

Spheres of influence: *Porphyromonas gingivalis* outer membrane vesicles

M.J. Gui, S.G. Dashper, N. Slakeski, Y-Y. Chen and E.C. Reynolds*

Running title: *P. gingivalis* vesicles

Keywords: *Porphyromonas gingivalis*, outer membrane vesicle biogenesis, membrane curvature, lipids/lipopolysaccharides, virulence, biofilm

Address: Oral Health Cooperative Research Centre, Melbourne Dental School, Bio21
Molecular Science and Biotechnology Institute, University of Melbourne, Victoria, Australia.

*Corresponding author

Oral Health Cooperative Research Centre, Melbourne Dental School, Bio21 Molecular
Science and Biotechnology Institute, University of Melbourne, Australia. Tel.: +61 3 9341
1500; E-mail: e.reynolds@unimelb.edu.au.

SUMMARY

Outer membrane vesicles (OMVs) are asymmetrical single bilayer membranous nanostructures produced by Gram-negative bacteria important for bacterial interaction with the environment. *P. gingivalis*, a keystone pathogen associated with chronic periodontitis produces OMVs that act as a virulence factor secretion system contributing to its pathogenicity. Despite their biological importance, the mechanisms of OMV biogenesis have not been fully elucidated. The ~14 times more curvature of the OMV membrane than cell outer membrane (OM) indicates that OMV biogenesis requires energy expenditure for significant curvature of the OMV membrane. In *P. gingivalis*, we propose that this may be achieved by upregulating the production of a certain inner or outer leaflet lipids, which causes localised outward curvature of the OM. This results in selection of A-LPS and associated CTD-family proteins on the outer surface due to their ability to accommodate the curvature. Deacylation of A-LPS may further enable increased curvature leading to OMV formation. *P. gingivalis* OMVs that are selectively enriched in CTD-family proteins, largely the gingipains, can support bacterial coaggregation, promote biofilm development and act as an intercessor for the transport of non-motile bacteria by motile bacteria. *P. gingivalis* OMVs are also believed to contribute to host interaction and colonization, evasion of immune defense mechanisms and destruction of periodontal tissues. *P. gingivalis* OMVs may be crucial for both micro and macronutrient capture, especially haem and probably other assimilable compounds for its own benefit and that of the wider biofilm community.

INTRODUCTION

Outer membrane vesicles (OMVs) are relatively small, discrete, spherical membranous structures produced by Gram-negative bacteria via vesiculation of the outer membrane (OM) during all growth phases (**Figure 1**; (Kulp and Kuehn 2010)). Many of these OMVs are released into the environment although a substantial amount can be retained on the cell surface. OMVs range in size from 50 - 300 nm in diameter and are composed of a single bilayer membrane that is derived from the OM (**Figure 1**). The inner layer is composed of phospholipids whilst the outer layer comprises lipopolysaccharide (LPS). Proteins, usually derived from the OM, make up the other major component of the OMVs (Ellis and Kuehn 2010; Kulp and Kuehn 2010). Although many studies have documented the production of OMVs over the last 50 years, their importance has been underestimated and OMVs have often been regarded as merely broken cells or artefacts. OMVs have been proposed to act as a highly sophisticated secretion system that can deliver an array of molecular effectors including those involved in cell-cell interactions, nutrient acquisition, host immune dysregulation and modulation, host-cell interaction and biofilm formation. Moreover, the ability of OMVs to disseminate far from the cell enables sessile biofilm-associated bacteria to extend their sphere of influence. Therefore, OMVs are major contributors to bacterial survival and virulence, and hence pathogenicity.

P. GINGIVALIS

Porphyromonas gingivalis is a non-motile, coccobacillus Gram-negative, asaccharolytic oral anaerobe that colonizes dental plaque biofilms in the human oral cavity (Darveau et al. 2012). Whilst our understanding of the roles particular oral bacterial species play in disease have changed over the past two decades, there is broad consensus that the highly proteolytic, amino acid fermenting *P. gingivalis* plays a significant role in either initiation and/or

progression of disease (Darveau 2010; Hajishengallis et al. 2012; Hajishengallis et al. 2011; Socransky et al. 1998). *P. gingivalis* possesses a number of virulence factors, many of which are associated with the surface of the cell including representatives of the C-terminal domain (CTD) family of proteins. A CTD protein is one that contains a C-terminal signal sequence that directs the protein once in the periplasm to the recently described type IX secretion system that secretes the protein, removes the CTD and attaches the protein to a novel glycolipid anchor, believed to be an anionic lipopolysaccharide (A-LPS) where they collectively form an electron dense surface layer (EDSL; (Chen et al. 2011; Curtis et al. 1999; Glew et al. 2012; Rangarajan et al. 2008; Veith et al. 2013). CTD proteins include the well-studied gingipains (RgpA/B and Kgp) (O'Brien-Simpson et al. 2004; O'Brien-Simpson et al. 2003), peptidyl arginine deiminase (Gully et al. 2014; Quirke et al. 2014), CPG70 (Chen et al. 2002), HPB35 (Shoji et al. 2010) and internalin-related proteins (Dashper et al. 2009). In addition, a range of lipoproteins, fimbriae and lipopolysaccharide are strongly associated with virulence.

OMV BIOGENESIS

Current evidence indicates that OMV biogenesis is a tightly controlled and regulated process, although the specific pathways are likely to vary between phyla, genera and even species and largely remain to be elucidated (Haurat et al. 2011; Shibata et al. 1999). Key characteristics of OMV biogenesis are proposed to include outward bulging of OM areas lacking membrane-peptidoglycan linkages, enrichment or exclusion of certain proteins and lipids from OMVs, and the capacity to upregulate OMV production without losing OM integrity (Kulp and Kuehn 2010). OMV biogenesis requires an upregulation of the synthesis of surface polysaccharides, in particular that of LPS, surface proteins, phospholipids and other OMV associated compounds. It is also likely that multiple pathways of OMV biogenesis occur,

even in the same bacterium, especially as heterogeneous vesicles have been detected in some species (Pérez-Cruz et al. 2015; Pérez-Cruz et al. 2013).

CURVATURE OF THE VESICLE MEMBRANE – THE ROLE OF LIPIDS

Like other Gram-negative bacteria, *P. gingivalis* produces OMVs (**Figure 2**). Highly pure preparations of *P. gingivalis* OMVs demonstrate that they have an average diameter of 80 nm in diameter of which 40 nm is comprised of the EDSL that is composed largely of the CTD family of extracellular proteins and A-LPS, so the diameter of the OMV membrane is ~40 nm (Veith et al. 2014). The overall diameter of a *P. gingivalis* cell is ~600 nm and the EDSL is 40 nm thus the diameter of the cellular OM is ~560 nm. Therefore the OMV diameter is ~7% that of the cell OM. The curvature of a sphere is defined as the reciprocal of the radius so as the size of the OMV or cell decreases the curvature of the asymmetric lipid bilayer increases. To form OMVs that have a diameter that is ~7% that of the whole cell the asymmetric OM leaflet must have a curvature 14 times that of the cell. Membrane curvature is an energy intensive process and the free energy (ΔG) required to form a spherical vesicle from a flat membrane is $\Delta G \sim 250\text{--}600$ kBT (where kBT is the thermal energy), and this is considered a non-spontaneous biological process (Bloom et al. 1991). Bacteria must therefore have evolved strategies to reduce the energy required for OMV formation as well as mechanisms to sort cargo onto/into the OMVs. A large differential in the amount or type of lipid between the inner and outer leaflets of the OMV must be created in the process of forming a highly curved OMV. Ordering one leaflet but not the other may be a means of inducing budding, for example by direct addition of lipids to one monolayer and not the other (Huttner and Zimmerberg 2001). This might cause an asymmetry of monolayer areas and thereby increase the intrinsic curvature of the whole bilayer, causing a curling of the membrane away from the side to which lipid is added, thus expanding the monolayer with more lipid and compressing

the monolayer with less lipid. A recent *in silico* study using a complex asymmetric plasma membrane model containing seven different lipids species demonstrated the spontaneous clustering of particular lipid species that resulted in localised curvature of the bilayer surface. The authors concluded that emergent nanoscale membrane organization may be coupled both to fluctuations in local membrane geometry and to interactions with proteins (Koldso et al. 2014). Based on the bilayer-couple model of OMV biogenesis in *Pseudomonas aeruginosa*, the hydrophobic signalling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) inserted into the outer leaflet of the OM can interact with lipid A component of LPS that contributes to a low rate of flip-flop between leaflets, causing PQS to expand the outer leaflet relative to the inner leaflet and consequently results in localized membrane curvature and ultimately vesiculation (Schertzer and Whiteley 2012). Therefore production of particular lipid species in the OM could result in their self-assembly in either the inner or outer leaflet, association with specific proteins and thus reduce the need for energy in OMV biogenesis.

Various studies have compared the phospholipid profiles of bacterial OM to their respective OMVs and shown that although there are many common phospholipids, OMVs may contain specific lipids profiles that differ from those in the OM (Kulkarni and Jagannadham 2014). A comparison of the phospholipid composition of *P. aeruginosa* OM and OMVs showed that phosphatidylethanolamine, phosphatidylglycerol and phosphatidylcholine were present in both OM and OMV however their relative abundances varied between the two. Phosphatidylethanolamine was the most abundant in the OM whereas phosphatidylglycerol was most abundant in the OMV (Tashiro et al. 2011). Furthermore, *P. aeruginosa* OMVs were enriched with saturated fatty acids compared with the OM which was composed of a fairly equal mixture of unsaturated and saturated fatty acids (Tashiro et al. 2011). OMVs from *A. actinomycetemcomitans* were found to

exclusively contain minor lipids that were not detected in OM samples and OMVs were enriched with cardiolipin (Kato et al. 2002).

In addition to distinctive phospholipid profiles, bacterial OMVs have also been shown to contain distinctive differences in the LPS profile when compared to that of their parent OM. LPS is a major structural element of the bacterial OM and has three constituent components: lipid A, core oligosaccharide and O antigen polysaccharide. Distinctive LPS profiles of OMV and OM preparations have also been reported in *P. aeruginosa* (Kadurugamuwa and Beveridge 1995), furthermore, the type and/or length of LPS O antigen has been reported to affect *P. aeruginosa* OMV development (Nguyen et al. 2003). *P. aeruginosa* synthesizes two forms of O antigen polysaccharide, a neutral form (A-band) and a negatively charged form (B-band), however only the negatively charged B-band LPS was detected in *P. aeruginosa* OMVs (Kadurugamuwa and Beveridge 1995). Thus, OMVs could be generated in regions where the B-band moiety is more abundant, bending the OM to release the charge repulsion generated by the negatively charged O antigen polysaccharide (Kadurugamuwa and Beveridge 1995). The role of the O antigen in OMV biogenesis was analyzed in several *P. aeruginosa* mutant strains and the mutant strain that only expressed A-band LPS secreted the least OMVs (Nguyen et al. 2003). Similar results were observed in *Escherichia coli* and *Salmonella typhimurium* (Smit et al. 1975). *P. gingivalis* also synthesizes two distinct O-antigen moieties, a negatively-charged LPS (A-LPS) and a neutral LPS (O-LPS) (Haurat et al. 2011). The LPS profile of the *P. gingivalis* OM and OMVs has been reported to be different, with A-LPS being more abundant on the OMVs than on the OM (Haurat et al. 2011). Additionally, the deacylated forms of lipid A were found to be particularly abundant in OMVs (Haurat et al. 2011). MALDI-TOF MS analysis of *P. gingivalis* lipid A extracted from whole cells showed that the major forms of lipid A were penta- or tetra-acylated species. However, lipid A isolated from the OMV preparations was

comprised mainly of tri-acyl species. These results suggest that the lipid A of OMV may undergo significant deacylation and dephosphorylation. This may be facilitated by LptO, which was also selectively enriched into *P. gingivalis* OMVs (Veith et al. 2014). LptO has structural homology to the 14-stranded β -barrel protein FadL and has been linked to the deacylation of lipid A (Chen et al. 2011). Deacylated and dephosphorylated lipid A would allow closer packing of the lipid A anchor of A-LPS-CTD protein conjugates and therefore would allow a tighter curvature of the OMV membrane. Further, other agents that enable a greater angle of lipid bilayer curvature apart from LPS may also promote OMV formation. For example, three OM lipoproteins (PG1823, PG2105 and PG2106) identified to be preferentially enriched into *P. gingivalis* OMVs were predicted to function together in forming or maintaining OMV structure, as two of them (PG1823 and PG2106) were predicted to form eight-stranded beta barrels (Veith et al. 2014). Similarly, in a transposon mutant screening of *E. coli*, two of the OMV deficient mutants had mutations in genes encoding cell-envelope-localized proteins (*ypjA* and *nlpA*), implicating these proteins in OMV biogenesis (McBroom et al. 2006). Despite a distinct difference in the lipid A compositions of several bacteria including *P. gingivalis*, an analysis of the closely related *Bacteroides fragilis* showed no difference in the lipid A composition between the OM and OMVs (Elhenawy et al. 2014) suggesting that there are multiple factors governing OMV biogenesis and they may be varied between species.

PROTEIN SORTING ONTO *P. GINGIVALIS* OMVS

OMV biogenesis has been likened to a highly sophisticated secretion system that allows the dispersal of protein complexes, as well as insoluble proteins, in the correct configuration and orientation. In this manner, cells are able to interact with a wide area of their environment, a particular advantage for sessile bacteria, such as *P. gingivalis*. OMV proteins include mainly

outer membrane proteins (OMPs) but also periplasmic proteins that are protected in the lumen of the OMVs. The protein composition and relative abundances of specific proteins of Gram-negative bacterial OMVs have been shown to differ from the surface of the cell in a number of species (Kulp and Kuehn 2010). It seems that only a selected set of proteins are packed in the OMVs. Recent evidence has shown that *P. gingivalis* has a mechanism to selectively sort specific OMPs onto OMVs.

By comparing the proteomes of the periplasm, cytoplasm and membrane fractions of *P. gingivalis* cells as well as that of OMVs, a total of 151 proteins were identified from OMVs. Of these 151 proteins, 109 were localised to the OMV membrane and 27 to the OMV lumen (Veith et al. 2014). Sorting of OMPs to the OMVs was determined by comparing the relative abundance of OMPs in the OMV and cell OM proteomes. CTD-family proteins and some lipoproteins were preferentially sorted to the OMVs while Ton-B dependent transporters and proteins with the OmpA peptidoglycan binding motif were preferentially retained on the cell OM (**Figure 3**; (Veith et al. 2014)). The selective enrichment of all CTD proteins on the OMVs relative to the cell membranes extends the results of Haurat *et al.* (2011) who found that the gingipains (RgpA/B and Kgp) were among the favored OMV cargo. This indicates that the specific exclusion and/or inclusion of proteins requires certain cues that dictate the proper sorting of the proteins onto the OM and OMVs, at the least, to differentiate between proteins to be retained on the OM or packaged onto OMVs to be secreted. By considering the very different curvature of OMVs compared to cells, this suggests that the major mechanism of CTD protein enrichment may be driven by A-LPS selection (see above) which as the anchor for CTD protein attachment will recruit CTD proteins and allow the necessary expansion of the EDSL to maintain an even coat of surface layer in OMVs. Since each CTD protein has its own anchor, more EDSL per unit membrane implies that the A-LPS anchor must be enriched relative to other lipids.

Four lipoproteins (PG0179, PG0180, PG0181 and PG0183) together with PG0182 are only found exclusively in the OMV fraction (Veith et al. 2014). Absence from the OM suggests a mechanism whereby these specific newly synthesized proteins are targeted for incorporation into the OM before immediate export via OMVs. OM lipoprotein interactions may play a significant, but as of yet unidentified, role in the mechanism of cargo selectivity and OMV biogenesis. Study of these proteins may reveal novel ties to OMV biogenesis as they are relatively abundant not only in *P. gingivalis* OMVs, but also other Gram-negative bacteria including *A. actinomycetemcomitans*, *Campylobacter jejuni* and *E. coli* (Nikaido 2003; Ueno et al. 2006). Furthermore, proteins that are strongly associated with peptidoglycan and IM are found to be retained in the OM suggesting that this may be a way to exclude certain proteins from being incorporated into OMVs (Veith et al. 2014).

Together, this suggests an upregulation of the production of a particular inner or outer leaflet lipid may result in outward curvature of the OM which results in selection of the A-LPS and associated CTD-family proteins on the outer surface as they are able to accommodate the curvature. Deacylation and dephosphorylation of A-LPS lipid A then further enables increased curvature leading to OMV formation.

REGULATION OF OMV BIOGENESIS BY ENVIRONMENTAL FACTORS

OMV biogenesis is an energy demanding process that is most beneficial to the cell under particular environmental conditions and therefore like all similar processes, it will be a tightly controlled, well-regulated and coordinated biological mechanism that enables crucial functions for the benefit of the producer.

OMV biogenesis can be influenced by environmental factors that cause cellular stress. Stress has been shown to increase OMV biogenesis and this helps bacteria to cope with harsh environments that threaten their survival (Kulp and Kuehn 2010; Manning and Kuehn 2011).

Environment stresses such as changes in pH and temperature, the availability of (micro)nutrients, bacteriophage infection, and interaction with host cells and other bacteria can increase OMV biogenesis and release from the cell surface (Chatterjee and Chaudhuri 2012; Deatherage and Cookson 2012). Further, an induced release of OMVs can be achieved by introducing stresses to bacteria such as by inhibiting protein synthesis, lysine starvation which causes autolytic cell wall degradation, increasing growth temperature and adding sublethal membrane active antibiotics to growing bacterial cultures (Collins 2011). The addition of serum into growth cultures may also induce the release of OMVs (Necchi et al. 2007). OMV production during stress can occur without any increase in cell lysis (Macdonald and Kuehn 2013; Schwechheimer et al. 2014).

Many of the genes responsible for the overproduction of OMVs in *E. coli* were found to be related to peptidoglycan synthesis, OMPs and the sigma E stress response pathway (McBroom et al. 2006). For example, a mutation in *degP*, which encodes a dual-function periplasmic serine protease-chaperone, caused substantially increased OMV production (McBroom et al. 2006). *degP* is upregulated by both the Cpx and sigma E stress response pathways, which are involved in the biogenesis of OMPs and the degradation of misfolded proteins (Meltzer et al. 2009). It was proposed that the *degP* mutant accumulates misfolded proteins in the periplasm which may cause increased envelope stress and/or bulging of the OM and, as a result, induce OMV biogenesis (McBroom and Kuehn 2007), a result similar to *degP* mutants of *P. aeruginosa* (Tashiro et al. 2009). In *V. cholera*, OMV production is increased by down-regulation of OmpA levels by the expression of the small RNA VrrA (Song et al. 2008). Small RNA functions by base-pairing with target mRNAs, and positively or negatively regulates translation and/or stability of these messages (Song et al. 2008). Because expression of the *vrrA* gene requires the membrane stress sigma factor E, the authors suggested that VrrA acts on *ompA* in response to periplasmic protein folding stress (Song et

al. 2008). The authors also proposed that harsh conditions induce the expression of VrrA, which represses OmpA synthesis and therefore stimulates OMV production and modulates infection of the host intestinal tract (Song et al. 2008). An autolysin mutant of *P. gingivalis* was shown to hypervesiculate (Hayashi et al. 2002). Autolysins are endogenous murein hydrolases that cleave covalent bonds in the cell wall peptidoglycan and are important for bacterial growth and cell division, including cell division, cell wall remodeling, and peptidoglycan turnover and recycling (Vollmer et al. 2008). Therefore, it was suggested that cell wall turnover may play a role in regulating OMV biogenesis (Vollmer et al. 2008). Considering all of these observations, OMV biogenesis occurs not only in bacteria growing under normal conditions, but is also a component of multiple stress responses, which is relevant to survival in their ecological niches.

MICRONUTRIENT CAPTURE

P. gingivalis is a haem auxotroph and preferentially obtains haem from haemoglobin through the activity of the gingipains (Dashper et al. 2004; Shi et al. 1999; Shizukuishi et al. 1995) and other cell surface haem-binding proteins (**Figure 3**; (Veith et al. 2014)). Strikingly many of the known and putative *P. gingivalis* OM iron-complex transport systems are composed of a haem binding lipoprotein coupled with a TonB-linked transmembrane transporter. The best studied example is the lipoprotein HmuY and HmuR, the TonB-linked transmembrane transporter (Olczak et al. 2001; Simpson et al. 2000). Expression of the entire *hmu* locus is upregulated, under haemin-limited growth (Dashper et al. 2009). Like HmuY, the haem-binding lipoprotein HusA is bound to the cell surface and once dimeric haem is bound, is proposed to deliver haem to HusB, the TonB-linked transmembrane transporter for transport to the periplasm (Gao et al. 2010). Although HmuY and HusA have the same function in haem capture, HusA has a higher binding affinity to haem compared to HmuY (Gao et al.

2010; Simpson et al. 2000; Wójtowicz et al. 2009). Several other haemin binding OMPs in *P. gingivalis* have been described including OMP26, OMP32, HBP35, HtrE (Tlr) and IhtB, many of which are expressed under low haemin growth conditions (Bramanti and Holt 1992; Dashper et al. 2009; Dashper et al. 2000; Shoji et al. 2010; Slakeski et al. 2000; Smalley et al. 1993). This redundancy emphasizes the importance of haem as an essential micronutrient for *P. gingivalis*.

Both the lipoproteins HmuY and IhtB were selectively enriched onto the *P. gingivalis* OMV surface whilst their cognate TonB-linked transmembrane transporters HmuR and IhtA remained on the cell surface (**Figure 3**; (Veith et al. 2014)). The preferential packaging of these haem binding lipoproteins and the gingipains on OMVs suggests an extension in their functionality is important for haem acquisition by acting in concert as haemophores to scavenge host haem by means of an OMV-dependent pathway. HmuY has been demonstrated to act as a haemophore, working together with the gingipains to acquire haem from haemoglobin (Smalley et al. 2011). Once *P. gingivalis* OMVs penetrate gingival tissue they cause tissue damage and instigate an inflammatory response (O'Brien-Simpson et al. 2009). The resulting inflammation and gingival exudate flow could then return the haem-loaded OMVs back to the biofilm allowing haem transfer to biofilm cells. In addition to providing a source of iron and protoporphyrin IX to *P. gingivalis*, these OMVs may also provide these micronutrients to many other subgingival plaque bacteria, thereby providing a community benefit that could allow the proliferation of other species. This could be one of the mechanisms that enable *P. gingivalis* to act as a keystone pathogen to produce dysbiosis.

NUTRIENT CAPTURE

It has been proposed that the OMVs carry out a 'social' function, as the oligo-, monosaccharides, peptides and amino acids resulting from the activity of OMV-located

hydrolytic enzymes are available for other bacteria to utilize (Elhenawy et al. 2014).

Members of the *Bacteroides* genus are capable of digesting different polysaccharides via OMV-packed hydrolases and the products of this digestion can support the growth of other species that are unable to degrade the polysaccharide (Rakoff-Nahoum et al. 2014).

Gingipains that are preferentially enriched in *P. gingivalis* OMVs may perform a similar function, hydrolysing host proteins to provide peptides and amino acids as community goods for other bacteria in the polymicrobial biofilm. Taken together, *P. gingivalis* OMVs may be crucial for both micro and macronutrient capture, especially haem, peptides and amino acids for its own benefit, as well as, a community-wide benefit.

COAGGREGATION AND BIOFILM DEVELOPMENT

Chronic periodontitis is a polymicrobial biofilm-mediated disease and *P. gingivalis* has been proposed to be a keystone pathogen that enables the proliferation of other subgingival bacteria (Darveau 2010; Hajishengallis and Lamont 2012). Direct synergistic interactions between *P. gingivalis* and other species including *Treponema denticola* and *Streptococcus gordonii* have been demonstrated in regard to biofilm development and nutrient exchange (Simionato et al. 2006; Tan et al. 2014; Zhu et al. 2013). It has been shown that OMVs are involved in biofilm formation of some bacterial species (Beveridge et al. 1997; Kulp and Kuehn 2010; Remis et al. 2010).

P. gingivalis OMVs allow the coaggregation of various bacteria, either onto the biofilm and/or out of the biofilm boundary. In polymicrobial biofilms formed by *P. gingivalis*, *T. denticola* and *Tannerella forsythia* a large number of OMVs were found on the surface of *P. gingivalis* cells (Zhu et al. 2013). It is now clear that *P. gingivalis* OMVs are selectively enriched with gingipains (**Figure 3**; (Haurat et al. 2011; Veith et al. 2014)).

Besides the catalytic domain, gingipains also contain non-catalytic adhesin domains and these

adhesins have been shown to be responsible for the interaction of *P. gingivalis* with other bacteria, including *T. denticola* (Ito et al. 2010; Kamaguchi et al. 2003a; Kamaguchi et al. 2003b). The enrichment of adhesins onto OMVs may be a contributing factor to the synergistic biofilm formation by *P. gingivalis* and *T. denticola* (Zhu et al. 2013).

P. gingivalis OMVs mediated the coaggregation of *Staphylococcus aureus* with *Streptococcus* spp., and the mycelium-type *Candida albicans*, *Actinomyces naeslundii*, and *Actinomyces viscosus* (Kamaguchi et al. 2003a). This study demonstrated the ability of other bacteria to coaggregate to oral biofilms on the tooth surface or in the gingival crevice when *P. gingivalis* was present via *P. gingivalis* OMVs (Kamaguchi et al. 2003a). Synergistic interactions between *P. gingivalis* and *T. forsythia* have been proposed, where alone *T. forsythia* attached and invaded host epithelial cells using BspA. However, when coaggregated with *P. gingivalis*, *T. forsythia* attached and invaded host epithelial cells independent of BspA because these processes were promoted by *P. gingivalis* OMVs (Inagaki et al. 2006). This study shows that *P. gingivalis* OMVs assisted in the enhancement of epithelial cell invasion by *T. forsythia* by mediating coaggregation of *T. forsythia* with *P. gingivalis* (Inagaki et al. 2006). A recent study on *P. gingivalis* OMVs revealed a potential new function of OMVs in biofilms, showing that OMVs can mediate coaggregation between motile bacteria such as spirochaetes and non-motile bacteria, leading to colocalization of the non-motile bacteria by a piggyback mechanism. Addition of *P. gingivalis* OMVs enabled coaggregation of *T. denticola* and *Lachnoanaerobaculum saburreum* via the *P. gingivalis* OMVs (Grenier 2013). This could enable the non-motile *L. saburreum* and potentially other species, to be transported by the motile *T. denticola* to a new environment.

Taken together, *P. gingivalis* OMVs not only aid in polymicrobial biofilm development by interaction with other bacteria through OMV-packed gingipains, they also support coaggregation that assists in attachment to host epithelial cells, as well as, acting as a

mediator that encourages the transport of non-motile bacteria by a motile bacteria, which may play a role in biofilm dispersion and proliferation.

OMVS AND THE HOST

OMVs are virulence factors used by pathogens to initiate and progress disease in the host. The key mechanisms through which OMVs produced by various bacterial pathogens induce inflammation and pathology in the host has been recently reviewed (Kaparakis-Liaskos and Ferrero 2015). So in this review we will focus purely on *P. gingivalis* OMVs. *P. gingivalis* OMVs are relatively small, stable, adhesive and protected from host-derived proteases. They have a large surface area to volume ratio of concentrated virulence factors, largely the gingipains, lipoproteins, and LPS (**Figure 3**). They can potentially facilitate host cell interactions deep in tissues that are not readily accessible to *P. gingivalis* whole cells accreted on the tooth root in a polymicrobial biofilm. OMVs can contribute to host colonization, evasion and dysregulation of immune defence mechanisms and may therefore have a key role in destruction of periodontal tissues.

IMMUNE RESPONSE AVOIDANCE

Studies on the role of OMVs in *P. gingivalis*' capability to evade specific host immune surveillance are largely limited to its major virulence factors, the gingipains associated with OMVs. The gingipains have been implicated in the dysregulation of the host's innate and adaptive immune responses. For example the gingipains can activate host proteinases (plasmin, thrombin), inactivate host proteinase inhibitors, activate cell PAR-2 receptors, cleave cell surface receptors and a range of cytokines and antibodies, promote vascular disruption and disarm the TLR2-MyD88 pathway blocking neutrophil phagocytosis (Duncan et al. 2004; Fleetwood et al. 2015; Hajishengallis et al. 2011; Lam et al. 2014; Maekawa et al.

2014; Vincents et al. 2011; Wong et al. 2010; Yongqing et al. 2011). Specifically, *P. gingivalis* OMVs can interfere with the protective action of human serum through the OMV-associated gingipains effective degradation of IgG, IgM and complement factor C3 (Grenier 1992). OMV-associated gingipains can also lead to hyporesponsiveness of macrophages to LPS by mediating cleavage of the LPS receptor CD14 which would decrease the cell's ability to trigger LPS-stimulated cytokine production (Duncan et al. 2004). Furthermore, OMV-associated gingipains have been shown to proteolytically degrade Jak proteins, crucial for signal transduction and membrane expression of major histocompatibility complex class I and II molecules induced by IFN- γ , resulting in the potential suppression of specific immune responses (Srisatjaluk et al. 2002). This immunosuppression would help enhance the survival of OMVs in the tissue and *P. gingivalis* and the rest of the plaque community (polymicrobial biofilm) in the periodontal pocket. Biological activities of other OMV-associated virulence factors should give a more comprehensive picture of how *P. gingivalis* OMVs affect the host immune system.

INTERACTIONS WITH ENDOTHELIAL AND EPITHELIAL CELLS

The ability of OMV-associated gingipains to degrade intracellular, signal transduction Jak proteins suggests that *P. gingivalis* OMVs can be internalized by host endothelial cells (Srisatjaluk et al. 2002). In this study Jak degradation most likely occurred following the rupture and release of OMVs from OMV-containing endosomes formed during their uptake by the endothelial cells (Srisatjaluk et al. 2002). *P. gingivalis* OMVs have been shown to invade epithelial cells and two different internalization mechanisms have been proposed (Furuta et al. 2009b; Tsuda et al. 2005) The first internalization mechanism is proposed to be an actin-mediated pathway controlled by phosphatidylinositol 3-kinase, which is dependent on caveolin, dynamin and Rac1 but independent of clathrin (Tsuda et al. 2005). *P. gingivalis*

OMVs may exploit host cell receptors, particularly $\alpha 5\beta 1$ integrin, for adherence and to trigger F-actin polymerization that induces OMV cellular engulfment (Tsuda et al. 2005; Tsuda et al. 2008). This mechanism of entry into host cells is employed by many other bacteria such as *E. coli* (Plancon et al. 2003) and *S. aureus* (Agerer et al. 2005). The second internalization mechanism is proposed to be fimbriae-dependent and mediated via a lipid raft endocytic pathway where OMVs are transported to lysosomes (Furuta et al. 2009b). This second mechanism has been shown to be dependent on phosphatidylinositol 3-kinase and Rac1 and involves various regulatory GTPases, but is independent of clathrin and dynamin (Furuta et al. 2009b). The differentiation factor influencing the internalization mechanism may be the size of the OMV particle as in one study (Tsuda et al. 2005) OMV-coated 1.0 μ m fluorescent beads were used and in the other (Furuta et al. 2009b) native OMVs 80 nm in diameter were used. Endocytic pathways have been shown previously to differ based on the size of the endocytic particle (Conner and Schmid 2003). Hence, differential sizes of *P. gingivalis* vesicles could contribute to host-cell interaction heterogeneity. Larger vesicles with a double-bilayer structure produced by several Gram-negative bacteria, designated as outer-inner membrane vesicles have been recently reported (Pérez-Cruz et al. 2015; Pérez-Cruz et al. 2013). The mechanism of OMV biogenesis of these “vesicles” is likely to be different to that of classical small diameter OMVs. . Further studies are required to elucidate the exact molecular mechanisms of interaction and internalisation of *P. gingivalis* OMVs into host cells, considering that adhesion of *P. gingivalis* to host cells is multimodal (Lamont and Jenkinson 1998) and involves a variety of cell surface and extracellular components, including fimbriae, proteases, hemagglutinins, and LPS (Cutler et al. 1995), which tend to be more abundant on OMVs (Haurat et al. 2011; Veith et al. 2014). Notwithstanding the exact internalization mechanism, host cell invasion by *P. gingivalis* OMVs would definitely increase their sphere of influence.

HOST TISSUE DESTRUCTION

P. gingivalis OMVs are able to stimulate host tissue destruction by heightening inflammatory responses and impeding wound healing and regeneration ability of periodontal tissues and blood vessels. Stimulation of E-selectin and ICAM-1 membrane expression on vascular endothelial cells by *P. gingivalis* OMVs can induce acute inflammation characterized by the accumulation of a large number of neutrophils in the gingival connective tissue (Srisatjaluk et al. 1999). Gingival inflammation can also be enhanced by *P. gingivalis* OMVs as they have been shown to stimulate nitric oxide production by macrophages (Imayoshi et al. 2011). The long survival period of *P. gingivalis* OMVs within lysosomes of host cells has been shown to result in degradation of cellular components and a significant impairment of cellular function. (Ferri and Kroemer 2001; Furuta et al. 2009a). Specifically, degradation of the cellular transferrin receptor and integrin-related signalling molecules, such as paxillin and focal adhesion kinase, by *P. gingivalis* OMV-associated gingipains resulted in depletion of intracellular transferrin and inhibition of cellular migration (Furuta et al. 2009a). Consequently, wound healing and regeneration of periodontal tissues may potentially be hindered (Kato et al. 2007). *P. gingivalis* OMVs have also been shown to inhibit fibroblast and endothelial cell proliferation in a dose-dependent manner, as well as suppress angiogenesis *in vitro* (Bartruff et al. 2005). The small diameter OMVs are more likely to penetrate the junctional and sulcular epithelium than the larger whole cells so they could cause a dense network of convoluted varicose capillaries through suppression of vasculature remodelling (Bartruff et al. 2005). This along with the host's attempt to revascularize would increase the number of degenerating blood vessels and compromise wound healing (Bartruff et al. 2005).

CONCLUSION

OMVs are produced by Gram-negative bacteria, including *P. gingivalis* to perform various functions, particularly in secretion of active biomolecules, nutrient acquisition, biofilm development and pathogenesis, in relation to host-cell interaction and disease progression. A novel selective sorting mechanism of specific OMPs and lipids into *P. gingivalis* OMVs has been proposed involving A-LPS. Nevertheless, the mechanisms of many aspects of OMV biogenesis, especially in relation to the inclusion and exclusion of various biomolecules are still to be determined. Therefore, future studies on the proteins that are selectively enriched in *P. gingivalis* OMVs and determination of their roles in OMV biogenesis together with investigation of the role of *P. gingivalis* OMVs in biofilm formation and development will be of great value in understanding the key role of this bacterium in periodontitis.

ACKNOWLEDGEMENTS

We are grateful for the technical assistance of Mrs Caroline Moore in the preparation of cells and OMVs for microscopic imaging. Dr. Simon Crawford for the SEM to visualise *P. gingivalis* W50 cells is gratefully acknowledged. Chris Owen is thanked for constructing a figure demonstrating *P. gingivalis* OMV biogenesis and their putative functions. Gui May Ju is supported by International Postgraduate Research Scholarship funded by the Australian Government under the University of Melbourne.

REFERENCES

- Agerer, F., Lux, S., Michel, A., Rohde, M., Ohlsen, K., and Hauck, C.R. (2005) Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. *J Cell Sci* 118: 2189-2200.
- Bartruff, J.B., Yukna, R.A., and Layman, D.L. (2005) Outer membrane vesicles from *Porphyromonas gingivalis* affect the growth and function of cultured human gingival fibroblasts and umbilical vein endothelial cells. *J Periodontol* 76: 972-979.
- Beveridge, T.J., Makin, S.A., Kadurugamuwa, J.L., and Li, Z. (1997) Interactions between biofilms and the environment. *FEMS Microbiol Rev* 20: 291-303.
- Bloom, M., Evans, E., and Mouritsen, O.G. (1991) Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Q Rev Biophys* 24: 293-397.
- Bramanti, T.E. and Holt, S.C. (1992) Effect of porphyrins and host iron transport proteins on outer membrane protein expression in *Porphyromonas (Bacteroides) gingivalis*: identification of a novel 26 kDa hemin-repressible surface protein. *Microb Pathog* 13: 61-73.
- Chatterjee, S.N. and Chaudhuri, K. (2012) *Outer membrane vesicles of bacteria*. Heidelberg: Springer.
- Chen, Y.Y., Cross, K.J., Paolini, R.A., Fielding, J.E., Slakeski, N., and Reynolds, E.C. (2002) CPG70 is a novel basic metalloprotease with C-terminal polycystic kidney disease domains from *Porphyromonas gingivalis*. *J Biol Chem* 277: 23433-23440.
- Chen, Y.Y., Peng, B., Yang, Q. et al. (2011) The outer membrane protein LptO is essential for the O-deacylation of LPS and the co-ordinated secretion and attachment of A-LPS and CTD proteins in *Porphyromonas gingivalis*. *Mol Microbiol* 79: 1380-1401.

- Collins, B.S. (2011) Gram-negative outer membrane vesicles in vaccine development. *Discov Med* 12: 7-15.
- Conner, S.D. and Schmid, S.L. (2003) Regulated portals of entry into the cell. *Nature* 422: 37-44.
- Curtis, M.A., Thickett, A., Slaney, J.M. et al. (1999) Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. *Infect Immun* 67: 3816-3823.
- Cutler, C.W., Kalmar, J.R., and Genco, C.A. (1995) Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. *Trends Microbiol* 3: 45-51.
- Darveau, R.P. (2010) Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol*: 481-490.
- Darveau, R.P., Hajishengallis, G., and Curtis, M.A. (2012) *Porphyromonas gingivalis* as a potential community activist for disease. *J Dent Res* 91: 816-820.
- Dashper, S.G., Ang, C.S., Veith, P.D. et al. (2009) Response of *Porphyromonas gingivalis* to heme limitation in continuous culture. *J Bacteriol* 191: 1044-1055.
- Dashper, S.G., Cross, K.J., Slakeski, N. et al. (2004) Hemoglobin hydrolysis and heme acquisition by *Porphyromonas gingivalis*. *Oral Microbiol Immunol* 19: 50-56.
- Dashper, S.G., Hendtlass, A., Slakeski, N. et al. (2000) Characterization of a novel outer membrane hemin-binding protein of *Porphyromonas gingivalis*. *J Bacteriol* 182: 6456-6462.
- Deatherage, B.L. and Cookson, B.T. (2012) Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect Immun* 80: 1948-1957.

- Duncan, L., Yoshioka, M., Chandad, F., and Grenier, D. (2004) Loss of lipopolysaccharide receptor CD14 from the surface of human macrophage-like cells mediated by *Porphyromonas gingivalis* outer membrane vesicles. *Microb Pathog* 36: 319-325.
- Elhenawy, W., Debelyy, M.O., and Feldman, M.F. (2014) Preferential packing of acidic glycosidases and proteases into *Bacteroides* outer membrane vesicles. *Curr Biol* 24: 40-49.
- Ellis, T.N. and Kuehn, M.J. (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol Mol Biol Rev* 74: 81-94.
- Ferri, K.F. and Kroemer, G. (2001) Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 3: E255-E263.
- Fleetwood, A.J., O'Brien-Simpson, N.M., Veith, P.D. et al. (2015) *Porphyromonas gingivalis*-derived RgpA-Kgp complex activates the macrophage urokinase plasminogen activator system: implications for periodontitis. *J Biol Chem* 290: 16031-42.
- Furuta, N., Takeuchi, H., and Amano, A. (2009a) Entry of *Porphyromonas gingivalis* outer membrane vesicles into epithelial cells causes cellular functional impairment. *Infect Immun* 77: 4761-4770.
- Furuta, N., Tsuda, K., Omori, H., Yoshimori, T., Yoshimura, F., and Amano, A. (2009b) *Porphyromonas gingivalis* outer membrane vesicles enter human epithelial cells via an endocytic pathway and are sorted to lysosomal compartments. *Infect Immun* 77: 4187-4196.
- Gao, J.-L., Nguyen, K.-A., and Hunter, N. (2010) Characterization of a hemophore-like protein from *Porphyromonas gingivalis*. *J Biol Chem* 285: 40028-40038.

- Glew, M.D., Veith, P.D., Peng, B. et al. (2012) PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. *J Biol Chem* 287: 24605-24617.
- Grenier, D. (1992) Inactivation of human serum bactericidal activity by a trypsinlike protease isolated from *Porphyromonas gingivalis*. *Infect Immun* 60: 1854-1857.
- Grenier, D. (2013) *Porphyromonas gingivalis* outer membrane vesicles mediate coaggregation and piggybacking of *Treponema denticola* and *Lachnoanaerobaculum saburreum*. *Int J Dent* 2013: 4.
- Gully, N., Bright, R., Marino, V. et al. (2014) *Porphyromonas gingivalis* peptidylarginine deiminase, a key contributor in the pathogenesis of experimental periodontal disease and experimental arthritis. *PLoS One* 9: e100838.
- Hajishengallis, G., Darveau, R.P., and Curtis, M.A. (2012) The keystone-pathogen hypothesis. *Nat Rev Microbiol* 10: 717-725.
- Hajishengallis, G. and Lamont, R.J. (2012) Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol* 27: 409-419.
- Hajishengallis, G., Liang, S., Payne, M.A. et al. (2011) Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe* 10: 497-506.
- Haurat, M.F., Aduse-Opoku, J., Rangarajan, M. et al. (2011) Selective sorting of cargo proteins into bacterial membrane vesicles. *J Biol Chem* 286: 1269-1276.
- Hayashi, J., Hamada, N., and Kuramitsu, H.K. (2002) The autolysin of *Porphyromonas gingivalis* is involved in outer membrane vesicle release. *FEMS Microbiol Lett* 216: 217-222.

- Huttner, W.B. and Zimmerberg, J. (2001) Implications of lipid microdomains for membrane curvature, budding and fission. *Curr Opin Cell Biol* 13: 478-484.
- Imayoshi, R., Cho, T., and Kaminishi, H. (2011) NO production in RAW264 cells stimulated with *Porphyromonas gingivalis* extracellular vesicles. *J Oral Dis* 17: 83-89.
- Inagaki, S., Onishi, S., Kuramitsu, H.K., and Sharma, A. (2006) *Porphyromonas gingivalis* vesicles enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of epithelial cells by "*Tannerella forsythia*". *Infect Immun* 74: 5023-5028.
- Ito, R., Ishihara, K., Shoji, M., Nakayama, K., and Okuda, K. (2010) Hemagglutinin/Adhesin domains of *Porphyromonas gingivalis* play key roles in coaggregation with *Treponema denticola*. *FEMS Immunol Med Microbiol* 60: 251-260.
- Kadurugamuwa, J.L. and Beveridge, T.J. (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol* 177: 3998-4008.
- Kamaguchi, A., Nakayama, K., Ichiyama, S. et al. (2003a) Effect of *Porphyromonas gingivalis* vesicles on coaggregation of *Staphylococcus aureus* to oral microorganisms. *Curr Microbiol* 47: 485-491.
- Kamaguchi, A., Ohyama, T., Sakai, E. et al. (2003b) Adhesins encoded by the gingipain genes of *Porphyromonas gingivalis* are responsible for co-aggregation with *Prevotella intermedia*. *Microbiology* 149: 1257-1264.
- Kaparakis-Liaskos, M. and Ferrero, R.L. (2015) Immune modulation by bacterial outer membrane vesicles. *Nat Rev Immunol* 15: 375-87.
- Kato, S., Kowashi, Y., and Demuth, D.R. (2002) Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. *Microb Pathog* 32: 1-13.

- Kato, T., Kawai, S., Nakano, K. et al. (2007) Virulence of *Porphyromonas gingivalis* is altered by substitution of fimbria gene with different genotype. *Cell Microbiol* 9: 753-765.
- Koldso, H., Shorthouse, D., Helie, J., and Sansom, M.S.P. (2014) Lipid clustering correlates with membrane curvature as revealed by molecular simulations of complex lipid bilayers. *PLoS Comp Biol* 10: e1003911.
- Kuehn, M.J. and Kesty, N.C. (2005) Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev* 19: 2645-55.
- Kulkarni, H.M. and Jagannadham, M.V. (2014) Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiology* 160: 2109-2121.
- Kulp, A. and Kuehn, M.J. (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol* 64: 163-184.
- Lam, R.S., O'Brien-Simpson, N.M., Lenzo, J.C. et al. (2014) Macrophage depletion abates *Porphyromonas gingivalis*-induced alveolar bone resorption in mice. *J Immunol* 193: 2349-62.
- Lamont, R.J. and Jenkinson, H.F. (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiology And Molecular Biology Reviews: MMBR* 62: 1244-1263.
- Macdonald, I.A. and Kuehn, M.J. (2013) Stress-induced outer membrane vesicle production by *Pseudomonas aeruginosa*. *J Bacteriol* 195: 2971-2981.
- Maekawa, T., Krauss, J.L., Abe, T. et al. (2014) *Porphyromonas gingivalis* manipulates complement and TLR signaling to uncouple bacterial clearance from inflammation and promote dysbiosis. *Cell Host Microbe* 15: 768-78.
- Manning, A.J. and Kuehn, M.J. (2011) Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiol* 11: 1-14.

- McBroom, A.J., Johnson, A.P., Vemulapalli, S., and Kuehn, M.J. (2006) Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *J Bacteriol* 188: 5385-5392.
- McBroom, A.J. and Kuehn, M.J. (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol* 63: 545-258.
- Meltzer, M., Hasenbein, S., Mamant, N. et al. (2009) Structure, function and regulation of the conserved serine proteases DegP and DegS of *Escherichia coli*. *Res Microbiol* 160: 660-666.
- Necchi, V., Candusso, M.E., Tava, F. et al. (2007) Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*. *Gastroenterology* 132: 1009-1023.
- Nguyen, T.T., Saxena, A., and Beveridge, T.J. (2003) Effect of surface lipopolysaccharide on the nature of membrane vesicles liberated from the Gram-negative bacterium *Pseudomonas aeruginosa*. *J Electron Microsc* 52: 465-469.
- Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 67: 593-656.
- O'Brien-Simpson, N.M., Pathirana, R.D., Walker, G.D., and Reynolds, E.C. (2009) *Porphyromonas gingivalis* RgpA-Kgp proteinase-adhesin complexes penetrate gingival tissue and induce proinflammatory cytokines or apoptosis in a concentration-dependent manner. *Infect Immun* 77: 1246-1261.
- O'Brien-Simpson, N.M., Veith, P.D., Dashper, S.G., and Reynolds, E.C. (2004) Antigens of bacteria associated with periodontitis. *Periodontol* 2000 35: 101-134.
- O'Brien-Simpson, N.M., Veith, P.D., Dashper, S.G., and Reynolds, E.C. (2003) *Porphyromonas gingivalis* gingipains: the molecular teeth of a microbial vampire. *Curr Protein Peptide Sci* 4: 409-426.

- Olczak, T., Dixon, D.W., and Genco, C.A. (2001) Binding specificity of the *Porphyromonas gingivalis* heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins. *J Bacteriol*: 5599.
- Pérez-Cruz, Delgado, L., López-Iglesias, C., and Mercade, E. (2015) Outer-inner membrane vesicles naturally secreted by Gram-negative pathogenic bacteria. *PLoS ONE* 10: e0116896.
- Pérez-Cruz, C., Carrión, O., Delgado, L., Martínez, G., López-Iglesias, C., and Mercade, E. (2013) New type of outer membrane vesicle produced by the Gram-negative Bacterium *Shewanella vesiculosa* M7(T): implications for DNA content. *Appl Environ Microbiol* 79: 1874-1881.
- Plancon, L., Du Merle, L., Le Friec, S. et al. (2003) Recognition of the cellular beta1-chain integrin by the bacterial AfaD invasin is implicated in the internalization of afa-expressing pathogenic *Escherichia coli* strains. *Cell Microbiol* 5: 681-693.
- Quirke, A.M., Lugli, E.B., Wegner, N. et al. (2014) Heightened immune response to autocitrullinated *Porphyromonas gingivalis* peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. *Ann Rheum Dis* 73: 263-269.
- Rakoff-Nahoum, S., Coyne, M.J., and Comstock, L.E. (2014) An ecological network of polysaccharide utilization among human intestinal symbionts. *Curr Biol* 24: 40-49.
- Rangarajan, M., Aduse-Opoku, J., Paramonov, N. et al. (2008) Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. *J Bacteriol* 190: 2920-2932.
- Remis, J.P., Costerton, J.W., and Auer, M. (2010) Biofilms: structures that may facilitate cell-cell interactions. *ISME Journal* 4: 1085-1087.
- Schertzer, J.W. and Whiteley, M. (2012) A bilayer-couple model of bacterial outer membrane vesicle biogenesis. *MBio* 3: e00297-11.

- Schwechheimer, C., Kulp, A., and Kuehn, M.J. (2014) Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiol* 14: 1-12.
- Shi, Y., Ratnayake, D.B., Okamoto, K., Abe, N., Yamamoto, K., and Nakayama, K. (1999) Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. *J Biol Chem* 274: 17955-17960.
- Shibata, Y., Hayakawa, M., Takiguchi, H., Shiroza, T., and Abiko, Y. (1999) Determination and characterization of the hemagglutinin-associated short motifs found in *Porphyromonas gingivalis* multiple gene products. *J Biol Chem* 274: 5012-5020.
- Shizukuishi, S., Tazaki, K., Inoshita, E., Kataoka, K., Hanioka, T., and Amano, A. (1995) Effect of concentration of compounds containing iron on the growth of *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 131: 313-317.
- Shoji, M., Shibata, Y., Shiroza, T. et al. (2010) Characterization of hemin-binding protein 35 (HBP35) in *Porphyromonas gingivalis*: its cellular distribution, thioredoxin activity and role in heme utilization. *BMC Microbiol* 10: 152-163.
- Simionato, M.R., Tucker, C.M., Kuboniwa, M. et al. (2006) *Porphyromonas gingivalis* genes involved in community development with *Streptococcus gordonii*. *Infect Immun* 74: 6419-6428.
- Simpson, W., Olczak, T., and Genco, C.A. (2000) Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of *Porphyromonas gingivalis*. *J Bacteriol* 182: 5737-5748.
- Slakeski, N., Dashper, S.G., Cook, P., Poon, C., Moore, C., and Reynolds, E.C. (2000) A *Porphyromonas gingivalis* genetic locus encoding a heme transport system. *Oral Microbiol Immunol* 15: 388-392.

- Smalley, J.W., Birss, A.J., McKee, A.S., and Marsh, P.D. (1993) Haemin-binding proteins of *Porphyromonas gingivalis* W50 grown in a chemostat under haemin-limitation. *J Gen Microbiol* 139: 2145-2150.
- Smalley, J.W., Byrne, D.P., Birss, A.J. et al. (2011) HmuY haemophore and gingipain proteases constitute a unique syntrophic system of haem acquisition by *Porphyromonas gingivalis*. *PLoS One* 6: e17182.
- Smit, J., Kamio, Y., and Nikaido, H. (1975) Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. *J Bacteriol* 124: 942-958.
- Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C., and Kent, R.L., Jr. (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* 25: 134-144.
- Song, T., Mika, F., Lindmark, B. et al. (2008) A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. *Mol Microbiol* 70: 100-111.
- Srisatjaluk, R., Doyle, R.J., and Justus, D.E. (1999) Outer membrane vesicles of *Porphyromonas gingivalis* inhibit IFN-gamma-mediated MHC class II expression by human vascular endothelial cells. *Microb Pathog* 27: 81-91.
- Srisatjaluk, R., Kotwal, G.J., Hunt, L.A., and Justus, D.E. (2002) Modulation of gamma interferon-induced major histocompatibility complex class ii gene expression by *Porphyromonas gingivalis* membrane vesicles. *Infect Immun* 70: 1185-1192.
- Tan, K.H., Seers, C.A., Dashper, S.G. et al. (2014) *Porphyromonas gingivalis* and *Treponema denticola* exhibit metabolic symbioses. *PLoS Pathogen* 10: e1003955.
- Tashiro, Y., Inagaki, A., Shimizu, M. et al. (2011) Characterization of phospholipids in membrane vesicles derived from *Pseudomonas aeruginosa*. *Biosci, Biotechnol, Biochem* 75: 605-607.

- Tashiro, Y., Sakai, R., Toyofuku, M. et al. (2009) Outer membrane machinery and alginate synthesis regulators control membrane vesicle production in *Pseudomonas aeruginosa*. *J Bacteriol* 191: 7509-7519.
- Tsuda, K., Amano, A., Umebayashi, K. et al. (2005) Molecular dissection of internalization of *Porphyromonas gingivalis* by cells using fluorescent beads coated with bacterial membrane vesicle. *Cell Struct Funct* 30: 81-91.
- Tsuda, K., Furuta, N., Inaba, H. et al. (2008) Functional analysis of alpha5beta1 integrin and lipid rafts in invasion of epithelial cells by *Porphyromonas gingivalis* using fluorescent beads coated with bacterial membrane vesicles. *Cell Struct Funct* 33: 123-132.
- Ueno, Y., Ohara, M., Kawamoto, T. et al. (2006) Biogenesis of the *Actinobacillus actinomycetemcomitans* cytolethal distending toxin holotoxin. *Infect Immun* 74: 3480-3487.
- Veith, P.D., Chen, Y.Y., Gorasia, D.G. et al. (2014) *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J Proteome Res* 13: 2420-2432.
- Veith, P.D., Nor Muhammad, N.A., Dashper, S.G. et al. (2013) Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. *J Proteome Res* 12: 4449-4461.
- Vincent, B., Guentsch, A., Kostolowska, D. et al. (2011) Cleavage of IgG1 and IgG3 by gingipain K from *Porphyromonas gingivalis* may compromise host defense in progressive periodontitis. *FASEB J* 25: 3741-50.
- Vollmer, W., Joris, B., Charlier, P., and Foster, S. (2008) Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* 32: 259-286.

- Wójtowicz, H., Guevara, T., Tallant, C. et al. (2009) Unique Structure and Stability of HmuY, a Novel Heme-Binding Protein of *Porphyromonas gingivalis*. *PLoS Path* 5: e1000419.
- Wong, D.M., Tam, V., Lam, R. et al. (2010) Protease-activated receptor 2 has pivotal roles in cellular mechanisms involved in experimental periodontitis. *Infect Immun* 78: 629-38.
- Yongqing, T., Potempa, J., Pike, R.N., and Wijeyewickrema, L.C. (2011) The lysine-specific gingipain of *Porphyromonas gingivalis* : importance to pathogenicity and potential strategies for inhibition. *Adv Exp Med Biol* 712: 15-29.
- Zhu, Y., Dashper, S.G., Chen, Y.Y., Crawford, S., Slakeski, N., and Reynolds, E.C. (2013) *Porphyromonas gingivalis* and *Treponema denticola* synergistic polymicrobial biofilm development. *Plos One* 8: e71727.

FIGURE LEGENDS

Figure 1. A modified schematic view of OMV biogenesis as proposed by Kuehn and Kesty (2005). OMVs consist of OM phospholipids and LPS and periplasmic proteins. Proteins and lipids of the IM and cytosolic content are excluded from OMVs. OMVs are likely to bud at sites lacking membrane-peptidoglycan linkages. Various cellular proteins associated with OM, IM, PG and Cyt are represented as coloured shapes (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane; (Cyt) cytosol.

Figure 2. A: SEM micrograph showing a *P. gingivalis* W50 cell with a large number of OMVs. **B:** Cryo-TEM micrograph showing *P. gingivalis* W50 OMVs and their surrounding EDSL.

Figure 3. A schematic of *P. gingivalis* OMV biogenesis and their putative functions. Pink symbols represent the RgpA/Kgp proteinase adhesin complexes and other type IX secretion system substrates. Purple symbols represent adhesive lipoproteins including HmuY and IhtB. Tan symbols represent TonB-linked transporters. Red symbols denote the abundance of A-LPS in the OMV membrane whilst yellow and green symbols indicate hypothetical proteins/compounds involved in biogenesis of the OMVs and the curvature of the membrane. Black represents the cell wall and blue/grey indicates Braun's proteins linking the cell wall to the outer membrane.