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Special article - Review

BACTERIAL EXTRACELLULAR VESICLES: THEIR NATURE AND ROLE IN HUMAN PATHOLOGY

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Abstract

Extracellular vesicles have been observed to bud from eukaryotic cells and bacteria. These membrane-bound, spherical structures range from 20 to 500 nm in diameter and enclose diverse molecular components. In eukaryotic cells, exosomes represent the prototypical extracellular vesicles. Gram-negative bacteria release outer membrane vesicles, whereas Gram-positive bacteria produce membrane vesicles despite their thick peptidoglycan-rich cell wall. More recently, outer-inner membrane vesicles have been described in both saprophytic and pathogenic bacteria. These vesicles carry proteins, lipids, enzymes, virulence factors, various types of RNA, and in some cases, DNA. Bacterial vesicles play critical roles in biofilm formation, antibiotic resistance, DNA transfer, delivery of virulence factors to eukaryotic cells, and immune response regulation. In this review we highlight the importance of these vesicles in human pathology, with particular emphasis on outer-inner membrane vesicles containing chromosomal DNA. We hypothesize that such vesicles may represent a novel adaptative strategy in non-sporulating bacteria to withstand environmental stress. This review is specifically aimed at biomedical professionals who are not specialists in microbiology.

Key words: bacteria, extracellular vesicles, outer-inner membrane vesicles

Resumen

Vesículas extracelulares bacterianas: su naturaleza e importancia en la patología humana

Las células eucarióticas y las bacterias liberan vesículas hacia el medio externo. Estas son estructuras esféricas cubiertas, al menos, de una membrana y con un diámetro aproximado de entre 20 y 500 nm. Las células eucarióticas producen exosomas, considerados prototipos de vesículas extracelulares. Las bacterias gramnegativas liberan vesículas de membrana externa, mientras que las bacterias grampositivas producen vesículas de membrana a pesar de su gruesa pared celular rica en peptidoglicano. Recientemente, fueron descriptas nuevas vesículas compuestas tanto por la membrana externa como por la membrana plasmática bacteriana, que se denominan O-IMVs. Todas ellas contienen proteínas, lípidos, enzimas, factores de virulencia, diferentes tipos de ARN y, algunas, ADN. Estas vesículas son importantes en la formación de biopelículas, la transferencia de ADN, especialmente asociada a la resistencia a antibióticos, la liberación de factores de virulencia y la regulación de la respuesta inmune. En este artículo destacamos la importancia de estas vesículas en la patología humana, con especial énfasis en las vesículas bacterianas O-IMVs que contienen ADN cromosómico y planteamos la hipótesis de que estas últimas podrían ser una

forma de resistencia de bacterias no esporuladas ante las condiciones adversas del medio. Esta revisión está especialmente dirigida a profesionales de la biomedicina no especialistas en microbiología.

Palabras clave: bacteria, vesículas extracelulares, vesículas de membrana externa-interna

KEY POINTS
Current knowledge

- Bacterial extracellular vesicles can be classified as Outer Membrane Vesicles in Gram-negative bacteria and Membrane Vesicles in Gram-positive bacteria. Both types are involved in cell-to-cell communication, biofilm formation, transport of virulence factors, antibiotic resistance and development of human diseases.

Article's contribution to current knowledge

- This article is intended for general practitioners without a strong connection with Microbiology. It addresses the relationship between bacterial extracellular vesicles and human infections, and a detailed description of the ultrastructure and functions of the recently discovered Outer-Inner Membrane Vesicles, using Enteroaggregative *Escherichia coli* as a prototype.

Extracellular vesicles (EVs) are nano or micro-sized particles, delimited by a lipid bilayer membrane, possibly released by all cell types[1]. Their size usually ranges from 20 to 500 nm, with an average diameter of approximately 100 nm, and they may be either empty or contain several contents[2]. Given their heterogeneity in size, origin, contents, and functions, the nomenclature of EVs remains somewhat confusing[3]. Moreover, it is uncommon to see just one type of EVs in a sample by transmission electron microscopy (TEM) but, instead, a mix of them even when derived from a single source[4]. Here we will use the terms "extracellular vesicles" to refer to vesicles produced by eukaryotic cells, as it is widely employed in the literature[5], in accordance with the recommendations of MISEV 2023[1], "outer membrane vesicles" (OMVs) to describe those EVs released by Gram-negative bacteria; and "membrane vesicles" (MVs) to designate EVs observed in Gram-positive bacteria.

The history of EVs has been thoroughly described by Couch et al[3]. Briefly, it was Charles Darwin who first hypothesized that cells of any type could produce particles able to establish a cell-to-cell communication, although at the time, this idea was not taken seriously by the scientific community[2,6]. In 1936, Chargaff and West speculated about the existence of particles involved in blood coagulation; their results were published in 1946[7] and those particles were later described as extracellular vesicles in 1967 by Wolf[8] using TEM. Subsequent studies demonstrated the presence of EVs released by alveolar cells[9], cartilage matrix[10] and thyroid cells[11], as well as the existence of multivesicular bodies[10]. EVs were also detected in body fluids and cancer cells[12]. In 1975, Dalton reported that EVs present in bovine serum were similar to those detected in epithelial cell lines[13]. This observation led to the recognition of EVs as structures with distinct characteristics, regardless of whether they are secreted by cells or present in fluids. All these studies described what are now considered exosomes. Therefore, we will regard exosomes as prototype extracellular vesicles produced by eukaryotic cells, while the main purpose of this review is to focus on microbial EVs, particularly for biomedical professionals who are not specialists in microbiology.

Summary of the methods employed to study extracellular vesicles

The first step in studying EVs (exosomes or bacterial vesicles) is to obtain them in sufficient quantity. This can be achieved, for example, by collecting the supernatant from cell cultures to isolate extracellular vesicles produced by eukaryotic cells, or by harvesting the broth in which bacteria have been grown. The specific methods employed depend on the purpose of each particular experiment, and every procedure has its own advantages and drawbacks[14]. In general, these approaches can be categorized into purification, size analysis, microscopic observation, biochemical and molecular characterization and, ultimately, functional assays.

Purification, size analysis and concentration

Precipitation using polyethylene glycol (PEG) or commercial kits followed by ultrafiltration through membranes with different molecular weight cutoffs can be employed. However, the purity of vesicles obtained by these methods is often low. Differential ultracentrifugation is the traditional gold standard method for vesicle isolation, but combinations incorporating density gradient centrifugation or size-exclusion chromatography have emerged as the gold standards for specific purification[14]. Other techniques based on immunoaffinity capture and microfluidic devices are also useful[15–17]. The size distribution and vesicle concentration can be assessed by nanoparticle tracking analysis (NTA), a method that combines the principles of Brownian motion and laser light scattering to study particles in liquid suspension[18].

Observation

Once vesicles have been purified, their observation by TEM is essential to define their ultrastructure, morphology, and size, and particularly to determine whether the sample contains a single vesicle type or a mixture of vesicles with different sizes and structures. Two TEM methods are commonly employed: negative staining and ultrathin sectioning. Both require specialized expertise, not only in sample preparation but also in image acquisition and interpretation. Negative staining provides a detailed resolution of vesicle surfaces and internal contents. In contrast, ultrathin sectioning requires purified vesicles to be centrifuged to form a pellet. Sections are obtained and stained with uranyl acetate and lead citrate, as described elsewhere[19,20]. This procedure allows vesicles to be clearly visualized in two-dimensional sections at different levels, revealing their contents and the trilaminar ultrastructure of the outer membrane. Scanning electron microscopy (SEM) can also be applied using standard procedures[21] providing three-dimensional images. Cryo-electron microscopy is arguably the best method for the study of vesicle ultrastructure as they are not fixed but frozen and imaged in near-native state[22].

Molecular and biochemical characterization

Vesicle proteins can be resolved in SDS-acrylamide gels, then blotted onto nitrocellulose or PVDF membranes, and detected with specific sera (Western blot), for example against CD63, CD81, CD9 in eukaryotic cells or outer membrane proteins in bacteria[23]. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) can be used for intravesicular RNA characterization[24], and PCR using specific primers can be employed to characterize single-stranded or double-stranded DNA[25]. Mass spectrometry can quantify and characterize lipids composing the single membrane that involves exosomes[26].

Functional assays

Once vesicles have been characterized ultrastructurally and biochemically, several functional assays can be performed. These include studies on vesicle uptake by cells, their internalization, cargo release, and interaction with cellular proteins[27]. Indirect immunofluorescence and confocal microscopy can be employed to visualize these processes[28]. Moreover, fluorescent labelling of exogenous EVs allows the assessment of vesicles biodistribution following inoculation in animals, with detection by whole-animal imaging[29]. Finally, gene expression and cellular signaling pathways activated by vesicles-cell interactions can be investigated using conventional methods[30,31].

Extracellular vesicles of eukaryotic cells

Eukaryotic cells produce a wide variety of EVs, including exosomes. Although, as stated above, the purpose of this review is to address the topic of bacterial exovesicle production, the description of exosomes produced by eukaryotic cells will provide a general idea of the complexity of exovesicle production mechanisms in general, since this aspect is less well known in bacteria. Exosomes are a heterogeneous group of rounded vesicles ranging from 40 to 160 nm in diameter (average ~100 nm) released by eukaryotic cells into the intercellular space and originated from the endoplasmic reticulum[32,33]. In contrast, ectosomes are vesicles -often larger than exosomes (50 nm to 1,000 nm in diameter) that bud directly from the plasma membrane[32]. The biogenesis of exosomes is a complex process that has been extensively reviewed[34–37]. Briefly, it begins with plasma membrane invagination through endocytosis, leading to the formation of early sorting endosomes (ESEs) that contain cell surface pro-

teins, extracellular proteins, lipids, metabolites and small molecules. These structures may also fuse with other ESEs derived from the endoplasmic reticulum, the trans-Golgi network or mitochondria, ultimately forming late sorting endosomes (LSEs) that incorporate both extracellular and intracellular components. Invagination of the LSEs membranes give rise to intraluminal vesicles (ILVs) which are precursors of exosomes. ILVs have vary in size and content and, eventually, may fuse with autophagosomes or form multivesicular bodies (MVBs). The fate of these MVBs will lead to their fusion either with lysosomes or with the inner face of the plasma membrane, resulting in the release of exosomes into the extracellular space. As a consequence of this process, free exosomes display several membrane biomarkers, such as tetraspanins (CD9, CD81 and CD63), integrins, flotillin, and ceramide, and contain a wide variety of proteins, lipids and nucleic acids. The latter include double-stranded, single-stranded, or mitochondrial DNA, and multiple types of RNAs, such as messenger, transfer and, especially, micro RNAs (miRNAs) which are small, non-coding RNA molecules of main importance in the regulation of gene expression[38]. The heterogeneity of exosome origin and cargo leads to different effects on recipient cells depending on receptor interactions, and may result in enhanced cell survival, immune modulation or, on the contrary, induction of apoptosis[32]. Exosomes participate in both physiological and pathological processes. For example, human semen contains free exosomes potentially associated with sperm maturation[39], trophoblastic cells can prevent placental infections by viruses through exosomes-released specific miRNAs[40] and human breast milk contains exosomes involved in immune modulation by miRNAs[41]. Of particular importance is the role of exosomes in immunity, as they contribute to macrophage homeostasis[42], viral pathogenesis, especially the Human Immunodeficiency Virus[43], and infections caused by bacteria, fungi, and parasites, with potential interactions with the OMVs described[44]. On the other hand, exosomes have been implicated in the pathogenesis of neurodegenerative, cardiac, and metabolic diseases and, especially in cancer[32,38,45] which are beyond the scope of this review.

Outer membrane vesicles in Gram-negative bacteria (OMVs)

The cell envelope of Gram-negative bacteria consists of an outer and inner membrane separated by a periplasmic space that contains a thin layer of peptidoglycan[46,47]. OMVs are spherical particles, 20-400 nm in diameter, released from the outer membrane during active bacterial growth[48,49]. OMVs were first hypothesized by Bishop *et al* in 1965 as extracellular glycolipids produced by *Escherichia coli*[50] and first observed by Chatterjee *et al* in 1967 using TEM[51]. Although OMVs were initially regarded as by-products of cell lysis[49,52], they are currently recognized as integral to bacterial physiology. OMVs contribute to interactions with host cells and other bacteria, mediate intercellular communication, promote biofilm formation and stimulate the immune system[49]. They contain outer membrane components as well as molecules from the periplasmic space, including proteins, enzymes, lipopolysaccharides (LPS), phospholipids, peptidoglycan, and nucleic acids[49]. Given that Gram-negative bacteria produce outer membrane proteins, it is not surprising that OMVs contain LPS and phospholipids, which compose their envelope with a similar -but not equal- nature with those of the bacterial outer membrane[53,54]. LPS not only exhibits the properties of an endotoxin but also may play an important role in the biogenesis of OMVs[55] including their shape and abundance. In addition, OMVs carry messenger RNA (mRNA), micro RNAs and other non-coding RNAs. Interestingly, DNA can be found both inside OMVs and attached to their surface, suggesting that it may be resistant to the adverse environmental conditions[56].

Three different hypotheses have been proposed to explain the mechanism of OMV generation. One suggests that the accumulation of peptidoglycan in the periplasmic space leads to increased pressure on the bacterial outer membrane, which, in turn, releases vesicles[57]. A second, less mechanistic hypothesis associates the bacterial outer membrane LPS composition with the higher or lower OMVs production[58]. A third possibility involves reduced anchoring of the bacterial outer membrane to the peptidoglycan layer through the Outer membrane protein A (OmpA) thereby facilitating vesicle for-

mation[57]. Other hypotheses exist but, whatever the mechanisms of OMVs releasing could be, the constructions of different bacterial mutants affecting OMVs shedding reveal that multiple genetic regulations occur, mainly secondary to the conditions of the environment where these bacteria live.

OMVs primarily contribute to bacterial survival and colonization by promoting biofilm formation, conferring antibiotic resistance, facilitating gene transfer, and even engaging in a form of "cannibalism". Under conditions of nutrient starvation, some OMVs can kill bacteria belonging to other species enabling the parental bacteria to persist when resources are scarce[49,52]. OMVs take part in the pathogenesis of numerous human infections, as extensively reviewed elsewhere[59]. Here, we summarize the role of OMVs produced by the most frequent Gram-negative bacteria involved in human diseases. Regarding this, OMVs derived from pathogenic *Escherichia coli* alter the expression of proteins involved in intestinal epithelial cell junctions (e.g., cadherin, claudin 2) leading to a potential disruption of the intestinal barrier and inducing a pro-inflammatory state that could cause "inflammatory bowel disease" (IBD)[60]. Enterohemorrhagic *Escherichia coli* O157:H7 releases OMVs containing LPS and Shiga toxins that pass from the intestinal lumen to the bloodstream and produce the clinical manifestations of hemolytic uremic syndrome[61]. OMVs from intestinal *E. coli* (including uropathogenic *E. coli*) have been shown to induce sepsis and septic-associated cardiomyopathy, even in the absence of live bacteria[62]. *Pseudomonas aeruginosa* is a widely distributed Gram-negative rod that exhibits resistance to many antibiotics and adverse environmental conditions. As an opportunistic pathogen, it is mainly associated with infections produced in immunocompromised patients, individuals with cystic fibrosis and burn wound infections. OMVs derived from *P. aeruginosa* can induce oxidative stress and autophagy in lung epithelial cells, as well as activate the NLRO3 inflammasome. Thus, these effects may account for part of the pathology caused by the parental bacteria[63]. Another Gram-negative pathogen, *Salmonella enterica*, also releases OMVs that produce structural alterations and inflammation of the ileum in experimental models[64]. *Vibrio cholerae* and *Campylobacter jejuni* are also important enteric pathogens, both of which produce OMVs containing virulence factors. *Vibrio cholerae* is the causative agent of cholerae, a severe intestinal disease transmitted by contaminated water and its main virulence factor -cholera toxin- has been detected into OMVs that enter intestinal cells[65]. Similarly, the cytolethal distending toxin (CDT), the major pathogenic factor of *C. jejuni*, is released within OMVs[66]. Of particular importance is the normal presence of non-sporulated anaerobic bacteria in the colonic lumen, for example, *Bacteroides fragilis*. These bacteria help to maintain colonic homeostasis and composition of the normal microbiota. This is achieved by the production of OMVs containing several hydrolases that can degrade complex polysaccharides into simpler molecules, then used by other gut bacterial species as a source of energy[67]. In the oral cavity, two Gram-negative bacterial species play a role in both local and systemic diseases. *Porphyromonas gingivalis*-derived OMVs contain several virulence factors such as gingipain, LPS and others, which elicit a strong inflammatory response mediated by nitric oxide, finally producing periodontal disease, at least in animal experimental models[59]. *Aggregatibacter actinomycetemcomitans* also colonizes the oral cavity, producing localized aggressive periodontitis through the virulence factor Ltx A present in OMVs[68]. It is well established that *Helicobacter pylori* colonizes the stomach causing chronic gastritis, peptic ulcer disease and gastric malignancies. However, this bacterium does not adhere tightly to the gastric epithelium but instead resides in the mucus layer that covers it. To achieve this, *Helicobacter pylori* first forms a biofilm and then secretes several virulence factors such as CagA, VacA, and OMPs, which are central to its pathogenesis. Several studies have demonstrated that those factors are present in OMVs released by the bacterium, targeting not only to the gastric mucosae but also the bloodstream, despite the fact that *H. pylori* does not invade the circulatory system[69,70].

In the respiratory tract, four bacterial species commonly associated with upper and/or lower respiratory diseases also produce OMVs: *Haemophilus influenzae, Moraxella catarrhalis, Legionella pneumophila* and *Bordetella pertussis*. *H. influenzae*-

derived OMVs not only participate in maintaining biofilms and augmenting blood-brain barrier permeability[71] but also facilitate evasion of the host immune response[72]; *Moraxella catarrhalis* OMVs indirectly produce tissue damage through neutrophils activation[59]; *Legionella pneumophila*, a causative agent of pneumonia, release OMVs that inhibit the fusion of the phagosome with the lysosome following intracellular infection, promote bacterial colonization, deliver virulence factors into host cells, and modulate the host immune response[73], and OMVs produced by *B. pertussis* carry LPS and toxins and adhere to lung cells[74]. Finally, several other bacteria associated with venereal diseases, such as *Neisseria gonorrhoeae,* or systemic infections with high mortality rates, such as *Yersinia pestis* (the causative agent of plague), *Acinetobacter baumanii* (a major cause of nosocomial infections), or *Borrelia budgorferi* (the etiological agent of Lyme disease), also release OMVs. These vesicles carry enzymes that confer resistance to antibiotics or virulence factors lethal to multiple host cell types[59].

Membrane vesicles in Gram-positive bacteria (MVs)

The first report describing the existence of MVs in Gram-positive bacteria was conducted in 1990[75]. The delay in studying these structures was probably due to the lack of an outer membrane in Gram-positive bacteria, thus leading to the assumption that they could not produce OMVs similar to those observed in Gram-negative bacteria. However, many Gram-positive bacteria do release extracellular vesicles, called "membrane vesicles" (MVs)[76]. It is well known that the cell wall of Gram-positive bacteria is characterized by a very thick peptidoglycan layer overlying the plasma membrane and the absence of a periplasmic space. Therefore, to explain how MVs are released, it has been hypothesized that some specific lipid-enriched regions of the plasma membrane act in concert with endolysins, reducing the thickness of peptidoglycan and enabling the production of 20-500 nm-width vesicles[48]. This process is regulated by a complex gene expression[77]. MVs contain proteins, peptidoglycan, phospholipids, DNA, RNA, and virulence factors, whose nature and characteristics have been clearly reviewed elsewhere[76], and their most relevant aspects are summarized here. While some of those proteins are conserved across bacterial species, others vary even in different isolates of the same species. Membrane lipoproteins are particularly important, as they are recognized by Toll-like receptor 2 -a member of the Toll-like receptor family, a group of pattern recognition receptors that play a central role in the innate immune system- and modulate the human immune response to certain infections. In addition, some enzymes within MVs can hydrolyze antibiotics, thereby enabling bacteria to survive under external stress conditions. The presence of bacterial genetic material into MVs protects it from the action of hydrolases and facilitates the transmission of miRNAs or bacterial DNA to eukaryotic cells leading to the impairment of host cell functions that -eventually- may determine their lysis through autophagy. Finally, the presence of virulence factors has obvious consequences for the host cells. Several Gram-positive bacteria pathogenic to humans have been shown to release MVs containing virulence factors. For example, *Staphylococcus aureus*, which causes abscesses, skin infections, pneumonia, osteomyelitis, sepsis, etc., produces vesicles containing α-hemolysin, leukocidins, lipases, nucleases and proteases as well as capsular polysaccharide and protein A all of which damage a variety of host cells and cell lines *in vitro*[78]. The major virulence factor of *Streptococcus pyogenes,* Streptolysin O, as well as NAD-glycohydrolase, have been found in MVs produced by this bacterium. These vesicles can invade human monocyte leukemia cells *in vitro,* leading to cell lysis[79]. This bacterium causes pharyngitis, and skin and soft tissue infections, and it is possible that MVs contribute to the pathogenesis of these diseases. *Streptococcus pneumoniae* is a Gram-positive, capsulated diplococcus, and a frequent cause of severe community-acquired infections such as pneumonia with or without sepsis, meningitis, otitis and sinusitis. One of its principal virulence factors, in addition to the capsule and IgA protease, is the pore-forming protein pneumolysin. In this regard, pneumolysin and other proteins have been identified in pneumococcal-derived MVs. When these vesicles were added to human lung cell cultures and

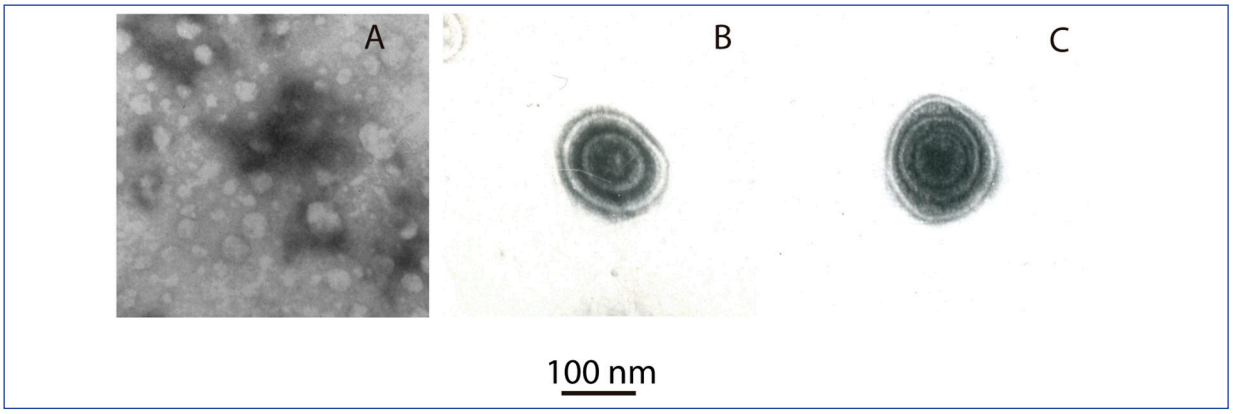
dendritic cells, vesicle internalization was observed along with cell damage resembling that observed in the pathogenesis of pneumonia, as well as modulation of the immune response[80]. MVs have also been described in *Enterococcus faecium,* a bacterial species that causes intra-hospital infections such as urinary tract infections, prostatitis, wound infections, endocarditis, and bacteremia. These vesicles, produced by bacteria isolated from several clinical samples, contain 11 virulence factors associated with extracellular matrix binding and biofilm formation, and other 11 proteins linked to antibiotic resistance, highlighting the contribution of MVs to bacterial pathogenesis[81]. *Listeria monocytogenes* is a Gram-positive intracellular rod that causes severe infections, including meningitis, and poses particular risks to pregnant women. After infecting host cells, these bacteria are engulfed into vacuoles and use the pore-forming toxin listeriolysin O together with phosphatidylinositol-specific phospholipase C to disrupt them and gain access to the cytoplasm. Their actin-based motility, forming a comet tail, enables the bacteria to invade neighboring cells without exposure to the intercellular medium. Interestingly, immuno-electron microscopy has revealed that listeriolysin O and phosphatidylinositol-specific phospholipase C are contained within MVs surrounding intracytoplasmic bacteria, thereby contributing to intracellular dissemination[82]. Human infections by the spore-forming *Bacillus anthracis* are uncommon but can occur after contact with contaminated animal products or through inhalation, and this pathogen has been exploited as a potent biological weapon. Edema toxin, protective antigen, lethal factor, and anthrolysin are important virulence factors of this bacterium, all of which have been detected in MVs released into the medium and in infected macrophages. These vesicles have also been employed to prepare experimental vaccines against *Bacillus anthracis* in mice[83]. *Mycobacterium tuberculosis* -like other mycobacteria- is neither Gram-positive nor Gram-negative, since the structure and composition of its cell wall are distinct. However, extracellular vesicles containing hydrophobic molecules, proteins, lipids, and glycolipids have been observed. Although the role of these vesicles remains unclear, they have been proposed to participate in host interactions, particularly through the modulation of human cellular immune response[84].

Outer-inner membrane vesicles (O-IMV) in Gram-negative bacteria

In 2013, Perez Cruz *et al* published the finding of a new type of vesicles in cultures of the Antarctic bacterium *Shewanella vesiculosa M7T* grown at 15ºC during the late log phase of the growth curve[85]. Unlike previously described OMVs and MVs, these vesicles are composed of both the outer membrane and the bacterial plasma membrane (inner membrane) and contain DNA and several cytoplasmic proteins. To explain their genesis, the authors proposed that protrusion of the outer and plasma membranes occurs following localized weakening of the peptidoglycan layer, thus allowing cytoplasmic components and DNA to become entrapped.

Enteroaggregative *E. coli* (EAEC) is a bacterium that causes acute and persistent diarrhea in children and adults following colonization of the intestinal epithelium through the formation of robust biofilms and the induction of inflammation by several virulence factors[86]. In our laboratory, while studying EAEC pili using negative-stained TEM samples, we observed vesicles budding from bacterial bodies that were also present in the broth and resembled O-IMVs. We characterized them employing the methods described above, and our results are described here as an example of the ultrastructure of O-IMVs released by a primary human pathogenic bacterium. As shown in Figure 1A, the vast majority of vesicles produced by EAEC appear to be typical OMVs of Gram-negative bacteria. Their size ranges from 20 to 50 nm and show no differences compared with those described elsewhere. However, a second, minor group of vesicles measured 100-120 nm in width and displayed double-bilayer structures with material enclosed between the two membranes were also seen (Fig. 1 B, C). Other authors described that O-IMVs account for no more than 0.1% of the total amount of vesicles released by Gram-negative bacteria[85] and, eventually, EAEC-derived O-IMV are not the exception. A more detailed observation of these O-IMVs by TEM, employing negative stained samples and ultrathin sections revealed that vesicles

Figure 1 | Negative staining of vesicles obtained from a 7-month-old Enteroaggregative *Escherichia coli* broth culture. Classic outer membrane vesicles are observed in panel A while in panels B and C, outer-inner membrane vesicles with a width of approximately 120 nm can be observed



were budding from the bacterial body directly as double-membrane structures with or without enclosed material (Fig. 2A). Moreover, some vesicles showed a thin connection between the released vesicle and the parental bacterial cell (Fig. 2B) strongly supporting the concept of active release of these structures as previously described by other researchers[85]. Figure 2C shows a high magnification image of a negative-stained O-IMV, displaying the outer membrane (OM), the inner membrane (IM), and a central core (C). In Figure 2D, the same structures can be observed in a thin section: the OM, the periplasmic space (PS), the IM and C. The structural similarities of O-IMVs revealed by two different TEM methods are evident. Fig. 2E is particularly interesting because it raises more questions than it answers: it depicts a thin section of a sample containing O-IMVs in which a C is visible, covered by two bilayer membranes: the OM and IM However, the most peripheral membrane (OM) appears to cover not only a complete vesicle but also to surround a second one. Thin sections observed by TEM yield 2-D figures that depend on where the ultramicrotome knife cuts, different to those obtained in the 3-D images observed by negative staining. Thus, it is very difficult to propose a full explanation of this biological phenomenon but, eventually it could lead to a complementary hypothesis regarding O-IMVs budding: the outer membrane could envelop more than one vesicle, while the plasma membrane encloses bacterial cytoplasmic proteins and DNA. Figure 3 shows a negatively stained O-IMV imaged directly at 200 000 X and furtherly amplified. It may be considered a prototype example of the ultrastructure of O-IMVs, consistent with the descriptions published by several other authors. The composition includes an OM, probably closely associated with the peptidoglycan layer (PG). Moving inward, the PS can be observed, followed by the inner (plasma) membrane (PM) and a C. Notably, an additional layer is observed on the outer membrane (fibrillar layer). Its ultrastructure appears fibrillar and may contain proteins and/or DNA, as reported by other authors in different bacteria[56]. Beyond their ultrastructural description, additional observations can be considered: EAEC-derived O-IMVs were mainly observed when bacterial growth at 37°C were in very late stationary phase, even in seven-month-old cultures without medium replacement. Vesicles were purified as described above using differential gradients and tracked by NTA. Proteomic analysis was performed employing two-dimensional gels, and mass spectrometry detected the presence of flagellin (FliC), OmpX and OmpA porins, HlyF hemolysin, Ag43 antigen, murein LPS, and several adhesins (unpublished data). Furthermore, EAEC vesicles contained bacterial chromosomal DNA, characterized by classical methods, TEM and Random Amplified Polymorphic DNA-Polymerase Chain Reaction DNA (RAPD-PCR). Notably, DNA-containing O-IMVs

Figure 2 | A and B: Negative staining of Outer-inner membrane vesicles budding from the body of Enteroaggregative *Escherichia coli* (EAEC). Note that in B, a thin structure connects the vesicle to the bacterial body. C: High magnification of a negative stained EAEC-derived Outer-inner membrane vesicle, where the outer membrane (OM), inner membrane (IM), and core (C) can be seen. D: The same kind of vesicle observed in a thin section stained with uranyl acetate and lead citrate. Trilaminar membranes are visible, and the ultrastructure corresponds to that observed in C. The outer membrane (OM), periplasmic space (PS), inner membrane (IM) and core (C) can be seen. E: Thin section stained with uranyl acetate and lead citrate showing a common outer membrane (OM) enclosing 2 vesicles, each with its own inner membrane (IM) and core (C)

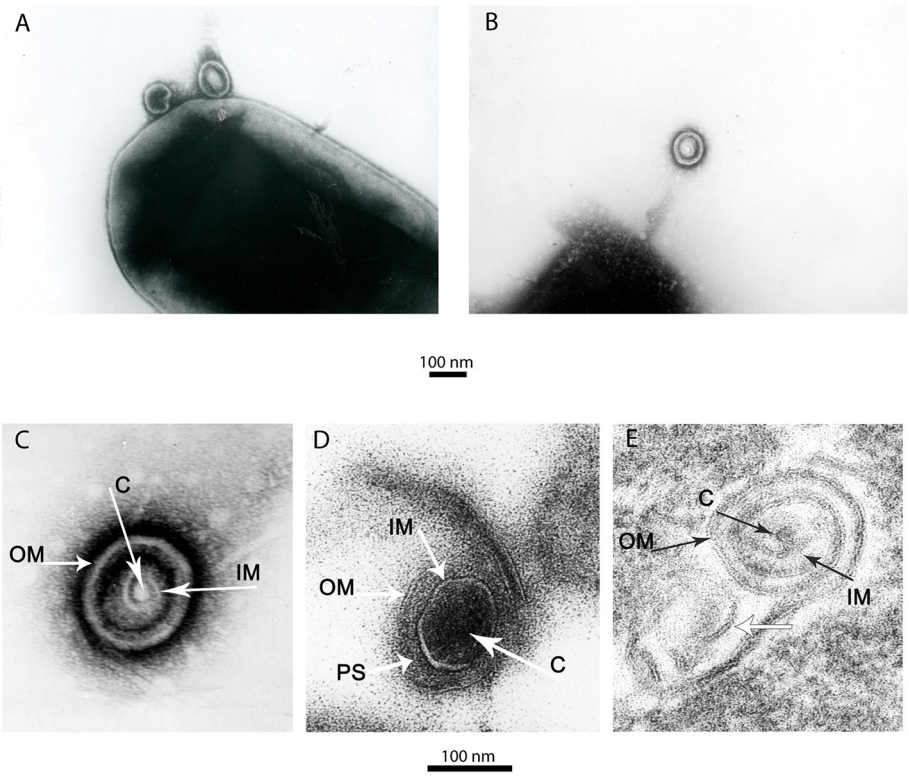
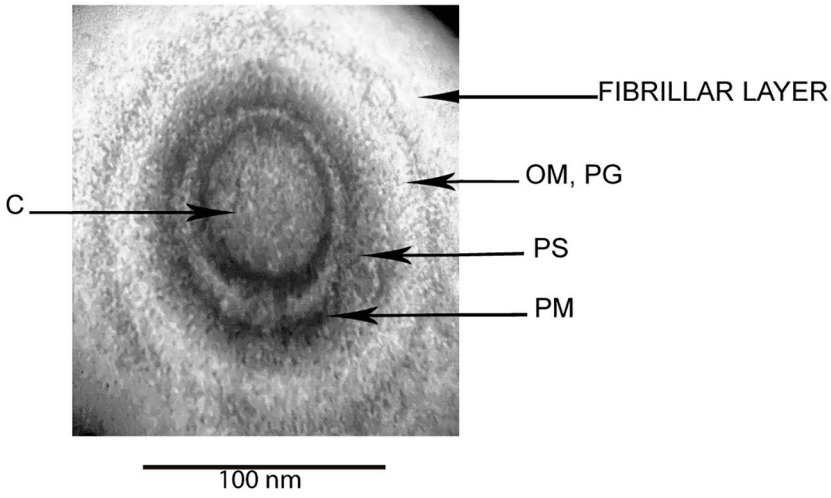


Figure 3 |Example of the ultrastructure of a typical negatively stained Outer-inner membrane vesicle observed at very high magnification by TEM: from outside to inside, a superficial fibrillary layer, the outer membrane (OM) and peptidoglycan (PG), the periplasmic space (PS) the plasma membrane (PM) and the central core (C) can be seen



are not unique to EAEC; they have also been observed in other pathogenic bacteria such as *Neisseria gonorrhoeae*, *P. aeruginosa* and *A. baumanii*[87]. In these three bacterial species, O-IMVs are released together with the classic OMVs described above and constitute only 1.2%, 0.5% and 0.2% of all vesicles[87]. In the referenced report, it was conclusively demonstrated that DNA is present within O-IMVs derived from *Neisseria gonorrhoeae* by ultrastructural immunolabelling. Of particular interest is the finding that, aside from DNA and multiple proteins, ATP is also contained in these vesicles, a result whose interpretation offers multiple possible implications.

Discussion

For decades, bacterial physiology and human bacterial diseases have been explained primarily through the secretion of toxins or other virulence factors to the surrounding medium. With the growing knowledge of the existence of bacterial extracellular vesicles, it is currently widely accepted that the release of toxins or nucleic acids from bacteria is more effectively protected from extracellular hydrolases when released within membrane vesicles. OMVs and MVs are considered as carriers of nucleic acids involved in the transmission of antibiotic resistance and are also critical in biofilm formation, induction of damage to human cells and modulation of the immune response. In contrast, O-IMVs represent a novel concept, and their role in nature and human pathology remains less well understood in comparison to that of OMVs and MVs. It is possible that O-IMVs share functions with the other bacterial vesicles, but the finding of chromosomal DNA in their core is particularly provocative. Substantial further efforts are needed to determine if the production of O-IMVs is a widespread phenomenon, as well as to elucidate the conditions and genetic regulation of their release. Given the content of chromosomal DNA, ultrastructure and persistence in broth after months of culture, we hypothesize here that O-IMVs could also represent a novel mechanism of resistance to adverse environmental conditions in non-sporulated bacteria. In any case, the discovery and characterization of bacterial extracellular vesicles of different types have deeply reshaped fundamental concepts in Bacteriology and, especially, in human infectious diseases.

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