Chapter 5
Functions of MVs in Inter-Bacterial Communication

Masanori Toyofuku, Yosuke Tashiro, Nobuhiko Nomura, and Leo Eberl

Abstract Bacterial communication depends on small molecules that are released into the environment and are perceived by other cells. Many of the bacterial communication molecules are hydrophobic and thus have a poor solubility in water. While it is well established that such molecules serve as bona fide signal molecules, very little is known on how these molecules travel in aqueous environments. In this chapter we will summarize the evidence that hydrophobic signals can be released by bacterial membrane vesicles (MVs), which also serve as vehicles for signal dispersal. Given that the signals are concentrated in MVs, which can target specific cell types, a new binary signaling mechanism has been proposed that is different from the classic diffusion-based signaling model. This has important implications on how bacteria communicate in natural aqueous habitats.

5.1 Introduction

Bacteria, including Gram-positive and Gram-negative bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa*, can communicate by the aid of signal molecules with each other to synchronize gene expression within the population, a phenomenon which forms the basis of coordinated group behaviors and is commonly referred to as quorum sensing (QS) (Miller and Bassler 2001; Whiteley et al. 2017). Many of the signals employed by bacteria are very hydrophobic and their solubility in water is very limited, raising the question of how these molecules can

M. Toyofuku (✉) · N. Nomura
Faculty of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan
e-mail: toyofuku.masanori.gf@u.tsukuba.ac.jp; nomura.nobuhiko.ge@u.tsukuba.ac.jp

Y. Tashiro
Applied Chemistry and Biochemical Engineering Course, Department of Engineering, Graduate School of Integrated Science and Technology, Shizuoka University, Hamamatsu, Japan
e-mail: tashiro.yosuke@shizuoka.ac.jp

L. Eberl
Department of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland
e-mail: leberl@botinst.uzh.ch

M. Kaparakis-Liaskos, T. A. Kufer (eds.), *Bacterial Membrane Vesicles*,
https://doi.org/10.1007/978-3-030-36331-4_5

travel between cells (Decho et al. 2011). In a seminal paper, Marvin Whiteley's group (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2009) showed that the *Pseudomonas aeruginosa* quinolone signal (PQS) is not only associated with membrane vesicles (MVs) but also stimulates MV formation through PQS intercalation into the outer membrane. Subsequent studies have provided compelling evidence that many different hydrophobic signal molecules, including long-chain *N*-acyl homoserine lactone (AHL) signals, are associated with and transported via MVs (Brameyer et al. 2018; Li et al. 2016; Toyofuku et al. 2017b). Given that the signal molecules are highly concentrated in MVs, the fusion of a single MV with a bacterial cell is often sufficient to trigger its quorum sensing (QS) response (Toyofuku et al. 2017b). Such a binary signaling mechanism is fundamentally different from the classic diffusion-based QS model both in terms of its kinetics and their socioecological consequences.

In this chapter we will give an overview of different bacterial signals such as PQS, *N*-hexadecanoyl homoserine lactone (C16-HSL), (z)-3-aminoundec-2-en-4-one (Ea-C_8-CAI-1), which have been reported to be associated with MVs, together with diffusible signal factor (DSF; cis-11-methyl-2-dodecenoic acid) and 2-cis-tetradecenoic acid (*Xylella fastidiosa* DSF; X*f*DSF) that regulate MV production (Fig. 5.1).

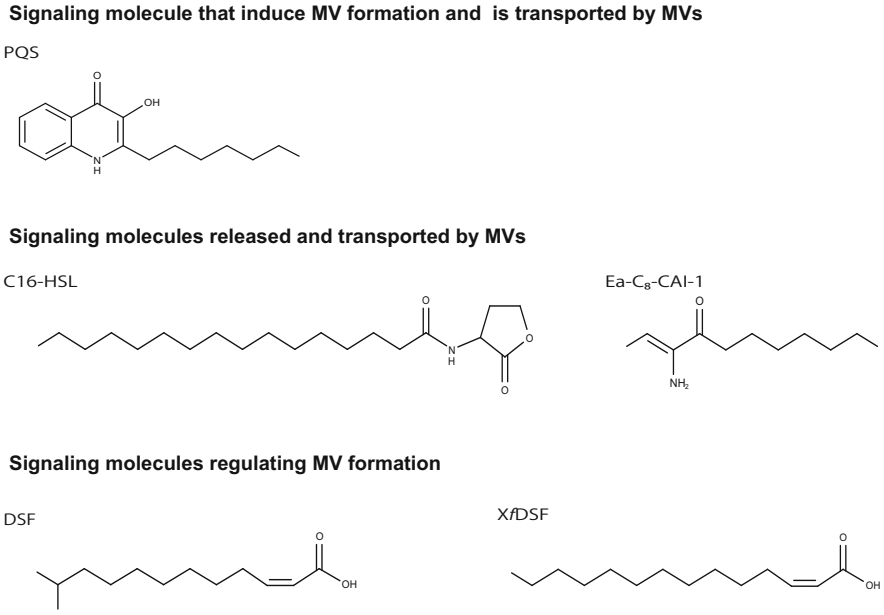

Fig. 5.1 Bacterial signal molecules associated with MVs. PQS can induce MV formation and is transported by MVs. Likewise, C16HSL and Ea-C8-CAI-1 are released and transported by MVs. DSF and X*f*DSF regulate MV formation by an unknown mechanism

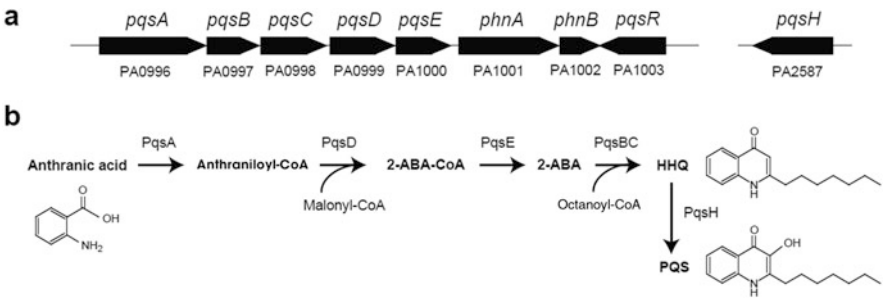

Fig. 5.2 The synthesis of *Pseudomonas* quinolone signal (PQS) in *P. aeruginosa*. (**a**) Genetic organization of the PQS gene clusters. (**b**) Proposed pathway for PQS biosynthesis from anthranilate. Biosynthesis of PQS requires PqsABCDE proteins and PqsH. 2-ABA-CoA, 2-aminobenzoylacetyl-CoA; 2-ABA, 2-aminobenzoylacetate; HHQ, 2-heptyl-4-hydroxyquinoline; PQS, 2-heptyl-3-hydroxy-4-quinolone

5.2 *Pseudomonas* Quinolone Signal

A wide variety of quinolone compounds are synthesized by animals, plants, and microorganisms, and those compounds are of medical interest because of their antiallergenic, anticancer, and antimicrobial activities (Heeb et al. 2011). *P. aeruginosa* produces at least fifty-five 2-alkyl-4(*1H*)-quinolones (AQs) and some of those compounds exhibit antimicrobial activities (Déziel et al. 2004). 2-heptyl-3-hydroxy-4-quinolone (known as PQS) is used as a signal in the quinolone-based QS system in *P. aeruginosa* (Pesci et al. 1999), in addition to the two main *N*-acyl homoserine lactone (AHL) signals *N*-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl homoserine lactone (C4-HSL). PQS and the two AHLs signals control expression of overlapping sets of hundreds of genes, many of which encode for virulence factors (Jimenez et al. 2012). PQS is synthesized from anthranilic acid by the products of the *pqsABCDE* operon and *pqsH* (Fig. 5.2) (Lin et al. 2018). The *pqsABCDE* operon is adjacent to the anthranilate synthase genes *phnAB* and the gene *pqsR,* which encodes the PQS receptor (Gallagher et al. 2002). The first step of PQS biosynthesis from anthranilate is conducted by PqsA, an anthranilate coenzyme A ligase (Coleman et al. 2008). Then, PqsD, which belongs to the FabH (β-ketoacyl-(acyl carrier protein) synthase III) protein family, synthesizes 2-aminobenzoylacetyl-CoA (2-ABA-CoA) from anthraniloyl-coenzyme A and malonyl-coenzyme A (Dulcey et al. 2013; Zhang et al. 2008). PqsE functions as a pathway-specific thioesterase, hydrolyzing 2-ABA-CoA to 2-aminobenzoylacetate (2-ABA) (Drees and Fetzner 2015). This reaction can be processed by the broad-specificity thioesterase TesB, explaining why the depletion of PqsE does not block the synthesis of 2-heptyl-4-hydroxyquinoline (HHQ) and PQS (Drees and Fetzner 2015). PqsBC are involved in adding an octanoyl moiety to 2-ABA to produce HHQ (Dulcey et al. 2013). Finally, the FAD-dependent mono-oxygenase PqsH converts HHQ into PQS (Déziel et al. 2004; Gallagher et al. 2002). Therefore, the deletion of *pqsH* abolishes PQS synthesis but the production of other AQs is continued.

5.2.1 Multifunctional PQS

PQS is the main signal among the AQs in *P. aeruginosa*. PQS binds to the LysR-type transcriptional regulator PqsR (also called MvfR) and its affinity to PqsR is higher than that of its precursor HHQ (Wade et al. 2005; Xiao et al. 2006). The PQS–PqsR complex regulates the expression of many genes, many of which encode functions related to motility, virulence, and biofilm formation (Déziel et al. 2005; Schuster et al. 2003). Interestingly, PQS-mediated transcriptional regulation can occur in a PqsR-dependent and PqsR-independent manner (Rampioni et al. 2010). In addition to PqsR, PQS directly interacts with other proteins, including MexG and RhlR as well as proteins involved in respiration (Baker et al. 2017; Dandela et al. 2018; Hodgkinson et al. 2016; Toyofuku et al. 2008).

It has been demonstrated that PQS can chelate ferric iron (Fe^{3+}) whereas HHQ, which is lacking the 3-hydroxy group of PQS, cannot (Bredenbruch et al. 2006; Diggle et al. 2007). PQS has a high affinity for iron and induces the expression of genes involved in the synthesis of the siderophores pyoverdine and pyochelin (Bredenbruch et al. 2006; Diggle et al. 2007). Iron-chelating activity of PQS also represses denitrification in *P. aeruginosa* as well as growth of some bacteria (Toyofuku et al. 2010; Toyofuku et al. 2008). In addition, PQS has been shown to exert immune modulatory and cytotoxic activities (Lin et al. 2018).

5.2.2 PQS Delivery Through MVs

AQs have generally a limited aqueous solubility. For example, the solubility of PQS is only 1 mg/L water (Lépine et al. 2003) and the octanol–water partition coefficient (logP) is 3.60 (Mashburn and Whiteley 2005). Despite its hydrophobic nature, PQS functions as a cell-to-cell communication signal in the aqueous solution. An elegant solution to this problem was offered by Mashburn and Whitley, who showed that about 80% of the total PQS is associated with MVs, in contrast to the homoserine lactone signals where only 1% of the signal was found to be within MVs (Mashburn and Whiteley 2005). The PQS carried by MVs was shown to be biologically active and can increase *pqs* gene expression, virulence factor production, and antimicrobial activities (Mashburn and Whiteley 2005; Tashiro et al. 2010b).

5.2.3 Vesiculation Is Stimulated by PQS

PQS is not only a constituent of MVs, but also stimulates MV formation in *P. aeruginosa* (Mashburn and Whiteley 2005). Consequently, MV production is decreased in mutants with inactivated *pqsA, pqsH*, or *pqsR* genes but can be restored by the exogenous addition of PQS (Mashburn and Whiteley 2005). The increase of

MV production in the *pqsR* mutant indicates that PQS-mediated MV formation is not due to PQS-dependent gene regulation. The structure of PQS is important for MV formation as HHQ is less effective (Mashburn-Warren et al. 2009). Interestingly, PQS can induce MV production also in many other bacterial strains (Horspool and Schertzer 2018; Mashburn-Warren et al. 2008; Tashiro et al. 2010a). More detailed biophysical experiments revealed that PQS stimulates outer membrane blebbing through intercalation into the outer membrane. When PQS is present at the extracellular milieu, it specifically interacts with the 4′-phosphate and acyl chains of lipid A of the lipopolysaccharide (LPS) (Mashburn-Warren et al. 2008). Salt bridges containing divalent cations (Mg^{2+} and Ca^{2+}) normally stabilize the negatively charged phosphates between adjacent LPS molecules (Kadurugamuwa and Beveridge 1996). PQS sequesters these cations and thereby increases the anionic repulsion between LPS molecules and creates membrane curvature (Mashburn-Warren and Whiteley 2006; Tashiro et al. 2012). Adding exogenous Mg^{2+} abolishes the effect of PQS on *Escherichia coli* MV formation, suggesting that excess cations can neutralize the repulsion of negatively charged LPS molecules (Tashiro et al. 2010a). Subsequent work resulted in a refined model, the so-called bilayer-couple model (Schertzer and Whiteley 2012), in which the specific interaction of PQS with LPS contributes to a low rate of flip-flops between the leaflets of the outer membrane. This leads to an expansion of the outer leaflet relative to the inner leaflet, which eventually gives rise to membrane curvature and MV formation. PQS has to be initially secreted from the cells via an unknown export mechanism to interact with the outer leaflet of the outer membrane (Lin et al. 2018) (Fig. 5.3). When strains PAO1 and PA14 were compared, PQS export and MV formation were significantly higher in the latter strain, even though equivalent amounts of total PQS were synthesized in both strains (Florez et al. 2017). While PQS accumulates in the outer membrane of PA14, in PAO1 most of the PQS accumulates in the inner membrane, presumably due to early saturation of the PQS export pathway (Florez et al. 2017). The differences in PQS localization among strains and its dependence on culture conditions may explain reports that could not confirm a role of PQS in MV formation (Macdonald and Kuehn 2013; Turnbull et al. 2016). Furthermore, PQS is not synthesized under anaerobic conditions as PqsH requires oxygen for PQS synthesis (Schertzer et al. 2010; Toyofuku et al. 2008). Molecules such as indole and its derivatives also repress MV formation in *P. aeruginosa* by inhibiting PQS biosynthesis (Tashiro et al. 2010c).

While PQS synthesis does not start before early stationary phase, MVs are already released during the exponential phase, suggesting that other routes for MV biogenesis exist (Tashiro et al. 2010b). The alternative sigma factor AlgU and the periplasmic protease MucD are related to the envelope stress pathway, and MV formation was shown to be increased when either of the genes were deleted (Tashiro et al. 2009). A mutation of *mucD* stimulates MV formation in PQS-deficient mutants, indicating that periplasmic stress-mediated MV formation is independent from PQS pathway. Similarly, MV formation is also induced in a *pqsA* mutant by other stresses, such as exposure to D-cycloserine, polymyxin B, or H_2O_2 (Macdonald and Kuehn 2013). Moreover, the peptidoglycan-associated outer membrane proteins

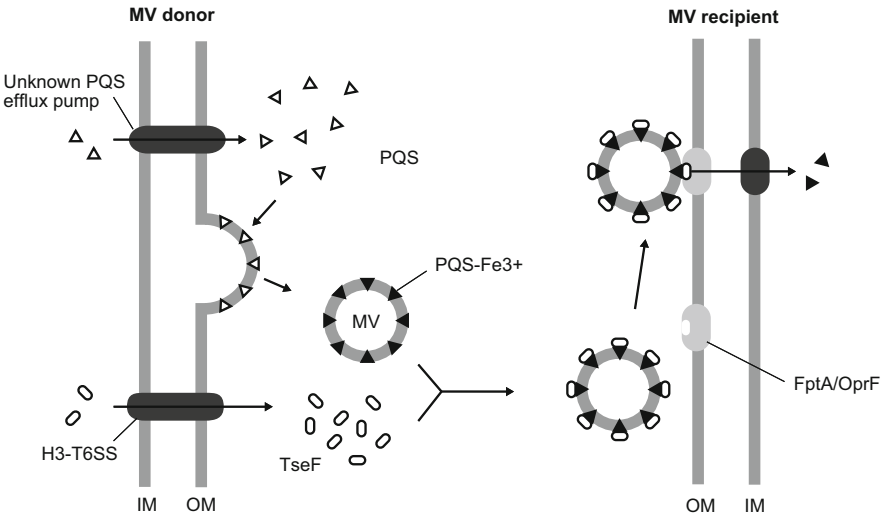

Fig. 5.3 Membrane vesicle-mediated PQS transfer and iron uptake. *Pseudomonas* quinolone signal (PQS) is synthesized in *P. aeruginosa* cytoplasm and moved out of the cell via a yet-to-be-defined export mechanism. PQS integrates the outer membrane of donor *P. aeruginosa*, resulting in membrane curvature and MV secretion. PQS chelates ferric iron and an extracellular protein TseF, which is secreted by the Type VI Secretion System H3 (H3-T6SS) interacts with PQS-Fe^{3+}. The complex of TseF and PQS-Fe^{3+} localized in MVs recognizes the pyochelin receptor FptA and the porin OprF on the recipient bacterial cell membrane, to facilitate the uptake of iron. OM, outer membrane; IM, inner membrane. The figure is modified from (Tashiro et al. 2019)

OprF and OprI affect MV formation (Wessel et al. 2013). Increased MV production by the *oprF* mutant was shown to be caused by upregulation of PQS production, while the effect of OprI on vesiculation is independent of PQS. Endolysin-mediated explosive cell lysis appears to be the main route for vesicle biogenesis in biofilms as well as under anoxic conditions in *P. aeruginosa* (Toyofuku et al. 2014; Turnbull et al. 2016).

5.3 *N*-Acyl Homoserine Lactone (AHL) Signals

The most common signaling molecules produced by Gram-negative bacteria are the AHLs, which are produced by hundreds of species mainly of the Proteobacteria. AHLs, which are typically synthesized by the LuxI-family enzymes, consist of a homoserine lactone ring and a fatty chain that contains 4–20 carbons that can have additional modifications (Arashida et al. 2018). These variations confer specificity to the signals, which are generally recognized by LuxR-type receptors. In general, the longer the fatty acid chain the more hydrophobic the signal is. The classic QS model assumes that the signals diffuse freely between cells to synchronize their gene expression when a particular population density has been attained. While the

short-chain AHLs such as C4-HSL were shown to freely diffuse in and out of bacterial cells, AHLs containing longer fatty acid chains often accumulate in the cell envelope and require transporters to be released (Buroni et al. 2009; Chan et al. 2007; Pearson et al. 1999). Given the low solubility of long-chain AHLs in water it is unclear how they are released and can travel to other cells.

5.3.1 Binary Signaling Involving MVs

In a recent study it has been demonstrated that *Paracoccus denitrificans* release C16-HSL through MVs (Toyofuku et al. 2017b). In this bacterium, cell aggregation and biofilm formation were inhibited in the presence of the signal. Of the extracellular C16-HSL, half of the molecules were found to be tightly associated with MVs, which were able to trigger the QS response in a *P. denitrificans* mutant deficient in signal production. Stimulation of MV formation increased C16-HSL release without affecting C16-HSL production. Importantly, the amount of C16-HSL associated with one MV was shown to be higher than the threshold concentration required to trigger the QS response of a single cell (Toyofuku et al. 2017b). This mechanism for cell–cell communication is fundamentally different from the classical QS model, which assumes the homogenous distribution of the signal in the medium (Miller and Bassler 2001). MVs deliver the signal molecules in high concentrations to target cells such that their QS response will inevitably be induced. For this reason, this MV-dependent signal dissemination mechanism was referred to as binary signaling (Toyofuku et al. 2017b) (Fig. 5.4).

This study provided evidence that hydrophobic AHL signals may be mainly released by MVs, which will also solubilize the signals in water (Toyofuku et al. 2017b). Likewise, the coral-associated bacterium *Vibrio shilonii* was shown to produce MVs that contain AHLs, although the structure of this signal remains to be identified (Li et al. 2016). The MV-dependent binary QS mechanism could be particularly relevant for trafficking hydrophobic signal molecules in open aqueous environments where free signals would be infinitely diluted.

5.3.2 Specific Signal Delivery Through MVs

One of the advantages of packaging signals into MVs is the possibility of a specific delivery to certain target cells, particularly relevant in polymicrobial communities. In such a situation, MVs would traffic the signals with high precision to the target cells and at the same time ensure that the signal is concentrated high enough that the quorum threshold of the receiver cell is attained.

Specific delivery was reported for *P. denitrificans,* for which it has been shown that MVs derived from this species fuse with a higher frequency with cells of their own species than those of other species (Toyofuku et al. 2017b). Furthermore, by using

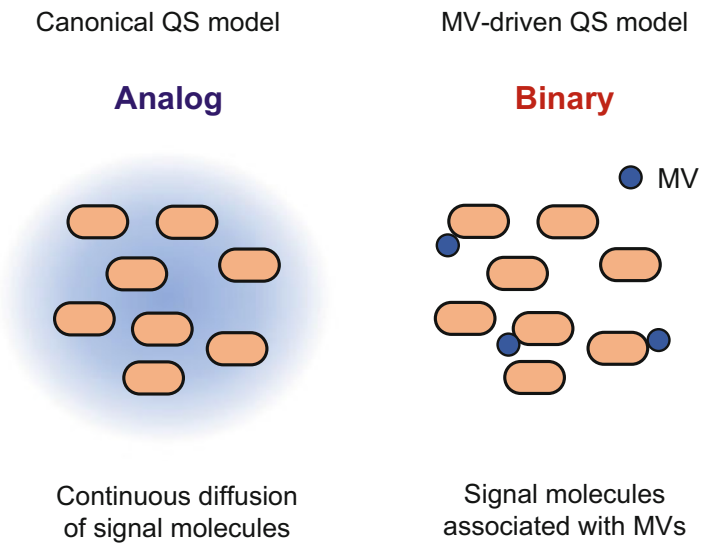

Fig. 5.4 Bacterial binary signaling involving MVs. *Left panel:* The canonical QS model assumes free diffusion of the signal molecule that will synchronize the gene expression within the bacterial population when its concentration has reached a certain threshold (analog signaling). *Right panel:* The MV-driven QS model involves MV-mediated delivery of the signal molecules in sufficiently high concentrations to target cells that their QS response will inevitably be induced (binary signaling)

AHL-reporter strains it was demonstrated that *P. denitrificans* responds equally well to free C16-HSL than to MV-associated C16-HSL, while *P. aeruginosa* does not respond well to MV-associated C16-HSL compared to free C16-HSL. It is currently not clear how MVs can be specifically delivered to target cells. Similar results of self-recognition by MVs have been observed in *Buttiauxella agrestis* for which gene transfer through MVs was only observed between cells of the same species (Tashiro et al. 2017). In this bacterium, the physiochemical properties of surfaces were critical for self-recognition by MVs. *B. agrestis* has a lower zeta-potential compared to other bacteria leading to a low energy barrier for fusion between MVs and *B. agrestis*. In the case of PQS of *P. aeruginosa*, TseF, a protein secreted by the type VI secretion system H3 (H3-T6SS), is involved in MV delivery (Fig. 5.3) (Lin et al. 2017). TseF is incorporated into MVs due to its direct interaction with iron-bound PQS (Lin et al. 2017). As TseF is captured by the Fe(III)-pyochelin receptor FptA and the porin OprF that is localized on the cell surface, PQS-Fe^{3+} loaded MVs are specifically delivered to *P. aeruginosa* cells. However, the mechanism of how PQS-Fe^{3+} is unloaded from MVs and is taken up by the cells is unknown. The presence of a protein like TseF on MVs that is recognized by surface receptors allows the specific delivery of the MV cargo to bacteria expressing the cognate receptor protein (Tashiro et al. 2019). These studies imply that the MV composition will influence the specificity of cargo delivery.

5.3.3 *Signal Piracy by MVs*

Besides releasing signals from the cell, MVs can also collect signal molecules from the environment and deliver them to a target bacterium (Morinaga et al. 2018). For example, *P. denitrificans* can sequester long-chain AHLs and exploit them to trigger its QS response. Although this bacterium primarily produces C16-HSL, it can respond to other long-chain AHLs such as C12-, C14- and C18-HSLs. When each signal was incubated with MVs derived from a *pdnI* mutant of *P. denitrificans,* which does not produce C16-HSL, the signals were adsorbed by the MVs. These AHL-loaded MVs were able to induce the QS response of the *P. denitrificans pdnI* mutant (Morinaga et al. 2018). It has been hypothesized that this signal piracy allows *P. denitrificans* to trigger its QS response in the presence of cooperating bacteria.

5.4 *cis*-2-Unsaturated Fatty Acids Signaling

Another group of quorum sensing signals are *cis*-2-unsaturated fatty acids. The first molecule belonging to this class of signals is the DSF, which was first reported to be produced by the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Barber et al. 1997). The biosynthesis and perception of the DSF signal molecule involves protein encoded by the *rpf* (regulation of pathogenicity factors) gene cluster (Dow 2017; Tang et al. 1991). DSF biosynthesis is dependent on the putative enoyl CoA hydratase Rpf. RpfB, a long-chain fatty acyl CoA ligase, appears to be involved in DSF turnover (Zhou et al. 2015). Cells perceive the DSF signal through RpfR or a two-component system, comprising the sensor histidine kinase RpfC and the HD-GYP domain response regulator RpfG (Deng et al. 2012; Suppiger et al. 2016). The HD-GYP domain of RpfG is a phosphodiesterase that hydrolyses the second messenger cyclic di-GMP. Upon binding of DSF to its receptor, the intracellular level of c-di-GMP is reduced by RpfC, which in turn regulates a broad range of biological functions, many of which are associated with virulence, biofilm formation, and stress tolerance. Subsequent work showed that DSF-family signals are produced by bacteria, which can use their signals even for interspecies communication (Boon et al. 2008; Deng et al. 2010). In addition, *cis*-2-dodecenoic acid, referred to as BDSF (***B****urkholderia* **d**iffusible **s**ignal **f**actor), was shown to inhibit the yeast-hyphal transition of *Candida albicans,* suggesting that this class of signals also have a role in interkingdom interactions (Boon et al. 2008).

5.4.1 *The Role of DSF Family Signals in MV Formation*

DSF family molecules are hydrophobic and little is known on how these signals are released from the cells and travel in aqueous environments. *X. fastidiosa* is a

bacterium that causes disease in a wide range of important crop plants. They can colonize specific areas of the foreguts of insect vectors that transmit the pathogen to their host plants, where they migrate and proliferate within xylem vessels. *X. fastidiosa* uses the signal X*f*DSF to suppress motility while stimulating the production of cell-surface adhesins, and thus is required for cell aggregation, surface attachment, and biofilm formation (Chatterjee et al. 2008). An *rpfF* mutant of *X. fastidiosa* that no longer produces X*f*DSF is hyper virulent to grapevine but the *rpfF* mutant is impaired in insect colonization and transmission. MV production in *X. fastidiosa* is suppressed by X*f*DSF (Ionescu et al. 2013). Interestingly, MV production was shown to influence bacteria–surface interactions (Ionescu et al. 2014). At a low population density, *X. fastidiosa* produces large numbers of MVs that inhibit attachment of the cells to the walls of xylem vessels and as a consequence the bacterium spreads in the plant host. On the other hand, when *X. fastidiosa* population is high, X*f*DSF accumulates and MV formation is suppressed and *X. fastidiosa* form a biofilm on the plant surface. Another study showed that the DSF also influences MV production in *Stenotrophomonas maltophilia,* an emerging multidrug resistant pathogen that is associated with bacteremia, pneumonia, and soft-tissue infections (Devos et al. 2015). In contrast to *X. fastidiosa,* however, DSF signal stimulates MV production in *S. maltophilia*. Vesiculation in this organism was also stimulated by BDSF but not by *cis*-2-decenoic acid (PDSF), a signal produced by *P. aeruginosa*.

MVs derived from one species can benefit other species and can play important roles in polymicrobial communities. For example, it was shown that MV formation in *S. maltophilia* can be induced by the β-lactam antibiotic imipenem, presumably through perturbation of the cell wall (Devos et al. 2015). The imipenem-induced MVs contain a β-lactamase, which degrades the antibiotic. These vesicles can protect other co-residing bacteria, including *P. aeruginosa* and *Burkholderia cenocepacia* that frequently coexist with *S. maltophilia* in the lungs of chronically infected cystic fibrosis (CF) patients (Devos et al. 2016). Naturally, signal-induced MVs do not contain β-lactamase, whereas imipenem-induced MVs do.

It has been postulated that membrane proteins that are regulated by DSF may influence MV biogenesis (Ionescu et al. 2014). However, the exact molecular mechanism of DSF-controlled MV formation remains to be elucidated.

5.5 CAI-1 Signaling

Another signal released through MVs is CAI-1 [(s)-3-hydroxytridecan-4-one] that is produced by many *Vibrio* species and is the predominant QS molecule of the human pathogen *Vibrio cholerae* (Brameyer et al. 2018). Other *Vibrio* species produce signal molecules with different acyl chain length and modifications (Ng et al. 2011). *Vibrio harveyi* produces Ea-C_8-CAI-1 and two additional signals, HAI-1 [*N*-3-(hydroxybutyryl)-homoserine lactone], and AI-2, a furanosyl borate diester that is widely spread among bacteria and is known as an inter-species signaling molecule

(Defoirdt et al. 2008). Each signal is perceived by specific membrane-bound hybrid sensor kinase. CAI-1 is sensed by CqsS, HAI-1 is sensed by LuxN, and AI-2 is sensed by LuxPQ.

5.5.1 CAI-1 Delivery Through MVs

While HAI-1 and AI-2 are both hydrophilic with LogP values of −0.94 and −1.25, respectively, CAI-1 is hydrophobic with a LogP of 3.05 (Brameyer et al. 2018). Due to its hydrophobicity, CAI-1 was thought to be partitioned into the lipid bilayer of the outer membrane. Indeed, CAI-1 was detected in MVs collected from stationary cultures of *V. harveyi* and was shown to activate the QS cascade in a CAI-1 mutant (Brameyer et al. 2018). In this study, MVs were fractionated by size and CAI-1 were detected using a CAI-1 reporter strain. Interestingly, only larger MVs (about 10% of all MVs) induced the QS response in the reporter strain. It is still unclear whether only the large MVs are able to fuse with neighboring cells or whether these MVs are formed through different routes and therefore have different contents and functions. *Vibrio* species are known to possess sheathed flagella at the pole of the cell. In *Aliivibrio fischeri*, rotation of the sheathed flagella generates MVs with smaller size than the ones produced by a mutant with a paralyzed flagellum (Aschtgen et al. 2016a). Such MVs may not contain signals and the different types of MVs generated from different routes may have different functions in *Vibrio* species.

5.6 Types of MVs and Their Role in Cell-to-Cell Communication

The main routes of MV formation in Gram-negative bacteria are blebbing of the outer membrane and cell lysis, and our knowledge regarding the production of Gram-positive MVs is still limited (Toyofuku et al. 2019). MVs were originally considered to be formed through controlled blebbing of the outer membrane without affecting the cell viability (Schwechheimer and Kuehn 2015). DNA damage was recently shown to induce MV formation through explosive cell lysis in *P. aeruginosa* and bubbling cell death in a Gram-positive bacterium, *B. subtilis* (Toyofuku et al. 2017a; Turnbull et al. 2016). Both processes are dependent on the enzymatic action of endolysins. These peptidoglycan (PG)-degrading enzymes are typically encoded in prophage regions and are required to lyse the host for phage release. In explosive cell lysis, the enzyme degrades the PG layer and consequently the cells round up and explode (Turnbull et al. 2016). The resulting shattered membrane fragments circularize and form MVs. It has been proposed that the composition and cargo of these MVs would differ from the ones that arise from blebbing and were therefore named EOMV (explosive outer membrane vesicles).

Outer-inner membrane vesicles (OIMV), which have recently been discovered in various bacteria, could also be generated through this route (Toyofuku et al. 2019). It is likely that the different genesis mechanisms lead to distinct MV types that serve particular functions. For example, evidence is accumulating that hydrophobic signal molecules have a high affinity to intercalate into the outer membrane, which may stimulate MV formation through blebbing (Horspool and Schertzer 2018). MVs generated via explosive cell lysis will randomly capture cellular materials such as DNA/RNA and cytoplasmic proteins as well as signal molecules. Small RNAs (sRNAs) are considered to be a universal "signal" across kingdoms (Cai et al. 2018), and MV types arising from explosive cell lysis and bubbling cell death appear to be the main carrier of this signal.

5.7 Interkingdom Signals Carried by MVs

Extracellular vesicles are released from cells of all domains of life and evidence is emerging that they play an important role in intra- and interkingdom interactions (Cai et al. 2018). An interesting example is the interaction between choanoflagellates and bacteria. Choanoflagellates are a group of eukaryotic microbes that are the closest living relatives of animals. *Salpongoeca rosetta* develops into multicellular rosettes from a single founding cell that undergoes serial rounds of oriented cell division. The initiation of rosette formation was found to be induced by bioactive lipids, including sulfonolidis and lysophosphatidylethanolamines, which are produced by the bacterium *Algoriphagus machipongonensis* (Alegado et al. 2012; Woznica et al. 2016). MVs isolated from *A. machipongonensis* cultures induce rosette formation, indicating that the inducing lipids are packed into MVs (Woznica et al. 2016). The involvement of bacterial MVs in host development is also well studied in the Hawaiian bobtail squid-*Vibrio fischeri* interaction. The LPS associated with MVs released by *V. fischeri* trigger morphogenesis of the light organ of the bobtail squid (Aschtgen et al. 2016a; Aschtgen et al. 2016b). In addition, recent work unraveled a role of MV-associated small bacterial RNAs in intercellular communication with eukaryotic cells (Tsatsaronis et al. 2018).

5.8 Concluding Remarks

In this chapter, we have reviewed recent studies that have demonstrated that hydrophobic signal molecules can be transported by MVs. The transport of signaling molecules by MVs has several advantages over simple diffusion, which in the case of highly hydrophobic molecules is not even possible. In MVs the signals are concentrated and this ensures that the QS response in recipient cells is induced (Toyofuku et al. 2017b). As fusion of vesicles with bacterial cells is a stochastic process, this may generate heterogeneous populations, with cells being either

induced or not. By contrast, the classic diffusion-based QS model assumes that upon reaching a threshold concentration the signals synchronize gene expression within all cells of the population to allow coordinated group behaviors (Miller and Bassler 2001). Another important difference is that free signals are accessible to the entire bacterial community, while evidence has emerged that MVs can deliver their signal contents to certain bacterial species and thus allow targeted communication (Tashiro et al. 2017; Toyofuku et al. 2017b). It will be a highly interesting line of research to investigate how MVs specifically fuse with certain bacteria and how these MV-associated signals are then delivered into the cytoplasm of the recipient cell. In addition, how MVs can deliver their cargo through the thick cell wall of Gram-positive bacteria has become a challenging question, and whether membrane vesicles produced by Gram-positive bacteria have a role in bacterial cell-to-cell communication remains to be investigated. It has also been shown that MVs can also sequester signals from the environment and this signal piracy may allow eavesdropping for cooperation partners in polymicrobial communities (Morinaga et al. 2018). MVs may even serve as "time capsules," as they protect the signals and thus allow bacteria to disperse their messages beyond their life span. The fact that some MV formation mechanisms are in fact based on bacterial cell death adds another interesting aspect to this idea (Toyofuku et al. 2019).

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