New Type of Outer Membrane Vesicle Produced by the Gram-Negative Bacterium *Shewanella vesiculosa* M7^T: Implications for DNA Content

Carla Pérez-Cruz,^a Ornella Carrión,^a Lidia Delgado,^a Gemma Martínez,^b Carmen López-Iglesias,^b Elena Mercade^a

Laboratori de Microbiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain^a; Crio-Microscòpia Electrònica, Centres Científics i Tecnològics, Universitat de Barcelona, Barcelona, Spain^b

Outer membrane vesicles (OMVs) from Gram-negative bacteria are known to be involved in lateral DNA transfer, but the presence of DNA in these vesicles has remained difficult to explain. An ultrastructural study of the Antarctic psychrotolerant bacterium *Shewanella vesiculosa* M7^T has revealed that this Gram-negative bacterium naturally releases conventional one-bilayer OMVs through a process in which the outer membrane is exfoliated and only the periplasm is entrapped, together with a more complex type of OMV, previously undescribed, which on formation drag along inner membrane and cytoplasmic content and can therefore also entrap DNA. These vesicles, with a double-bilayer structure and containing electron-dense material, were visualized by transmission electron microscopy (TEM) after high-pressure freezing and freeze-substitution (HPF-FS), and their DNA content was fluorometrically quantified as 1.8 ± 0.24 ng DNA/ μ g OMV protein. The new double-bilayer OMVs were estimated by cryo-TEM to represent 0.1% of total vesicles. The presence of DNA inside the vesicles was confirmed by gold DNA immunolabeling with a specific monoclonal IgM against double-stranded DNA. In addition, a proteomic study of purified membrane vesicles confirmed the presence of plasma membrane and cytoplasmic proteins in OMVs from this strain. Our data demonstrate the existence of a previously unobserved type of double-bilayer OMV in the Gram-negative bacterium *Shewanella vesiculosa* M7^T that can incorporate DNA, for which we propose the name outer-inner membrane vesicle (O-IMV).

In recent years, many studies have been conducted on outer membrane vesicles (OMVs) produced by Gram-negative bacteria (1). It is now commonly accepted that these small spherical structures (20 to 250 nm) are extruded from the outer membrane of the cell and thus contain bacterial lipids, outer membrane proteins, periplasmic content, and other insoluble components that are delivered to the environment to accomplish several functions. OMVs are involved in pathogenesis, interspecies communication, biofilm formation, nutrient acquisition, and DNA transfer (2, 3, 4, 5, 6, 7, 8, 9, 10). 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 (11, 12, 13, 14, 15, 16, 17, 18, 19). Although reported in several studies, the presence of DNA in OMVs has remained difficult to explain, particularly since all the vesiculation mechanisms proposed to date rule out the presence of any cytoplasmic membrane and therefore of any cytoplasmic components (1, 6, 18, 20). Clearly, OMVs encapsulate DNA, but the mechanism of DNA packaging into OMVs has not been conclusively demonstrated (1, 9, 16).

Various models have been proposed to explain DNA packaging into OMVs (4, 9, 16). In one model, extracellular DNA released after bacterial lysis is internalized in the vesicles by a mechanism similar to that used in bacterial transformation. Renelli and coworkers reinforced this model by demonstrating that OMVs can take up “naked” plasmid DNA (16). They also proposed that exogenous DNA can be internalized by the opening and closing of a small proportion of OMVs, but the amount of DNA detected could not be explained if this was the only active mechanism. Another model involves the incorporation of DNA into OMVs before their release. In this case, it is assumed that the DNA some-

how passes from the cytoplasm through the plasma membrane to be encapsulated within an OMV once in the periplasm. Although these models have not been sufficiently backed up by experimental evidence, it has been suggested that probably both these naturally occurring mechanisms are involved in DNA incorporation into OMVs (8, 9, 16).

A third model was proposed by Kadurugamuwa and Beveridge (4) to explain the presence of some cytoplasmic constituents in natural and gentamicin-induced OMVs from *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, the images provided by transmission electron microscopy (TEM) were inconclusive, and the existence of a new type of double-layered membrane vesicle was not demonstrated.

Since their study, methods for analyzing bacteria with thin-section TEM have greatly improved (21). Vitrification of specimens by high-pressure freezing (HPF) and freeze-substitution (FS) preserves structural details almost in the “native” state, and

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Address correspondence to Elena Mercade, mmercade@ub.edu.

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this approach is also theoretically the best for preserving structure under conditions compatible with immunogold labeling (22). Our research group has used TEM after HPF and FS to show that cold-adapted Antarctic bacteria produce huge amounts of OMVs that accumulate in their extracellular matter (23, 24). Specifically, *Shewanella vesiculosa* M7^T was named on the basis of its considerable capacity for producing OMVs (25), which makes it a potentially excellent model for studying the vesiculation process. Previous structural studies with this strain allowed us to observe that this Gram-negative bacterium produces different types of OMVs, including conventional vesicles with a single bilayer and another, more complex type with a double bilayer and containing electron-dense material.

We here report TEM, cryo-TEM, and proteomic studies conducted to confirm a new model of vesiculation in *S. vesiculosa* M7^T that leads to the formation of a different type of outer membrane vesicle with a double-bilayer structure, which encapsulates DNA and thus could be involved in DNA transfer.

MATERIALS AND METHODS

Bacteria and growth conditions. All studies were performed with the cold-adapted Antarctic bacterium *Shewanella vesiculosa* M7^T, which grows at temperatures ranging from -4°C to 34°C . For TEM, *Shewanella vesiculosa* M7^T was grown on Trypticase soy agar (TSA) (Oxoid) for 5 days at 15°C , and for OMV isolation, the strain was grown on Trypticase soy broth (TSB) (Oxoid) for 2 days at 15°C , unless otherwise specified. For liquid cultures, an orbital shaker at 100 rpm was used.

High-pressure freezing and freeze-substitution (HPF-FS) of *S. vesiculosa* M7^T. For the TEM observation of *S. vesiculosa* M7^T cells, electron microscopy analysis of Epon-embedded thin sections was performed as described by Frias et al. (23). Briefly, bacterial colonies were cryoimmobilized using a Leica EMPact high-pressure freezer (Leica, Vienna, Austria). Frozen samples were freeze-substituted in a Leica EM automatic freeze substitution (AFS) system (Leica, Vienna, Austria), where the substitution was performed in pure acetone containing 2% (wt/vol) osmium tetroxide and 0.1% (wt/vol) uranyl acetate at -90°C for 72 h. The temperature was gradually increased ($5^{\circ}\text{C}/\text{h}$) to 4°C , held constant for 2 h, and then finally increased to room temperature and maintained for 1 h. Samples were washed for 1 h in acetone at room temperature and infiltrated in a graded series of Epon-acetone mixtures: 1:3 for 2 h, 2:2 for 2 h, 3:1 for 16 h, and pure Epon 812 (Ted Pella, Inc.) for 30 h. Samples were embedded in fresh Epon and polymerized at 60°C for 48 h.

Sectioning and electron microscopy analysis. Ultrathin sections were cut with a Leica UCT ultramicrotome and mounted on Formvar carbon-coated copper grids. Sections were poststained with 2% (wt/vol) aqueous uranyl acetate and lead citrate and examined in a Tecnai Spirit electron microscope (FEI Company, Netherlands) at an acceleration voltage of 120 kV. Vesicle size was determined with the program analySIS (Soft Image System, Switzerland).

Isolation and purification of OMVs from culture medium. OMVs from *S. vesiculosa* M7^T naturally secreted into the medium were collected from broth cultures (500 ml) in the late log phase by an adaptation of the method described by McBroom and coworkers (26). The cells were pelleted by centrifugation at $10,000 \times g$ for 10 min at 4°C , and the supernatant was filtered through $0.45\text{-}\mu\text{m}$ -pore-size filters to remove remaining bacterial cells. OMVs were obtained by centrifugation at $38,400 \times g$ for 1 h at 4°C in an Avanti J-20 XP centrifuge (Beckman Coulter, Inc.). Pelleted vesicles were resuspended in 25 ml of 50 mM HEPES (pH 6.8) and filtered through $0.45\text{-}\mu\text{m}$ -pore-size Ultrafree spin filters (Millipore). Vesicles were again pelleted as described above and finally resuspended in an adequate volume of 50 mM HEPES, pH 6.8.

Vesicles were further purified for proteomic analysis using a method adapted from that described by Horstman and Kuehn (3). The crude vesicle sample was adjusted to 45% OptiPrep density gradient medium

(Sigma) in 10 mM HEPES–0.85% (wt/vol) NaCl (pH 6.8) (HEPES–NaCl). OptiPrep gradients were layered over the 4-ml crude vesicle sample and centrifuged ($100,000 \times g$, 20 h), and fractions of equal volume were removed sequentially from the top. A portion of each fraction was visualized by 12% SDS-PAGE and Coomassie blue staining. Fractions containing vesicles were pooled, dialyzed with 10 mM HEPES, and concentrated with Amicon ultracentrifugal filter devices (Millipore).

DNA immunolabeling of OMV thin sections. *S. vesiculosa* M7^T OMVs were isolated as described above. Before carrying out HPF-FS and immunolabeling, vesicle samples were treated with 50 $\mu\text{g}/\text{ml}$ DNase I (Sigma) and 10 mM MgCl_2 (1 h at 37°C) to remove the DNA outside the vesicles. HPF-FS of OMVs was done as described above but with the following modifications to perform further immunolabeling. After HPF, substitution was performed in pure methanol containing 0.5% (wt/vol) uranyl acetate at -90°C for 80 h. The temperature was gradually increased ($5^{\circ}\text{C}/\text{h}$) to -50°C . Samples were then washed for 3.5 h in methanol at -50°C and infiltrated in a graded series of HM20-methanol mixtures: 1:3 for 3 h, 2:2 overnight, 3:1 for 6 h, and 3 changes of pure HM20 (Electron Microscopy Sciences) for 48 h. Samples were polymerized at -50°C for 24 h by indirect UV irradiation (360 nm), followed by further hardening at room temperature for 48 h. Ultrathin sections were obtained as described above. Colloidal gold DNA immunolabeling was carried out as follows. Unless specified, washing steps were carried out by floating the grids face down on drops. Grids with sections were floated on 0.1 M phosphate-buffered saline (PBS) for 3 min. The grids were blocked on 0.1 M PBS–50 mM glycine and rinsed with 0.1 M PBS, again with 5% bovine serum albumin (BSA) (Sigma)–0.1 M PBS (1 drop for 3 min and 1 drop for 12 min), and again with 1% BSA–0.1 M PBS (1 drop for 8 s). Next, the grids were incubated with monoclonal IgM specific for double-stranded DNA (dsDNA) (clone AC-30-10; Novus Biologicals, Littleton, CO), diluted 1/2 in 1% BSA–0.1 M PBS, for 1 h. Grids were washed for 5 min on 5 drops of 0.25% Tween 20–0.1 M PBS followed by 3 min on 1 drop of 1% BSA–0.1 M PBS. After that, grids were incubated for 30 min with a secondary goat anti-mouse antibody coupled to 12-nm colloidal gold (lot 84359; Jackson) diluted 1/20 in 1% BSA–0.1 M PBS. Grids were washed with 0.1 M PBS followed by double-distilled water and then floated on 1% glutaraldehyde–0.1 M PBS for 5 min. Grids were rinsed with double-distilled water and dried with filter paper. Finally, grids were poststained with 2% uranyl acetate–methanol for 5 min, 70% methanol for 3 min, 50% methanol for 3 min, and 30% methanol for 3 min, jet-washed with double-distilled water, air dried for 20 min, stained with lead citrate for 2 min, and jet-washed with double-distilled water. Several controls were used. First, the dsDNA monoclonal antibody was omitted. Second, a primary IgM monoclonal antibody to *Plasmodium falciparum* (clone 11B7; Acris Antibodies GmbH) with no affinity for dsDNA was also employed. Third, the grids were preincubated at 37°C for 6 h with 1 mg/ml DNase I (Sigma) in PBS plus 7 mM MgCl_2 , and then the grids were thoroughly washed with water before immunolabeling with dsDNA monoclonal antibody.

Fluorometric quantification of DNA. Surface-associated DNA and DNA contained within OMVs were quantified by the PicoGreen assay (Molecular Probes). For this purpose, OMVs were collected independently from three exponentially growing cultures (500 ml), and DNA was measured as described by Renelli et al. (16) with some modifications. For each experiment, two 30- μg protein samples from OMVs were prepared. One was pretreated with 50 $\mu\text{g}/\text{ml}$ pancreatic DNase I (Sigma) and 10 mM MgCl_2 (1 h at 37°C) to digest DNA located outside the OMVs, after which the DNase was inactivated. Both OMV samples were lysed with 0.125% Triton X-100 solution. Samples were further processed according to the manufacturer's instructions, and fluorescence was measured in a Hitachi F-2000 fluorescence spectrophotometer. Results are expressed as mean $\pm$ standard deviation (SD).

Cryo-TEM. Isolated OMVs were visualized by cryo-transmission electron microscopy (cryo-TEM). A thin aqueous film was formed by dipping and withdrawing a bare specimen grid with OMVs resuspended in deionized water. Glow-discharged holey carbon grids were used. The excess

liquid was blotted with filter paper, leaving thin sample films spanning the grid holes. These films were vitrified by plunging the grid into ethane, which was kept at melting point with liquid nitrogen using a Vitrobot (FEI Company, Eindhoven, Netherlands), and the sample was maintained before freezing at 100% humidity and at room temperature. The vitreous sample films were transferred to a Tecnai F20 microscope (FEI Company, Eindhoven, Netherlands) using a Gatan cryotransfer system (Gatan Inc., Pleasanton, CA). Cryo-TEM visualizations were carried out at a temperature between -170°C and -175°C and an accelerating voltage of 200 kV. Images were acquired using low-dose imaging conditions and an Eagle 4k $\times$ 4k charge-coupled device (CCD) camera (FEI Company, Eindhoven, Netherlands). The different types of vesicles were analyzed, and the proportion of each was measured from digital images using the analySIS software (Soft Image System, Switzerland).

Proteomic analysis. A proteomic analysis of purified OMVs was carried out using one-dimensional (1-D) SDS-PAGE and nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described by Frias et al. (23). Briefly, proteins were in-gel digested with trypsin, and then tryptic peptides were extracted from the gel matrix, pooled, and dried in a vacuum centrifuge. Purified peptides were injected for chromatographic separation in a reverse-phase capillary C_{18} column, and the eluted peptides were subsequently analyzed on a nano-ESI-QTOF mass spectrometer (Q-TOF Global, Micromass-Waters). Data generated in PKL file format were submitted for database searching in the MASCOT server. For the database search, only tandem mass spectra of +2 and +3 charged ions were searched against the NCBI nonredundant protein sequence database. Proteins were identified by using the probability-based Mowse score, and individual ions scores of >53 indicate identity or extensive homology ($P < 0.05$). Bacterial protein subcellular localization was estimated with the software PSORTb v3.0.2 (27).

RESULTS

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. 1A and B). These vesicles were derived from the outer membrane of *S. vesiculosa* M7^T cells, as can be clearly observed in Fig. 1A. The vesicle membranes showed the same bilayer structure, width, and staining profile as the outer cell membrane. In addition, the OMVs were surrounded by the same fringe of fine fibers as the cells and contained a low-electron-density similar material to that in the periplasmic space of the cells. Unexpectedly, however, in some *S. vesiculosa* M7^T cells we noted the formation of membrane vesicles in which both the outer membrane (OM) and plasma membrane (PM) were extruded. During this vesiculation process, we observed that cytoplasmic content (CC) also became entrapped within the vesicle (Fig. 1C to F). Vesicles 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. 1C to F clearly depict. Inside this inner membrane, we observed an electron-dense material similar to that seen in the cell cytoplasm. Although much less common, these singular double-layered vesicles were apparent in many of the sections analyzed. They were observed more frequently out of the cell (Fig. 1D and F), and in a few cases it was possible to visualize the double-bilayer vesicles precisely at the moment of formation and before they were detached from the cell (Fig. 1C and D). Double-bilayer vesicles were observed in normal cells and not in those that were dividing. They seemed to bud preferentially from cell polar or subpolar sites but were also visualized elsewhere in the cell. It was remarkable that when ob-

served in TEM sections, several of the observed double-bilayer vesicles showed an elongated shape at the time of formation and expulsion from the cell, but once liberated, vesicles became almost round.

The presence of double-bilayer OMVs was also 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. 2, white arrow) predominated in all observed fields, but double-bilayer OMVs were also observed (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 determine their DNA content before and after DNase treatment. Vesicles were retrieved by high-speed centrifugation from three exponentially growing cultures, and DNA was quantified using the PicoGreen assay, which detects double-stranded DNA with minimal interference of fluorescence due to RNA or single-stranded DNA. 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 of OMVs with an antibody specific for double-stranded DNA. Isolated OMVs from exponentially growing cultures were first treated with DNase before cryoimmobilization and freeze-substitution (HPF-FS) to eliminate DNA present outside the vesicles, and then the DNA gold-immunolabeling technique was applied to thin sections of OMVs. TEM observations of Lowicryl HM20 thin sections of *S. vesiculosa* M7^T OMVs also showed the presence of both types: (i) conventional or single-bilayer OMVs that were rarely marked with gold and contained non-electron-dense material (Fig. 3A) and (ii) OMVs with two bilayer membranes (Fig. 3B). The latter showed an external bilayer membrane that corresponded to the cell OM and an inner membrane also showing a bilayer structure that corresponded to the cell PM, as depicted in Fig. 1. These double-bilayer vesicles were filled with an electron-dense material, and most of them exhibited a highly visible gold mark (Fig. 3B). As expected, the gold marker was not seen outside the vesicles due to previous DNase treatment of OMVs before high-pressure freezing (Fig. 3A and B). To check that gold immunolabeling was specific, we performed several control experiments. First, sections were treated only with the secondary antibody, and no gold marking was observed (Fig. 3C). Second, we used a primary IgM antibody with no affinity for DNA and found that double-bilayer OMVs had no gold mark at all (Fig. 3D). Finally, when grids with sections were preincubated at 37°C for 6 h with 1 mg/ml DNase and then immunolabeled with the anti-DNA antibody, gold markers were not detected, showing that the DNA within double-bilayer vesicles was degraded by DNase treatment (Fig. 3E).

To identify protein components of *S. vesiculosa* M7^T-derived OMVs and determine their subcellular localization, we used a proteomic approach with 1-D SDS-PAGE and nano-LC-MS/MS analysis. For the proteomic analysis, isolated OMVs were further

FIG 1 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 observed in panel C but once 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).

purified on an OptiPrep density gradient to remove contaminants. The protein profile and negative staining of *S. vesiculosa* M7^T purified OMVs are shown in Fig. 4. In Fig. 4B, we can see a double-bilayer OMV, but as OMVs can collapse on negative staining, this is not a good technique for a clear analysis of structural details. Protein bands (Fig. 4A) were excised from the gel and digested with trypsin. Peptides were separated by liquid chromatography and subsequently analyzed using a nano-ESI-QTOF mass spectrometer. Data were submitted for database searching in a MASCOT server and were searched against the NCBI nonredundant protein sequence database. The subcellular localization of proteins was analyzed using the PSORTb v3.0.2 program (24). The genome sequence for this bacterium is not available, and pro-

teins were putatively identified by their similarity to proteins from related species. Only 46 proteins were identified despite achievement of good mass spectra with more peptides, which was probably due to the significant differences between the protein sequence of this species and those of its counterparts in the database. The putative functions and subcellular localization of the proteins identified in OMVs from *S. vesiculosa* M7^T are summarized in Table S1 in the supplemental material. As expected, the identified vesicular proteins were mainly from the outer membrane (OM) (69.57%), with most of them being TonB-dependent receptors and porins involved in inorganic ion transport and metabolism. The two prominent bands observed in 1-D SDS-polyacrylamide gels belonged to this category (Fig. 4A, see arrows), with the first

FIG 2 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.

one corresponding to a TonB-dependent receptor (first protein listed in Table S1 in the supplemental material), while the second band included more than one protein, also mostly from the family of TonB-dependent receptors (proteins from rows 2 to 7 in Table S1 in the supplemental material). The few periplasm proteins identified (P) (4.35%) were mainly proteases. Another peptidase was localized as extracellular protein (EC) (2.2%). The proteomic study also identified the presence within *S. vesiculosa* M7^T-derived OMVs of cytoplasmic membrane proteins such as cytochrome *c* oxidase and nucleoside transporters (CM) (6.5%) and cytoplasmic proteins such as F₀F₁ ATP synthase and Na⁺-translocating NADH-quinone reductase (C) (4.3%). The subcellular localization of some proteins, mainly hypothetical, was unknown (U) (13%).

DISCUSSION

Numerous studies, particularly on pathogenic bacteria, have shown that OMVs can contain DNA and, in some cases, transfer it to other bacteria (9, 11, 12, 14, 15, 16, 17, 19). The discovery that OMVs can function as a lateral DNA transfer mechanism in bacteria has important implications. 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 (9, 19). 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 (1, 4, 8, 9, 16).

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) (25). This strain can grow at temperatures ranging from −4°C to 34°C, and one of its prominent traits is an ability to produce a huge amount of natural OMVs from solid or liquid cultures without any inducing factors such as the addition of membrane-perturbing agents. Consequently, *S. vesiculosa* M7^T is an excellent bacterium for exploring the vesiculation process.

Structural analysis of the strain by TEM gave us an insight into a possible mechanism that would explain the presence of DNA inside OMVs from a Gram-negative bacterium. What drew our attention was that among the large number of single-bilayer OMVs produced by the *S. vesiculosa* M7^T cells, we could see a different type of vesicle surrounded by a double bilayer and containing electron-dense material. We were able to repeatedly visualize this new type of double-bilayer OMV by TEM after HPF-FS in many thin sections of cells of this strain.

It was first necessary to rule out that these new vesicles were artifacts of the microscopic technique. Sample cryoimmobilization by rapid cooling and freeze-substitution is an accepted approach to visualizing samples very close to the native structure (21) and is also one of the best methods for preserving structure under conditions compatible with immunogold labeling (22). Some shrinkage of the specimen is often inevitable and modifications at the molecular level can occur, but what cannot be attributed to the technique is the appearance of vesicles, either with one or with two bilayers. It was particularly remarkable that for some *S. vesiculosa* M7^T cells, both types of vesicles were captured at the very moment they were protruding from the cells. Even for the conventional OMVs described to date, it has proved extremely difficult to visualize vesicles at this stage of formation, and practically no quality images are available in the numerous published reports (8, 15, 20, 28). These double-bilayer vesicles were repeatedly observed in sections of *S. vesiculosa* M7^T cells grown on solid media and among vesicles harvested from liquid cultures in the exponential growth phase. Moreover, double-bilayer vesicles isolated from liquid media were also observed using the cryo-TEM technique, in which the specimen was undoubtedly “native” and free of chemical artifacts. The reliable images produced by state-of-the-art TEM techniques allowed us to confirm that *S. vesiculosa* M7^T produces both single-bilayer and double-bilayer OMVs.

The images we obtained of double-bilayer OMVs of *S. vesiculosa* M7^T corroborate one of the models proposed by Kadurugamuwa and Beveridge (4) to explain how certain macromolecules in *Pseudomonas aeruginosa*, such as cytoplasmic enzymes and DNA, are exported via membrane vesicles. These authors proposed that a transient breach in the peptidoglycan during OMV development leads to the formation of vesicles that contain both inner and outer membranes as well as cytoplasmic constituents such as DNA. Indeed, micrographs produced in the current study match the model proposed by Kadurugamuwa and Beveridge (Fig. 5). The mechanism of formation of what they call “complicated” membrane vesicles containing cytoplasmic content implies a localized and transient action of autolysins that weakens the peptidoglycan layer just at the point where OMVs are blebbing. Therefore, the formation of these vesicles supposes an overproduction of peptidoglycan hydrolases, and the authors proved that *P. aeruginosa*-derived OMVs were enriched with hydrolyzing enzymes, providing vesicles with a lytic activity against other bacteria. Similarly, OMVs of *S. vesiculosa* M7^T induced cell lysis when brought into contact with several Gram-positive Antarctic bacteria, suggesting the presence of hydrolytic enzymes (data not shown).

P. aeruginosa-derived OMVs with cytoplasmic content that were overproduced after gentamicin treatment were larger than conventional OMVs (4). Although OMV size is generally accepted to be highly variable (20 to 250 nm) (1, 21), we observed that double-bilayer vesicles of *S. vesiculosa* M7^T were also larger than

FIG 3 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 of the cell (OM) and the inner layer to the plasma membrane of the cell (PM). Vesicles are filled with an electron-dense material, and 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 with no gold mark at all. (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 a dsDNA IgM and a secondary antibody coupled to gold. No gold mark is observed. Bars, 200 nm.

FIG 4 (A) Protein profile of *S. vesiculosa* M7^T purified OMVs using 12.5% 1-D SDS-PAGE. Numbers on the left correspond to molecular weight (MW) in thousands. Arrows indicate the two prominent bands detected. (B) Negative-staining micrograph from purified *S. vesiculosa* M7^T OMVs. A double-bilayer vesicle can be observed with an inner layer that corresponds to the plasma membrane (PM) and an outer layer corresponding to the outer membrane (OM). Bar, 50 nm.

single-bilayer vesicles, with diameters commonly ranging from 100 to 250 nm, which suggests that the two types of OMVs undergo different formation processes.

Of all the OMVs observed in *S. vesiculosa* M7^T, 0.1% were quantified by cryo-TEM as double bilayered, which seems reasonably accurate, since a higher proportion could compromise cell viability. We should take into account that the formation of these double-bilayer vesicles implies a perturbation of the integrity of the peptidoglycan layer, resulting in the loss of both cytoplasmic membrane and content, which could also lead to cell death. This proportion was estimated among vesicles retrieved from exponentially growing cultures to avoid cell lysis, which could generate membrane fragments of bacterial envelopes that reseal in solution. We should also point out that no opened vesicles were observed in the cryo-TEM analysis of OMVs from *S. vesiculosa* M7^T. In future studies it will be interesting to determine if the proportion of double-bilayer OMVs from *S. vesiculosa* M7^T depends on the growth phase and growth conditions for this strain.

The proteomic study of OMVs from *S. vesiculosa* M7^T also confirmed the presence of proteins and enzymes from the plasma membrane and cytoplasm, thus corroborating that both inner membrane and cytoplasmic content were included in these new

vesicles. Other accurate proteomic studies of OMVs from Gram-negative bacteria have also reported the presence of enzymes from the plasma membrane and cytoplasm but these studies have not clarified the mechanism involved (1, 6, 28, 29).

Fluorometric DNA detection confirmed that *S. vesiculosa* M7^T is capable of exporting dsDNA inside vesicles. The DNA content of natural OMVs from this Antarctic strain was higher than values reported for those of *Pseudomonas aeruginosa* (16) and several strains of *Escherichia coli* O157:H7 (15). This can be explained by the particular ability of *S. vesiculosa* M7^T to produce these double-layered OMVs with cytoplasmic content and the capacity of the strain to form a huge amount of vesicles in general. The amount of DNA within OMVs seems to be a variable parameter within the same bacterial strain and among different strains of the same bacterial species (15), and this variability was also detected in *S. vesiculosa* M7^T.

The TEM immunolabeling technique with an antibody highly specific for dsDNA detection clearly demonstrated that the DNA quantified in *S. vesiculosa* M7^T-derived OMVs was mostly packaged in double-layered vesicles. DNA content reported in OMVs from different Gram-negative bacteria has been quantified using other methods, but its presence inside the vesicles has never been visualized (4, 15, 16) before the present work.

The DNA detected in OMVs from *S. vesiculosa* M7^T was small (≈ 600 bp), and no plasmids from this strain were detected inside vesicles (data not shown). For the moment, it is difficult to propose a role for these double-bilayer OMVs from *S. vesiculosa* M7^T or to demonstrate if they are involved in DNA transfer within the strain or between other related strains in the Antarctic environment, mainly because a genomic sequence of this strain is not available yet and no genes related to enzymes, virulence factors, or other proteins have been identified inside OMVs.

We can conclude that *S. vesiculosa* M7^T naturally produces a previously undescribed type of OMV that contains not only the outer membrane of the cell but also its plasma membrane and cytoplasmic content, with the consequent ability to entrap DNA. We propose the name outer-inner membrane vesicle (O-IMV) for this new type of double-bilayer OMV. This finding is important because it corroborates a model proposed by Beveridge's group to explain how cytoplasmic components and DNA can be incorporated into OMVs before they are released from the cell. Future work will be directed to demonstrating the existence of this new type of double-bilayer vesicle in pathogenic bacteria for which DNA transfer through OMVs has been already reported.

FIG 5 (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 that is leaving the cell, and DNA can thus 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.

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