Selective Sorting of Cargo Proteins into Bacterial Membrane Vesicles*

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In contrast to the well established multiple cellular roles of membrane vesicles in eukaryotic cell biology, outer membrane vesicles (OMV) produced via blebbing of prokaryotic membranes have frequently been regarded as cell debris or microscopy artifacts. Increasingly, however, bacterial membrane vesicles are thought to play a role in microbial virulence, although it remains to be determined whether OMV result from a directed process or from passive disintegration of the outer membrane. Here we establish that the human oral pathogen Porphyromonas gingivalis has a mechanism to selectively sort proteins into OMV, resulting in the preferential packaging of virulence factors into OMV and the exclusion of abundant outer membrane proteins from the protein cargo. Furthermore, we show a critical role for lipopolysaccharide in directing this sorting mechanism. The existence of a process to package specific virulence factors into OMV may significantly alter our current understanding of host-pathogen interactions.

The formation and trafficking of membrane vesicles are essential processes in eukaryotes. These structures are formed to store, traffic, or digest cellular components, and virtually all of the organelles of the eukaryotic cell, including mitochondria and chloroplasts, are able to form vesicles (1, 2). In contrast, in prokaryotes, vesicles have historically been simply regarded as cell debris or microscopy artifacts (3). It has only relatively recently been acknowledged that the outer membrane vesicles (OMV) produced via blebbing of the outer membrane (OM) of Gram-negative bacteria possess multiple functional roles (4). OMV production is increased upon exposure of bacterial cells to harsh conditions, such as the addition of chemical stressors to medium or the host environment during infection (5, 6). This has been shown in the Caenorhabditis elegans model system, where an abundant amount of OMV accumulated during infection with Pseudomonas aeruginosa (7). An increase in OMV production under stressful conditions is correlated with bacterial survival (5, 6). Furthermore, OMV constitute protective environments for cargo proteins, which inside the OMV are not accessible to proteases produced by neighboring eukaryotic and prokaryotic cells (8–10). Like extracellular eukaryotic vesicles, OMV can mediate cell-cell communication (4, 10). Incubation of purified P. aeruginosa OMV with eukaryotic cells lead to the fusion of the OMV with lipid rafts present in eukaryotic membranes, with the concomitant release of multiple virulence factors into the host cytosol (11). In other cases, whole OMV were internalized and incorporated into the trafficking network of the host cells (9, 12). Based on these observations, it has been proposed that OMV act as long distance toxin delivery devices (4, 11). However, it remains to be determined whether OMV result from a directed process or by passive disintegration of the OM.

LPS is the main component of the outer leaflet of the bacterial OM and therefore also constitutes the outermost layer of OMV. LPS is composed of lipid A, a core oligosaccharide, and a long polysaccharide chain named O antigen or O polysaccharide. The human oral pathogen Porphyromonas gingivalis, a major causative agent of chronic periodontitis, produces two classes of LPS, carrying either neutral (O-LPS) (13) or negatively charged (A-LPS) O antigen chains (14, 15). In other bacterial species containing both neutral and negatively charged O polysaccharides, only the latter has been found in the OMV. For this reason it has been suggested that an interaction between negatively charged O antigen chains contributes to OMV formation (16).

In this work we establish that P. gingivalis has a mechanism to selectively sort OM proteins into OMV. This process results in the preferential packaging of gingipains, a group of proteases that constitute a major virulence factor of P. gingivalis, and in the exclusion of abundant OM proteins from the protein cargo. Furthermore we show that mutations affecting the LPS result in aberrant protein sorting into the OMV.

Experimental Procedures

Bacterial Strain, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are shown in supplemental Table S1. All of the P. gingivalis strains were

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2 The abbreviations used are: OMV, outer membrane vesicle(s); OM, outer membrane.
grown either on blood agar plates containing 5% defibrinated horse blood or brain-heart infusion broth supplemented with hemein (5 µg ml⁻¹) and menadione (1 µg ml⁻¹) in an anaerobic atmosphere of 90% N₂, 5% H₂, and 5% CO₂. Clindamycin HCl was added to 5 µg ml⁻¹ for selection of ermF in P. gingivalis. Tetracycline (1 µg ml⁻¹) was added for the selection of the complemented strains. Escherichia coli strains were grown in LB.

**Constructions of Bacterial Strains**—Construction and complementation of P. gingivalis mutants were performed as described previously (14, 17) and are detailed in the supplemental data (supplemental Fig. S1).

**LPS Analysis** (Immunoblotting/Silver Staining)—LPS was prepared as described previously by Marolda et al. (18), and the details are given in the supplemental data. The LPS was run on 15% SDS-PAGE and visualized by the silver staining method described by Tsai and Frasch (19). P. gingivalis LPS was also analyzed by Western blotting, using monoclonal mouse α-A-LPS (MAb1B5) (20). After incubation with a secondary goat anti-mouse IRDye-800CW antibody (LI-COR Biosciences), the blots were scanned with an Odyssey infrared imaging system (LI-COR Biosciences).

**OMV Purification**—Cells corresponding to 10 OD₆₀₀ units of overnight cultures of P. gingivalis wild type and mutant strains were removed from the suspension by centrifugation at 6,000 × g. The supernatants were filtered through a 0.22-µm-pore-size PVDF membrane (Millex GV, Millipore) to remove residual cells. OMV were recovered from the resulting filtrates by ultracentrifugation at 100,000 × g for 3 h at 4 °C (Optima L-90K Ultracentrifuge; Beckman Coulter) and resuspended in 150 µl of PBS (16).

**Large Scale OMV Purification**—250 ml of 24-h cultures of P. gingivalis W50 (OD₆₀₀ equal to 2.0) were centrifuged at 9000 rpm at 4 °C, and the supernatant was filtered using a 0.22-µm-pore-size filter. The filtrate was subjected to ultracentrifugation at 140,000 × g for 1.5 h (Sorvall Ultracentrifuge). The supernatant was discarded, the pellet was washed with sterile PBS, and the ultracentrifugation step was repeated. The vesicle pellet was resuspended in distilled water and freeze-dried. 14.5 mg of vesicles were obtained from 250 ml of culture.

**OM Purification**—The cells of overnight cultures were harvested by centrifugation at 15,000 × g for 10 min at 4 °C. The pellets were gently resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 50 mM MgCl₂ containing complete EDTA-free protease inhibitor mixture (Roche Applied Science) and then lysed by sonication. The membranes were collected by ultracentrifugation at 100,000 × g for 1 h at 4 °C. The OM were isolated by differential extraction with the same buffer and 1.5% (v/v) Triton X-100 and incubated at 20 °C for 1 h. The OM fractions were recovered by centrifugation at 100,000 × g for 1 h at 4 °C (21).

The previous protocol was modified for the preparation of OM in the absence of protease inhibitor. After the sonication step, half of the supernatant was subjected to ultracentrifugation to recover the OM (OM t₀ fraction), as described above. The other half was incubated at 37 °C for 2 h, before continuing with the preparation (OM t₂ₜ fraction).

**Analysis of the OM and OMV**—Purified OM and OMV were run on 10% SDS-PAGE and stained with Coomassie Brilliant Blue or analyzed by Western blotting, using monoclonal antibody αRagB (mAb B15) (22). Protein bands were excised from gels. This analysis was performed from at least three independent sample preparations to ensure reproducibility.

**Mass Spectrometry**—The excised protein bands were in-gel digested using sequencing grade modified trypsin (Promega) (23). Peptide fragments were eluted from the gel piece, desalted using ZipTip C₁₈ (Millipore) according to the supplier protocol, and dissolved in 0.1% formic acid. A hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, Q-TOF Premier (Waters), equipped with a nanoACQUITY Ultra performance liquid chromatography system (Waters) was used for MS/MS analyses of the peptides, and the resulting mass spectra were used for the identification of the proteins by the Mascot search engine using the NCBI nr data base (50) (supplemental Table S2).

**Preparation of Lipid A for Analysis by MALDI-MS**—Lipid A from vesicles was prepared in duplicate using 10 mg of vesicles for each preparation using the procedure of Yi and Hackett (24). The lipid A was resuspended in 50 µl of water. MALDI-TOF MS was performed using a Bruker Microflex instrument fitted with a nitrogen laser operating at 337 nm using pulsed extraction in negative linear mode. Lipid A was analyzed using Norharmane (9-H-pyrido[3,4]indole) in methanol at a concentration of 10 mg/ml in methanol as the matrix, 0.5 µl of lipid A suspension together with 0.5 µl of matrix solution was applied to the MALDI plate and allowed to air dry. The instrument was calibrated using the peptides Des-Arg₁ bradykinin (mass, 904.0), angiotensin 1 (mass, 1296.5), and neurotensin (mass, 1672.3), and average masses were used throughout.

**Transmission Electron Microscopy**—3 µl of the OMV preparations were adsorbed onto carbon-coated copper grids (3 min). Liquid excess was discarded, and the samples were negatively stained with 2% (w/v) uranyl acetate for 3 min and evaluated in a Morgagni (FEI) transmission electron microscope.

**Imaging of Bacterial Cells with Atomic Force Microscopy (AFM)**—The cells of P. gingivalis W50 were strongly bound to the surface of glass slides coated with 3-aminopropyltrimethoxysilane (Genorama, Asper Biotech, Tartu, Estonia) as described in the supplemental data. The AFM imaging was performed using a Molecular Force Probe 3D from Asylum Research (Santa Barbara, CA) controlled with IGOR PRO software (WaveMetrics, Portland, OR). All of the AFM images were acquired in tapping mode to avoid the surface damage that usually accompanies contact mode imaging of soft samples as detailed in the supplemental data. Every set of AFM experiments was conducted with new tips.

**RESULTS**

**Mutation in porS Flippase Affects A-LPS Biosynthesis**—The O antigen repeating units are generally assembled onto undecaprenyl phosphate at the cytoplasmic side of the inner membrane. Completed subunits are translocated to the periplasm.
by a flippase. In this compartment, the subunits are polymerized and subsequently transferred to the lipid A core by the WaaL ligase (25). We reasoned that a flippase-deficient strain would be useful to determine whether *P. gingivalis* O antigen chains contribute to OMV formation. The *porR* locus is involved in A-LPS biosynthesis (15, 26); however, not all of the genes in that locus have been characterized (supplemental Fig. S1). PorS is encoded in the *porR* locus and presents homology to members of the Wzx flippase family (27, 28). First, we tested the flippase activity of *porS*. Flippases generally have relaxed specificities toward the translocated substrates (28, 29). We confirmed a flippase function for PorS by demonstrating its ability to restore O antigen biosynthesis and *Campylobacter jejuni* protein N-glycosylation reconstituted in an *E. coli* flippase-deficient strain (the details are given in supplemental data and supplemental Fig. S2). We then generated a *porS*-deficient strain (*porS*) and its corresponding complemented (*porS+*) strain. The effect of *porS* inactivation on LPS production was determined by comparing the LPS of wild type and mutant strains by SDS-PAGE, followed by silver staining and Western blot analysis using a monoclonal antibody that specifically recognizes A-LPS (Fig. 1). The silver-stained gel showed the characteristic ladder corresponding to smooth LPS in the wild type and *porS+* strains (Fig. 1A, lanes 1 and 3, respectively). However, the inactivation of *porS* caused a dramatic loss of reactivity toward the monoclonal antibody α-A-LPS, although a residual amount of A-LPS was synthesized in this strain (Fig. 1B, lane 2). Other putative flippases present in *P. gingivalis*, for example the product of *PG0117*, could be responsible for the partial substitution of A-LPS biosynthesis in the *porS−* strain. The *porS* mutant continued to exhibit silver-stained bands corresponding to low molecular weight LPS molecules, not detected with α-A-LPS, which most likely represent O-LPS (Fig. 1, lanes 3 and 4). These experiments demonstrated that cells carrying a mutation in the *porS* flippase displayed a negligible amount of the anionic A-LPS compared with the wild type strain but still produced O-LPS.

**LPS O Antigens Are Not Essential for OMV Biogenesis**—To analyze whether A-LPS was required for OMV formation, we also included in our experiments a strain carrying a mutation in the *waaL* gene, which encodes the O antigen ligase. This strain has been already characterized and lacks both O antigens and produces only core lipid A (14). OMV from the wild type, *porS−*, *porS+*, and *waaL−* strains were purified and analyzed by transmission electron microscopy (Fig. 2A). All four strains were able to produce OMV of similar size, indicating that neither of the two *P. gingivalis* O antigens is required for
OMV Cargo Selection

**FIGURE 3.** *Mutation in LPS results in aberrant protein sorting into the OMV.* A, purified OM from all four strains analyzed by SDS-PAGE showing no major difference in their composition. B, SDS-PAGE of purified OMV. The protein composition was anomalous in the strains displaying altered O antigens (lanes 2 and 4). C, samples of purified OM were analyzed by Western blot using a monoclonal antibody recognizing RagB. Comparable levels of RagB were found in all OM preparations. D, Western blot showing presence of RagB only in OMV of A-LPS-deficient strains (lanes 2 and 4). Lane 1, wild type; lane 2, porS+/−; lane 3, porS+/−; lane 4, waaL−.

OMV formation. Purified OMV were treated with protease K, and the LPS composition was analyzed by SDS-PAGE followed by silver staining and Western blot using a monoclonal antibody directed against A-LPS (Fig. 2B). A-LPS molecules were detected in purified wild type and porS+/− OMV, but as observed in whole cell analysis, porS+/− OMV carried mainly O-LPS and only minor amounts of A-LPS. As expected, only lipid A core was found in waaL− purified OMV. Our results indicated that O antigens do not play any key role in vesicle formation in *P. gingivalis.*

**OMV Cargo Sorting Is a Directed Process**—We subsequently analyzed the wild type OMV protein content. OMV proteins were separated by SDS-PAGE and visualized by Coomassie staining. We found that only a limited subset of proteins was present in the wild type OMV (Fig. 3B and supplemental Fig. S3B, lane 1) relative to the numerous proteins found in the purified OM of the same strain (Fig. 3A and Fig. S3A, lane 1). MS/MS analysis of the most abundant proteins in wild type OMV revealed that these bands (supplemental Fig. S3), with one exception, corresponded to different forms of the gingipains (Table 1 and supplemental Table S2). Interestingly, only OM and no periplasmic or cytosolic proteins were identified in the OMV. These results prompted us to postulate the existence of a mechanism enabling *P. gingivalis* to selectively pack certain OM proteins, mainly gingipains, into the OMV and to exclude other abundant OM proteins, such as PG0694 and PG0695, from the OMV (Table 1).

**Mutation in LPS Affects OMV Protein Sorting**—We then evaluated the possibility that modifications in the O antigen could affect OMV protein content. Unexpectedly, OMV of the porS− and the waaL− strains carried additional proteins (Fig. 3B, lanes 2 and 4, respectively) that were not detected in the wild type or the porS+/− OMV (Fig. 3B, lanes 1 and 4, respectively and Table 1 and supplemental Table S2). Particularly prominent were two proteins, RagA and RagB (RagA/B) (Fig. 3, B and D). RagA/B are immunodominant surface proteins of *P. gingivalis* (22, 30, 31). RagA is an integral OM protein that exhibits all of the typical features of a TonB-linked OM receptor, whereas RagB is a lipoprotein (32). These two proteins are thought to form a complex in the OM that is responsible for nutrient uptake (31). The exact function of these proteins has yet to be determined. Previously, Murakami *et al.* (33) have shown that RagA/B are not detected in *P. gingivalis* ATCC 33277 OMV.

The presence of additional proteins in the OMV of the mutant strains could be an indirect consequence of changes in the characteristics of the LPS. Although the mutations generated do not affect the LPS moieties in closest contact with the OM, loss of O antigen might alter the physicochemical properties of the OM or result in envelope stress. This could lead to misfolding and degradation of certain proteins in the OM or potentially to the overexpression of RagA/B in the mutant strains. To analyze this possibility, the protein composition of the OM of the four strains was analyzed by SDS-PAGE (Fig. 3A and supplemental Fig. S3A), and the major proteins were identified by MS/MS (supplemental Table S2). Except for the absence of the gingipains in the waaL mutant and the increase in the contents of OMP28 in the OM of both mutants, no significant differences were found, ruling out the possibility that O antigen loss leads to a generalized destabilization of the OM (Fig. 3A). The absence of gingipains in the OM of the waaL− mutant strain may be a consequence of a defect in gingipain OM anchoring mechanisms, as reported in a porR mutant (26). SDS-PAGE and Western blot analysis of the OM showed equivalent amounts of RagA/B in the four strains (Fig. 3, A and C), indicating that inclusion of RagA/B in the OMV of the A-LPS mutant strains may not be result of misfolding, degradation, or overexpression of these proteins induced by O antigen modification. Instead, our results support a model in which A-LPS integrity is required for proper protein sorting into the OMV.

**RagA/B Are Not Gingipain Substrates**—As mentioned above, gingipains were not detected in the OM or in the OMV of the waaL− strain (Fig. 3, A and B, lane 4, and supplemental Table S2). Previously it has been reported that the porR locus is not only required for A-LPS formation but also for gingipain glycosylation and maturation: certain glycan moieties are common to these glycosylated proteases and to A-LPS (15, 20, 26). If RagA/B were gingipain substrates, the presence of RagA/B in the OM purified from porS− and the waaL− strains might simply reflect reduced gingipain activity in those strains rather than the absence of a specific sorting mechanism. To analyze this possibility, we purified wild type OM in the presence and absence of protease inhibitors as described under “Experimental Procedures.” After incubating
the OM preparation without protease inhibitors for 2 h at 37 °C, RagA/B were still present as confirmed by MS/MS and Western blot analysis (supplemental Fig. S4, A and B). Other protein bands disappeared when the OM were purified without protease inhibitors, demonstrating that the proteases were active during this procedure (supplemental Fig. S4A). The proteins susceptible to proteolysis were identified by MS/MS as PG1414, PG0694, and PG0695. This result demonstrated that RagA/B are not gingipain substrates, and therefore their presence in the OMV of the porS mutant cannot be attributed to a reduction of gingipain activity in the OMV.

**OMV Carry LPS Molecules with Long Sugar Chains and Decayed Lipid A**—OMV protein cargo selection requires a certain degree of compartmentalization of the OM, which is necessary for the selective recruitment of proteins into the regions where the OMV will be formed. Our results indicated that A-LPS plays a crucial role in this sorting process. For this reason, we hypothesized that LPS from the OM and the OMV may have different composition. We purified and analyzed the LPS from *P. gingivalis* OM and OMV. Comparing the silver staining LPS bands, we observed that the low molecular weight LPS molecules from both preparations presented similar intensity (Fig. 4A). However, it became evident that LPS molecules carrying long polysaccharide chains were more abundant in OMV than in OM (Fig. 4A). By Western blot analysis, we showed that the long LPS molecules reacted with αA-LPS monoclonal antibody (Fig. 4B), which indicated that OMV are enriched in A-LPS. In addition, we examined the lipid A component of the LPS. Analysis of *P. gingivalis* lipid A extracted from whole cells by MALDI-TOF MS gave the expected clusters of peaks corresponding to bis-P-pentacyl, mono-P-penta-acyl, mono-P-tetra-acyl, non-P-penta-acyl, and non-P-tetra-acyl species (Fig. 4C). However, MALDI-TOF MS of lipid A isolated from the vesicle preparations from *P. gingivalis* W50 did not show the characteristic cluster of peaks (Fig. 4D). There were a few weak signals in the lower mass/charge (m/z) region of the spectrum that, on the basis of molecular weight, may correspond to bis-P-tri-acyl, mono-P-tri-acyl, and non-P-tri-acyl species (Fig. 4D). These results suggest that the lipid A of OMV may undergo significant deacylation.

**Multiple OMV Are Generated throughout the Surface of a Single *P. gingivalis* Cell**—We subsequently analyzed the surface of a single *P. gingivalis* wild type cell by AFM. A rough and heterogeneous surface and multiple structures compatible in shape and size with OMV were visualized (Fig. 5). It is tempting to speculate that some of the structures seen are OMV in formation, which could be visualized because of the existence of LPS patches of different lengths on the surface. OMV were detected at multiple sites throughout the surface of a single live bacterial cell. Interestingly, OMV appeared to be of comparable size regardless the location in which they were generated.

**DISCUSSION**

Although vesicle production in Gram-negative bacteria was reported several decades ago, the mechanism(s) involved in their biogenesis remains unknown. Circumstantial evidence that virulence factors are enriched in the OMV of other Gram-negative bacteria has previously been reported (34–38). We have found that in *P. gingivalis* OMV, only virulence factors (mainly gingipains) were enriched, suggesting the existence of a specific cargo selection process. Certain forms of the gingipains are thought to exert a role in tissues distant from the site of the infection, and therefore it seems logical that these proteases are preferentially packed into OMV. In contrast, in agreement with their proposed roles in nutrient uptake or metabolite exchange, some porins (PG0694 and PG0695) and RagA/B (the remaining dominant OM proteins) are completely excluded from the OMV and remain in the OM (31, 33, 39). The specific protein packing process into OMV was aberrant in two LPS mutant strains. This is best exemplified by the presence of RagA/B in the OMV of these strains. Interestingly, despite its enrichment in the OM of the LPS mutant strains, OMP28 was not detectable in the OMV of any strain. This suggests that this protein is somehow excluded from the OMV.

We propose that *P. gingivalis* has developed a mechanism that utilizes LPS to enable the preferential packing of a select subset of proteins into the OMV. OMV are formed and secreted throughout the surface of *P. gingivalis* cell (Fig. 5). To recruit and exclude particular proteins into membrane regions where OMV will be generated, some

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### TABLE 1

Summary of the most abundant proteins identified by MS/MS in purified wild type OM and in purified OMV from wild type and mutant strains

| Most abundant protein bands identified by MS/MS | OM (wild type) | Wild type | porS | porS' | waaL |
|-----------------------------------------------|---------------|----------|------|------|------|
| Kgp (K-gingipain)                             | +             | +        | +    | +    | ND   |
| RagA/B (R-gingipain)                          |               |           |      |      |      |
| RagA (TonB-dependent OM receptor)             | +             | +        | +    | +    | ND   |
| RagB (TonB-dependent OM binding protein)      |               |           |      |      |      |
| PG1626 (possible OM-associated protein F58)   |               |           |      |      |      |
| PG1414 (TonB-linked OM receptor PG47)         |               |           |      |      |      |
| PG1028 (probable OM lipoprotein P61)          |               |           |      |      |      |
| PG0694 (OmpA/Omp40)                          |               |           |      |      |      |
| PG0695 (OmpA/Omp41)                          |               |           |      |      |      |
| PG0027 (probable integral OM protein P40)    |               |           |      |      |      |

+ indicates present. ND, not detected.
degree of compartmentalization of the bacterial surface has to be achieved. It has been proposed that microdomains, enriched in certain types of lipids and proteins, perform a localized activity during cell division and sporulation (40). For example, the phospholipid phosphatidylethanolamine is localized to the membranes of the polar septum during cell division and to the engulfment and forespore membranes at different sporulation stages (41). Likewise, cardiolipin is enriched in the spores (41, 42). Theoretically, OM domains could be created by the formation of patches of different LPS molecules sorted according to polysaccharide composition or length. OM proteins could then be selectively recruited or excluded from those regions, thereby compartmentalizing the OM. Our model predicts that LPS from the OM and the OMV will have different composition. We analyzed the LPS profiles from \textit{P. gingivalis} OM and OMV of wild type bacteria and found that OMV are enriched in high molecular weight A-LPS molecules. In addition, we determined that the OMV lipid A is particularly abundant in tri-acyl species. OM patches with distinct LPS and protein composition could be the result of self-organization of LPS molecules and the proteins destined for packing into the OMV. In this case, the proteins could be compartmentalized as a consequence of their affinities toward a specific glycan moiety or the overall charge or length of the LPS molecules. Alternatively, a putative sort-
ing factor could simultaneously recognize recruiting or exclusion signals in the target proteins and, through a lectin domain, a sugar in the A-LPS. The existence of exclusion signals could explain why certain proteins (such as PG1028 and PG0694) are not packed in the OMV of any strain. This model is analogous to the role of galectin in eukaryotes that contribute to the protein sorting process and stabilization of lipid domains (super rafts) from which endosomal vesicles are secreted (43). Other factors could contribute to the assembly of OM microdomains and therefore influence protein cargo selection. For example, it has been proposed that the lipid composition is different in OM and OMV (35, 36). In addition, OM compartmentalization could be coupled to LPS and/or OM protein translocation processes (44).

It has been shown that OMV produced by *P. gingivalis* have proteolytic activity, mainly because of the presence of gingipains (34) and that this enables the degradation of host proteins, including collagen, elements of the complement cascade, and cell receptors (45, 46). Furthermore, *P. gingivalis* OMV can be internalized and subsequently digest cell components resulting in cellular malfunction (12). OMV may also be responsible for the presence of *P. gingivalis* antigens in preterm delivery placentas (47). It would be interesting to evaluate the impact in the virulence of the presence of RagA/B in the OMV. However, in the mutant strains used in this study not only the protein cargo has been affected but also the LPS composition, and in the case of *waaL*, the gingipains were not detected in the OMV. The pleiotropic effect of *pors* mutagenesis would complicate the interpretation of such virulence experiments.

In eukaryotes, vesicles constitute an essential mechanism for trafficking of cell material between different cell organelles and between the cell and its surroundings. The cells have developed several mechanisms to pack the appropriate cargo into these vesicles. It has been demonstrated that mitochondria-derived vesicles fuse to and deliver a specific cargo into a population of peroxisomes (1). In addition, archaea also produce and secrete vesicles with defined cargos. Proteins homologous to components of the endosomal sorting complex required for transport have been found in vesicles secreted by * Sulfolobus* species (48). Based on those observations, it is tempting to speculate that OMV formation with defined protein cargo in prokaryotes may be an ancient process related to vesicle formation in eukaryotes.

It remains to be demonstrated whether our model for OMV cargo selection in *P. gingivalis* can be extended to other species, which may explain previous reports showing that certain toxins are enriched in the OMV compared with the OM in diverse pathogenic species (5, 16, 35, 36, 38). Proper OMV cargo selection could be critical for pathogenesis, and therefore our results identify new opportunities for intervention against pathogenic bacteria. In addition, understanding how proteins are selectively packed into OMV may be useful to improve current OMV-based vaccines, which have already been used in humans in different parts of the world (49).

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