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4. Outer membrane vesicles from cold-adapted antarctic bacteria

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Abstract. Many Gram-negative, cold-adapted bacteria from the Antarctic environment produce large amounts of extracellular matter with potential biotechnological applications. Transmission electron microscopy (TEM) analysis after high-pressure freezing and freeze substitution (HPF-FS) showed that this extracellular matter is structurally complex, appearing around cells as a netlike mesh, and composed of an exopolymeric substance (EPS) containing large numbers of outer membrane vesicles (OMVs). Isolation, purification and protein profiling via 1D SDS-PAGE confirmed the outer membrane origin of these Antarctic bacteria OMVs. In an initial attempt to elucidate the role of OMVs in cold-adapted strains of Gram-negative bacteria, a proteomic analysis demonstrated that they were highly enriched in outer membrane proteins and periplasmic proteins associated with nutrient processing and transport, suggesting that the OMVs may be involved in nutrient sensing and bacterial survival. OMVs from Gram-negative bacteria are known to play a role in lateral DNA transfer, but the presence of DNA in these vesicles has remained difficult to explain. A structural study of *Shewanella vesiculosa*

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M7^T using TEM and Cryo-TEM revealed that this Antarctic Gram-negative bacterium naturally releases conventional one-bilayer OMVs, together with a more complex type of OMV, previously undescribed, which on formation drags along inner membrane and cytoplasmic content and can therefore also entrap DNA.

Introduction

Some of the world's most primitive ecosystems are found in Antarctica, where environmental conditions are so extreme that, in terms of average values, it constitutes the world's coldest, driest, and windiest continent. Among the few organisms that can survive in such a harsh environment are numerous types of bacteria that have developed various strategies for cold adaptation. Many polar cold-adapted Gram-negative bacteria produce extracellular polymeric substances (EPSs) that enhance their growth and survival in natural systems [1,2]. EPSs have been found to modify the physicochemical environment of bacterial cells, participate in cell adhesion to surfaces and retention of water, favor the sequestration and concentration of nutrients, retain and protect extracellular enzymes against cold denaturation and also act as cryoprotectants [2]. Although studies of bacterial EPSs have singled out exopolysaccharides as their major chemical constituent, it has become clear that they are also composed of other molecules such as proteins, lipids, and nucleic acids. An important finding is the abundant presence of particulate structures such as outer membrane vesicles (OMVs) within the extracellular matter that bacteria secrete to the environment, which may be the source, in part, of the aforementioned compounds [3-6].

OMVs are produced by a wide variety of Gram-negative bacteria during the course of normal metabolism and cell growth. These spherical bilayered lipid vesicles, ranging in size from 25 to 250 nm, are produced when small portions of the bacterial outer membrane (OM) bulge away from the cell and are released (Fig.1). Consequently, they are primarily composed of lipopolysaccharides (LPS), periplasmic proteins, outer membrane proteins and phospholipids [7].

Described more than a decade ago, OMVs are currently seen as a new form of secretion [7]. Studies that were initially conducted on pathogenic bacteria and the delivery of toxins are now mainly focused on the mechanism of vesicle formation and their pathophysiological roles [5]. Although observed for many years, the process by which Gram-negative bacteria produce OMVs is still unknown [7,8]. Comparative studies of the protein profiles of OMVs and outer membranes (OMs) show high similarity, although OMVs are enriched with some proteins while others are entirely absent, suggesting that the vesicles are not randomly extruded from the outer

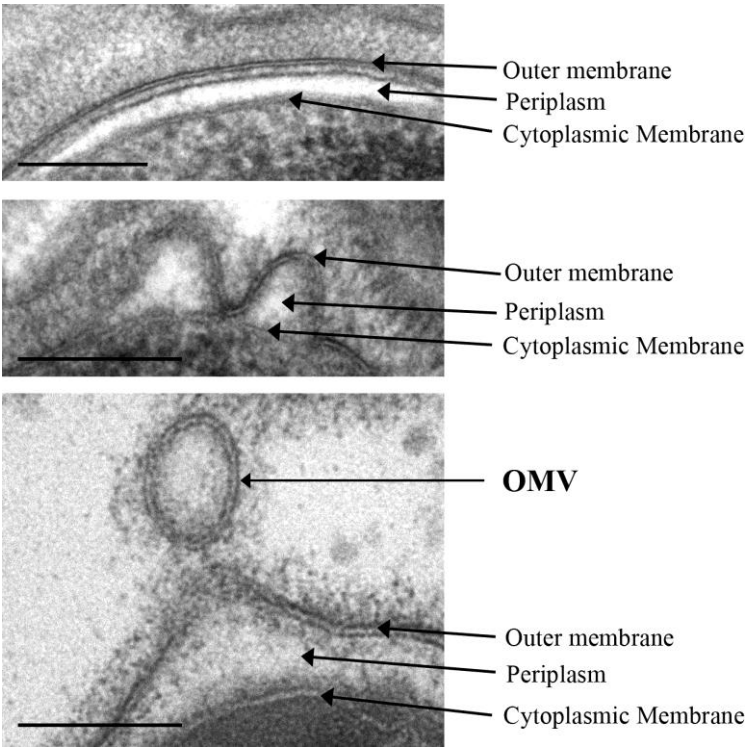

Figure 1. Process of formation of an outer membrane vesicle (OMV). In the first micrograph we can observe a portion of the cell envelope from the Gram-negative bacterium *Shewanella vesiculosa* M7^T. In the second one the outer membrane starts to protrude at different points of the cell. Finally the third micrograph shows a released OMV.

cell membrane, nor are they a product of cell lysis. In addition, extra bands can be detected in many OMVs corresponding to soluble periplasmic proteins. In brief, it has been established that the biogenesis of OMVs must be a well-regulated biological mechanism that leads to heterogeneous packaging of proteins and lipids, and varies according to certain physiological situations and the bacterial species [8].

Once secreted, OMVs can spread away from the cell and accomplish several biological functions in the surrounding environment and in other cells: they are involved in pathogenesis, interspecies communication, nutrient acquisition, innate bacterial defenses and horizontal gene transfer [9]. Since

OMV release seems to be a conserved mechanism across Gram-negative bacteria and represents a high energy cost, it must play an essential role in cell survival. The biogenesis and biological roles of OMV are described in several up-to-date reviews, so we will not be revisiting these issues here [7,9]. The presence of OMVs in non-pathogenic bacteria is less studied and although multiple functions can be envisaged, such as cell-to-cell signaling, biofilm formation and bacterial survival, their role remains unclear and deserves further study.

The Antarctic environment is a rich source of new microorganisms with unknown properties, which have aroused growing interest throughout the scientific community in recent years. Our research group has isolated several cold-adapted microorganisms from water and marine sediments collected in the Antarctica area of the South Shetland Islands, which have been characterized as new species in distinct taxonomic groups [10-14]. A predominant feature of these bacterial colonies is their mucoid appearance, owing to the accumulation of large amounts of extracellular matter. Our study clearly shows the structural complexity of the extracellular matter of these Antarctic bacteria [5].

1. Structural analysis of the extracellular matter secreted by cold-adapted antarctic bacteria

As mentioned above, one of the major adaptations to cold by Antarctic bacteria is the production of abundant extracellular matter, which in recent years has received considerable attention for its potential biotechnological applications. Exopolymeric substances, which are an integral part of the extracellular matter of many polar bacteria, have been chemically characterized, but the structural characterization of extracellular matter has been limited, partly by the difficulty of preserving these highly hydrated structures.

TEM analysis of ultrathin sections has been extensively used in the characterization of bacterial cell and envelope structures. The proper preparation of biological samples for electron microscopy is crucial for maintaining the original structure and avoiding the presence of artifacts. For many years the highly hydrated bacterial exopolymers escaped detection by electron microscopy using conventional techniques due to their low affinity for heavy metal stains, as well as their marked propensity to collapse and be removed during the dehydration preparatory steps at room temperature [4,15]. Our knowledge of extracellular matter was improved by the introduction of high-pressure freezing-fixation (HPF) and freeze-substitution

(FS) techniques. In HPF, biological samples are processed at a very low temperature, the principle objective being the ultrarapid solidification of water into an amorphous glassy state to prevent ice crystal formation. Then, FS allows water to be substituted with chemical fixatives and dehydrating agents at low temperatures (-80°C) to minimize secondary ice crystal growth [16]. The resulting biological specimens are embedded in plastic resins and are therefore amenable to standard thin-sectioning techniques at room temperature [15,16]. The use of these methods in bacterial studies has greatly improved ultrastructural preservation of extracellular matter.

The psychrotolerant strain *Pseudoalteromonas antarctica* NF3^T is a Gram-negative bacterium isolated from muddy soil samples of Antarctica that secretes large amounts of a mucoid exopolymer with a high protein content [3]. We examined the structure of *P. antarctica* NF3^T and the extracellular matter it secretes by conventional methods and compared this with information obtained by TEM after HPF-FS, and Epon resin embedding. When *P. antarctica* NF3^T was observed by standard chemical fixation, dehydration, and embedding techniques, cytoplasmic content was not uniformly distributed; holes and completely voided cells were frequent, and although *P. antarctica* colonies are extremely mucoid, no capsular or exopolymeric material was observed around the cells (Fig. 2A). However, examination of *P. antarctica* after HPF-FS revealed other, previously undetectable, structural features. Immediately striking was the absence of ice-crystal damage to the cell structure, particularly when one considers that no cryoprotectants were used, indirectly pointing to the cryoprotectant

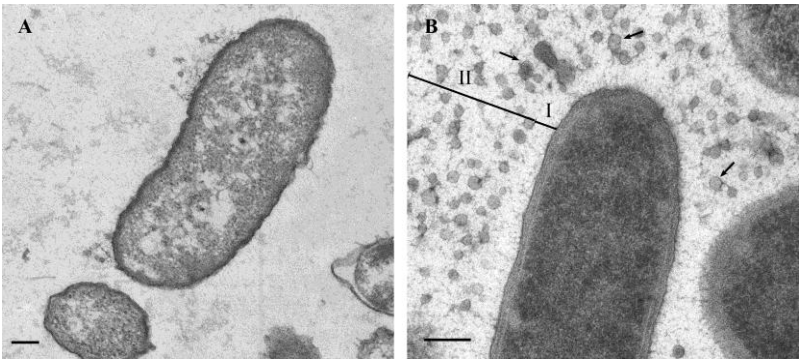

Figure 2. TEM of ultrathin sections from *P. antarctica* NF3^T prepared by conventional embedding methods (A) and prepared by high-pressure freezing and freeze substitution (B). I, halo of fine fibers. II, fibers beyond cells. Arrows indicate OMV Bars, 100 nm.

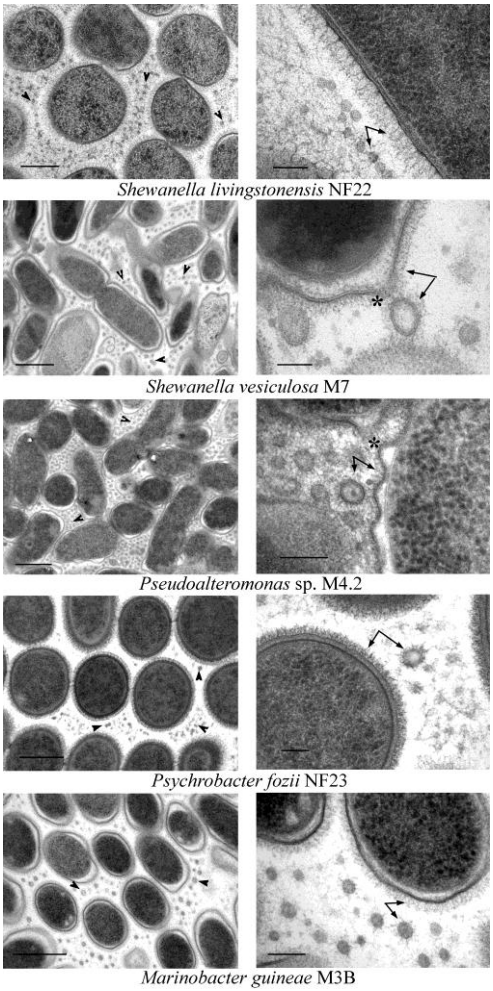

Figure 3. TEM micrographs of ultrathin sections from five cold-adapted Antarctic bacteria prepared by HPF-FS. The left images show a general view of cells as well as large amounts of membrane vesicles (arrow heads). The right images show magnified views of cells surrounded by a halo of fibers of perpendicular orientation (arrows with double arrowhead). The right images also show the same bilayered structure around the vesicles and the bacterial outer membrane. Asterisks mark protrusions in the outer membrane. The perpendicular fibers can also be observed around vesicles and the outer membrane of cells (arrows with one arrowhead). Bars in the left images are 200 nm; bars in the right images are 100 nm. (From Microb. Ecol. 2010. 59:476-486).

role of the extracellular matter of *P. antarctica.* Cells appeared turgid with homogeneously distributed cytoplasmic content and well-differentiated envelope profiles, and their surfaces were completely covered with a distinctive halo of fine fibers standing perpendicular to the cell wall. This fine fibrous fringe corresponded to the presence of a capsular material within the extracellular matter secreted by *P. antarctica* NF3^T. In addition to the closely adherent halo, a polymeric material composed of a netlike mesh of more randomly arranged fibers extended far beyond the cells (Fig. 2B). A noteworthy feature observed in ultrathin sections of *P. antarctica* cells by HPF-FS was the large quantity of OMVs immersed in this netlike mesh.

In view of these results, we also analyzed the structure of other cold-adapted Antarctic bacteria by TEM after HPF-FS [5]. Five strains (*Shewanella livingstonensis* NF22^T, *Shewanella vesiculosa* M7^T, *Pseudoalteromonas* sp. M4.2, *Psychrobacter fozii* NF23^T, and *Marinobacter guineae* M3B^T) were selected for their mucoid colony morphology, which is associated with the production of abundant exopolymeric substances. A complex composition was again found in all analyzed extracellular matter, with the presence of ordered fibrous material external to the outer membrane and large amounts of cell-derived membrane vesicles, which have not been previously described for members of the genera *Psychrobacter* and *Marinobacter* (Fig. 3).

The Antarctic bacterial OMVs showed the typical characteristics described for these structures. They appeared as spherical bilayered lipid vesicles extruded from regions of the bacterial OM with diameters ranging from 20 to 200 nm. TEM analysis also indicated that the OMVs were covered with the same capsular polymeric fibers as those found around cells. In our strains, this could facilitate the formation of a netlike mesh, which would allow OMVs to be retained near cells and also to adhere to surfaces or to other cells.

2. Proteomic study of OMVs from cold-adapted Antarctic bacteria

In an initial attempt to elucidate the role of OMVs of Antarctic bacteria and their relation with cold adaptation, a proteomic analysis of the main proteins of OMVs produced by different Antarctic strains was performed. Although the full proteome analysis of Gram-negative bacteria OMVs has proved a useful approach to characterizing individual proteins and their functions, most of these studies have focused on pathogenic bacteria and little is known about the roles of OMVs in the Antarctic environment [3].

As mentioned above, OMVs are secreted during the normal growth of bacteria. Additionally, we observed that OMVs from cold-adapted bacteria are not found in isolation but within a surrounding netlike mesh. It should be emphasized that other components such as fimbriae and flagella may be present in this environment, so the choice of methods for obtaining and purifying the OMVs is crucial when studying their composition and functions if errors in interpretation are to be avoided [8].

The first step in obtaining purified OMVs is to separate bacterial cells from culture broths by low-speed centrifugation (6,000-10,000x g; 10 min). Clarified supernatants have to be filtered through small pore size filters (0.22-0.45 μm) to remove any remaining cell. OMVs are then usually isolated from cell-free supernatants by high-speed centrifugation (≥40,000x g, 1h). At this point, there are other components in the culture medium that co-sediment with the OMVs, such as flagella, products from cell debris and soluble extracellular macromolecules. To characterize a pure population, OMVs must be separated from other supernatant material. Typically, this is accomplished by velocity or equilibrium density gradient centrifugation, which separates molecules on the basis of their buoyant density. Thus, the different components of the first pelleted OMVs are resuspended in a small volume of buffer and loaded at the bottom of an equilibrium density gradient such as 45%-20% iodixanol (100,000x g, 14 h). The different components distribute along the gradient and concentrate where their density matches with the surrounding solution. The OMVs usually float higher than the soluble proteins or flagella [7,8]. After the centrifugation, bands can sometimes be separated simply by observation with the naked eye. Otherwise, small portions of the gradient have to be removed and the presence and purity of the OMVs is verified by SDS-PAGE electrophoresis and TEM observation after negative staining.

Since OMVs are derived from Gram-negative bacterial OM, they are composed mainly of LPS, OM proteins and phospholipids. However, OMVs are not simply fragments of the OM released to the external medium; they also contain periplasmic proteins and in some cases are enriched with specific proteins while others are excluded, suggesting that they function as a distinct secretory pathway [7]. As in other bacteria, SDS-PAGE analysis of OMV proteins from Antarctic strains showed different banding profiles from those of OM, suggesting some kind of protein sorting during vesicle formation (Fig. 4) [8].

A proteomic analysis of purified OMVs from three Antarctic bacteria, *Pseudoalteromonas antarctica* NF3^T, *Shewanella livingstonensis* NF22^T and *S. vesiculosa* M7^T, was performed to characterize the main proteins present in these vesicles [3,5,6]. Beforehand, isolated OMVs were further purified on

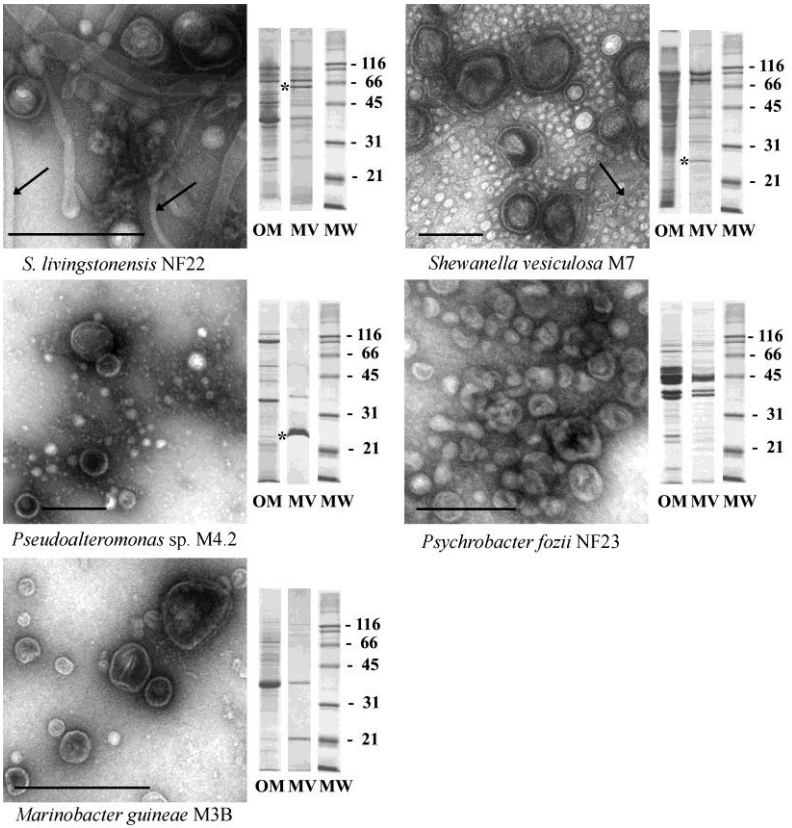

Figure 4. Negative-staining micrographs from membrane vesicles isolated from five Antarctic bacteria cultures, and Coomassie blue-stained SDS-PAGE (12%) protein profiles of outer membrane fractions (OM) and membrane vesicles (MV) from each strain. The molecular mass marker (MW) is expressed in kilodaltons. Asterisks indicate polypeptides that were overexpressed in membrane vesicle preparations. Arrows indicate flagella. Bar 200 nm. (From Microb. Ecol. 2010. 59:476-486).

an Optiprep® density gradient to remove contaminants including flagella. Proteins from purified OMVs were separated using 12.5% 1D SDS-PAGE. Protein bands were excised from the gel and digested with trypsin. Peptides were separated by liquid chromatography and subsequently analyzed on a nano-ESIQTOF mass spectrometer. Data were submitted for database searching in MASCOT server, and were searched against the NCBI non redundant protein sequence database.

The identification of OMV proteins from Antarctic strains, a challenging task since no peptide mass data were available for these new bacteria, was carried out by cross-species peptide mass finger printing. The proteomic analysis revealed the presence of OM and periplasmic proteins qualitatively similar to other OMVs characterized in Gram-negative species. Sequences of vesicle proteins matched those of multi-function proteins such as proteolytic enzymes, transport receptor and binding proteins, secretion proteins, polysaccharide biosynthesis proteins, enzymes involved in bacterial cell wall degradation, putative porins, proteins that participate in electron transport, adhesins and proteins involved in protein folding (Fig. 5).

The most abundant proteins detected were putative TonB-dependent receptors. This family of OM beta-barrel proteins is mainly involved in the uptake of molecules that are too large to diffuse through the porins, chiefly iron siderophores and vitamin B12 [5]. The overexpression of these proteins is thought to be a survival mechanism in nutrient-limiting conditions, since they could play a role in sensing nutrients and importing them into the cell. Nutrient limitation could also explain the presence of abundant proteins putatively identified as bifunctional UDP-sugar hydrolase/ 5′-nucleotidase periplasmic precursors, phosphate-binding periplasmic protein precursors, and phosphate-selective porins O and P. Phosphorous is an essential component of macromolecules and bacteria need an optimal supply of

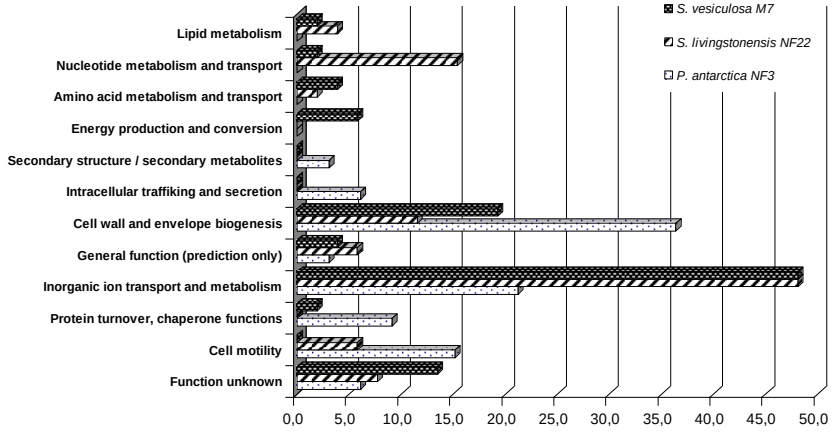

Figure 5. Functional classification of the proteins identified in the proteomic analyses of three Antarctic bacteria OMV. Results are expressed as percentage of the total protein identified.

phosphorous from the environment. These precursors and porins may play roles in nucleotide salvage and in phosphorous metabolism [3].

Proteomic analysis also revealed the presence of putative proteolytic degradative enzymes in OMVs. Their presence could contribute to the degradation of high molecular weight compounds present in the organic matter common to marine environments, which are largely unavailable for direct uptake by marine bacteria for catabolic and biosynthetic purposes [17]. Additionally, it has been suggested that the presence of these hydrolytic enzymes within OMVs confers a predatory activity, allowing the bacteria to kill other micro-organisms and use the lysis products to grow [9].

In the psychrotolerant bacterium, *S. livingstonensis* NF22^T, the growth temperature seemed to influence the amount and morphology of OMVs [5]. Quantification and TEM observation revealed that at low temperatures (4ºC), OMVs were more abundant, smaller (mean value 26.6 nm in diameter) and more regular in size than at higher temperatures (18 ºC) (mean value 40.2 nm in diameter). The overexpressed proteins in OMVs detected at low temperatures (mostly homologues of TonB-dependent receptors, porins, and phosphate-binding periplasmic protein precursors), are essentially related with membrane transport. Cold induction of these proteins may counteract the low diffusion rate of solutes at low temperatures. At this stage, however, it is difficult to infer a function for cold-adapted bacterial OMVs mainly because Antarctic growing conditions cannot be reproduced in the laboratory and this implies that some of the identified proteins and their concentrations may differ from those in natural environments.

3. New type of outer membrane vesicles: Implications in DNA content

Numerous studies, particularly on pathogenic bacteria, have shown that OMVs can contain DNA and, in some cases, transfer it to other bacteria [7,9,18-21]. The mechanism is a plausible one, since vesicles can protect DNA from degradation outside the cell and also favor DNA transmission between bacteria by association with cell envelopes [20]. Despite the great interest generated by the presence of DNA in bacterial OMVs, the mechanisms by which DNA is internalized in these vesicles are still not clear [21], particularly since all the vesiculation mechanisms proposed to date rule out the presence of any cytoplasmic membrane and therefore of any cytoplasmic components [7].

Among various models proposed to explain DNA packaging in OMVs [19,21], one involves the release of extracellular DNA after bacterial lysis is

internalized in the vesicles by a mechanism similar to that used in bacterial transformation. Another model involves the incorporation of DNA into OMVs before their release, assuming that the DNA somehow passes from the cytoplasm through the plasma membrane to be encapsulated within an OMV once in the periplasm. Nevertheless, neither of these models have been sufficiently backed up by experimental evidence [21]. A third model was proposed by Kadurugamuwa and Beveridge [19] to explain the presence of some cytoplasmic constituents in natural and gentamicin-induced OMVs of *Pseudomonas aeruginosa*. They suggested that the peptidoglycan layer can be weakened by autolysins and that transient and localized breaches in the peptidoglycan can lead to the formation of what they called complicated OMVs, which contain both inner and outer membranes as well as cytoplasmic components such as DNA. However, TEM images were inconclusive, and the existence of a new type of double-layered membrane vesicle was not demonstrated.

Shewanella vesiculosa M7^T is an Antarctic psychrotolerant Gram-negative bacterium isolated by our research group from marine sediments collected on Deception Island (South Shetland Islands) [13]. This strain produces a huge amount of natural OMVs from solid or liquid cultures without any inducing factors such as the addition of membrane-perturbing agents. Initial structural analysis of the strain gave us an insight into a possible mechanism that would explain the presence of DNA inside OMVs of a Gram-negative bacterium.

In TEM observations of *S. vesiculosa* M7^T sections obtained after HPF-FS, we repeatedly noted the presence of two different types of OMVs secreted from this strain. The first were conventional OMVs, surrounded by a bilayer membrane and with diameters ranging between 25 and 200 nm (Fig. 6A and B). These vesicles were derived from the OM of *S. vesiculosa* M7^T cells, as can be clearly observed in Fig.6A, with their membranes showing the same bilayer structure, width, and staining profile as the cell OM. The OMVs were also surrounded by the same fringe of fine fibers as the cells and contained material of low electron density similar to that in the cell periplasmic space. Unexpectedly, however, in some *S. vesiculosa* M7^T cells we noted the formation of membrane vesicles in which the plasma membrane (PM) was extruded as well as the OM. During this vesiculation process, we observed that cytoplasmic content (CC) also became entrapped within the vesicle (Fig. 6C to 6F). OMVs formed in this way had diameters of between 100 and 250 nm and two bilayer membrane structures, i.e., the external membrane derived from the cell OM and an inner membrane corresponding to the cell PM, as Fig. 6C to 6F clearly depict. Inside this inner membrane, we observed an electron-dense material similar to that seen in the cell

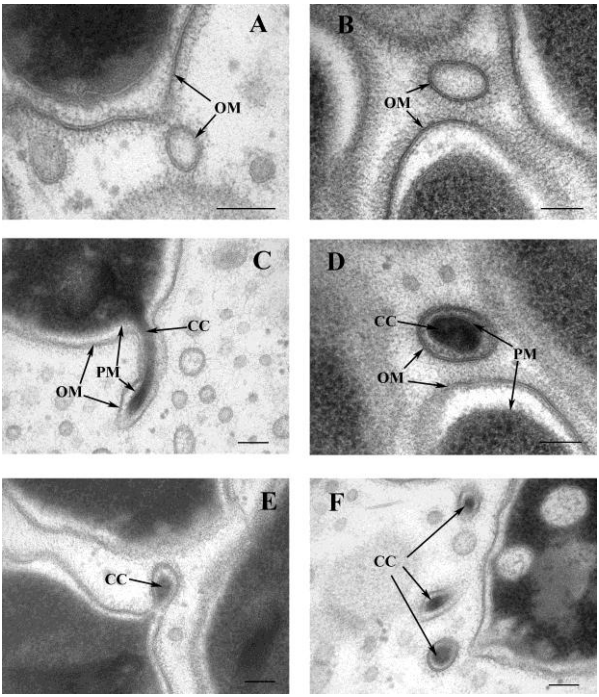

Figure 6. TEM micrographs of ultrathin sections from *S. vesiculosa* M7^T prepared by HPF-FS. (A and B) A view of OMVs extruded from cells. Only one bilayer is observed around the vesicles, with the same structure as the outer membrane (OM) of the cell (arrows). (C) OMVs being released from cells and dragging the plasma membrane (PM) and a portion of the cytoplasmic content (CC) in addition to the OM. (D) The same type of vesicle is depicted in panel C but once it is outside the cell. (E and F) More views of OMVs that on release have incorporated CC surrounded by the PM. Bars, 100 nm (A, C, E) and 200 nm (B, D, F). (From Appl. Environ. Microbiol. 2013.79(6):1874-81).

cytoplasm. Although much less common than the conventional OMVs, these singular double-layered vesicles were apparent in many of the sections analyzed.

The presence of these double-bilayer OMVs was confirmed by cryo-TEM of thin frozen films of isolated OMVs. For this purpose, total OMVs from *S. vesiculosa* M7^T were isolated from liquid cultures. Vesicles were collected from exponentially growing cultures to avoid the presence of lysed cells. Single-bilayer OMVs (Fig.7, white arrow) predominated in all observed

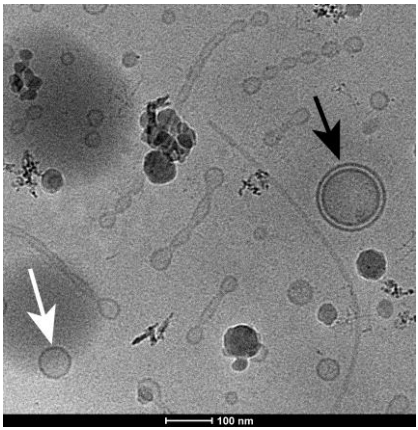

Figure 7. Isolated OMVs from *S. vesiculosa* M7^T observed by cryo-TEM. Two types of OMVs can be seen: most vesicles have a single membrane (white arrow), but occasionally vesicles with two membranes are observed (black arrow). Bar, 100 nm. (From Appl. Environ. Microbiol. 2013.79(6):1874-81).

fields, but double-bilayer OMVs were also present (black arrow), always with a round shape, unlike those in TEM sections. After counting 9,000 vesicles visualized by cryo-TEM of thin frozen foils, we found that 0.1% of the total vesicles corresponded to new double-bilayer OMVs.

OMVs obtained from liquid cultures of *S. vesiculosa* M7^T were also used to quantify their DNA content before and after DNase treatment, using the PicoGreen assay. The DNA content of OMVs was 2.1 ± 0.4 ng DNA/μg OMV protein before DNase treatment and 1.8 ± 0.24 ng DNA/μg OMV protein afterwards. This result confirmed that most DNA was inside the vesicles and not surface-associated, since approximately 85% remained after DNase treatment.

To further characterize OMVs from *S. vesiculosa* M7^T and verify that DNA was within the vesicles rather than surface-associated in a DNase-resistant manner, we performed immunogold labeling with an antibody specific for double-stranded DNA. Isolated OMVs from exponentially growing cultures were first treated with DNase before cryoimmobilization and HPF-FS to eliminate DNA present outside the vesicles, and then the immunogold technique to label DNA was applied to Lowicryl HM20 thin sections of *S. vesiculosa* M7^T OMVs. Subsequent TEM observations showed the presence of both types of vesicles: (i) conventional or single-bilayer OMVs, which were rarely marked with gold and contained non-electron-dense material (Fig. 8A) and (ii) OMVs with two bilayer membranes (Fig. 8B).

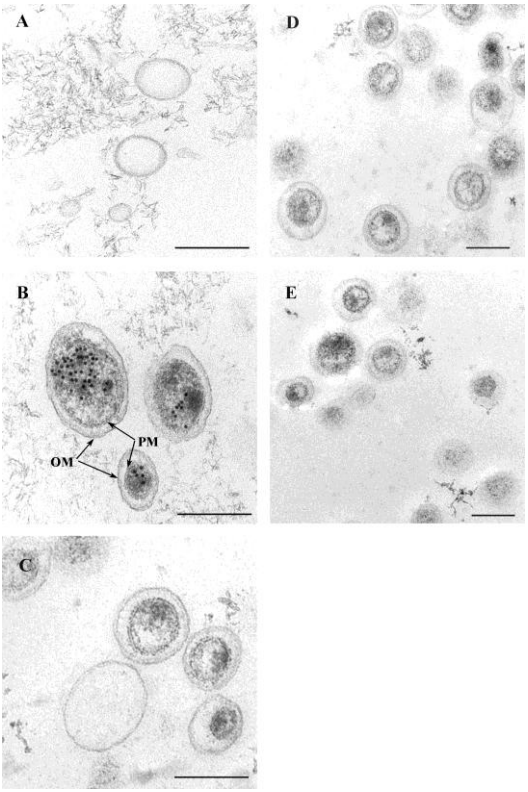

Figure 8. DNA immunolabeling on Lowicryl HM20 thin sections of isolated OMVs from *S. vesiculosa* M7^T. (A) TEM micrograph showing single-bilayer OMVs immunolabeled with a monoclonal IgM specific against dsDNA and a secondary goat anti-mouse antibody coupled to 12-nm colloidal gold. No gold mark or electron-dense material is observed inside these vesicles. (B) TEM micrograph showing double-bilayer OMVs immunolabeled like the vesicles in panel A. The outer layer corresponds to the outer membrane (OM) of the cell and the inner layer to the plasma membrane (PM) of the cell. Vesicles are filled with an electron-dense material, and a gold mark is visualized inside the inner layer. (C) TEM micrograph of OMVs labeled only with the secondary antibody. Single- and double-bilayer OMVs are visualized without any gold marking. (D) TEM micrograph of OMVs labeled with a primary IgM monoclonal antibody to *Plasmodium falciparum* with no affinity to DNA and a secondary antibody coupled to 12-nm colloidal gold. No gold mark is observed. (E) TEM micrograph of OMVs from grids preincubated with 1 mg/ml DNase I and then immunolabeled with IgM dsDNA and a secondary antibody coupled to gold. No gold mark is observed. Bars, 200 nm. (From Appl. Environ. Microbiol. 2013.79(6):1874-81).

The latter showed an external bilayer membrane that corresponded to the cell OM and an inner membrane also with a bilayer structure that corresponded to the cell PM, as depicted in Fig. 6. These double-bilayer vesicles were filled with an electron-dense material, and most of them exhibited a highly visible gold mark (Fig. 8B). As expected, the gold marker was not seen outside the vesicles due to previous DNase treatment before HPF (Fig. 8A and B). To check that gold immunolabeling was specific, we performed several control experiments (see Fig. 8C, D and E).

Several proteomic studies have described the presence of cytoplasmic and PM proteins inside Gram-negative bacterial OMVs, although all the vesiculation mechanisms proposed so far rule out the presence of such components [7,9]. To identify protein components of *S. vesiculosa* $M7^T$-derived OMVs we used a proteomic approach with 1-D SDS-PAGE and nano-LC-MS/MS analysis and their subcellular localization was analyzed using the PSORTb v3.0.2 program [22]. Along with the proteins mentioned in the previous section, the proteomic study also identified the presence of cytoplasmic membrane proteins within *S. vesiculosa* $M7^T$-derived OMVs, such as cytochrome c oxidase and nucleoside transporters (CM) (6.5%), and cytoplasmic proteins such as FoF1 ATP synthase and Na+-quinone reductase (C) (4.3%).

This previously undescribed type of OMVs naturally produced by *S. vesiculosa* $M7^T$, containing not only the cell OM but also PM and CC, with the consequent ability to entrap DNA, were named outer-inner membrane

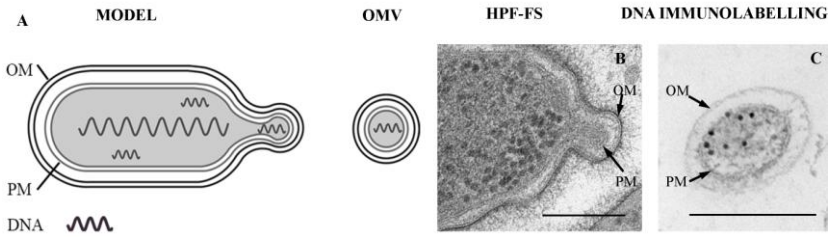

Figure 9. (A) Model proposed for the formation of new O-IMVs in Gram-negative bacteria and packaging of DNA. Plasma membrane and cytoplasmic content are included in the vesicle leaving the cell, thus allowing DNA to be incorporated. (B) TEM micrograph of an *S. vesiculosa* $M7^T$ cell supporting the model in panel A. (C) TEM micrograph showing an isolated double-bilayer vesicle from this strain after immunolabeling with a dsDNA antibody. OM, outer membrane; PM, plasma membrane; OMV, outer membrane vesicle; HPF-FS, high-pressure freezing and freeze-substitution. Bars, 200 nm. (From Appl. Environ. Microbiol. 2013.79(6): 1874-81).

vesicles (O-IMV). This important finding corroborates a model proposed by Beveridge's group to explain how CC and DNA can be incorporated into OMVs before they are released from the cell [19].

4. Conclusions

A common feature of most cold-adapted Antarctic bacteria is the abundant production of complex extracellular matter, which probably performs more than one function. The net-like mesh surrounding the cells composed of polymers and protein-charged OMVs most likely can modify the physicochemical properties around the cells, creating a micro-environment that may promote bacterial survival in a harsh environment. For instance, capsular polymers may have a cryoprotectant role and help in cell adhesion, and the polymeric fibers around vesicles could preserve the net-like mesh structure. On the other hand, the huge amount of OMVs entrapped in this mesh could be a source of different proteins with diverse functions, such as nutrient sensing, transport, and polysaccharide biosynthesis, as well as adhesins and degradative enzymes, which may help to concentrate nutrients around the cells.

We have demonstrated the existence of a previously unobserved type of double-bilayer OMV in the Gram-negative bacterium *Shewanella vesiculosa* $M7^T$ that can incorporate DNA. The presence of DNA inside bacterial OMVs and the possibility that these structures constitute a new mechanism of lateral gene transfer have important implications in several areas, including prokaryotic evolution and in particular the transfer of antibiotic resistance genes or virulence genes within bacteria. For this reason, these new OMVs deserve further study, and future work will be directed to demonstrating their existence in pathogenic bacteria for which DNA transfer through OMVs has been already reported.

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