constructive suggestions.

The authors would like to acknowledge all the scientists whose work has contributed to this review and to apologize to those colleagues whose work has advanced the field of bacterial membrane vesicles but has not been cited in this review.

FUNDING

Research in the authors' laboratories is supported by grants from the Swedish Research Council (2015-03007, 2015-06824, 2016-06598, 2018-02914, 2019-01720 and 349-2007-8673), the Kempe Foundations (JCK-1724 and JCK-1728), the Swedish Cancer Society (2017-419) and the Faculty of Medicine at Umeå University (Strategic Research Grant 2019-2021).

Conflict of interest. The authors declare no conflict of interest.

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