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Production of Outer Membrane Vesicles in a Clinical Strain of *Aeromonas hydrophila*

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Abstract

Some *Aeromonas* species are pathogenic in humans. Their pathogenic mechanism is multifactorial and is attributed to several putative virulence factors, toxins and secretion systems. Outer membrane vesicles have been described as a new secretion system in Gram-negative bacteria. In this work, the production of outer membrane vesicles is demonstrated in an *in vitro* culture of *Aeromonas* by transmission electron microscopy. In addition, an isolation and purification technique is developed. The results indicate the presence of this secretion system in *Aeromonas*.

Keywords: *Vesicles; Outer membrane; Bacteria; Secretion systems; Aeromonas*

Abbreviations: OMV: Outer membrane vesicle; OM: outer membrane; IM: inner membrane; PS: Periplasmic space; TEM: Transmission Electron Microscopy

Introduction

Aeromonas is a bacterial genus of clinical and veterinary importance. In humans, this bacterium is present in intestinal and extraintestinal infections including bacteremia, septicemia, and soft tissue infections [1].

Like other bacteria recognized as pathogenic, *Aeromonas* has a variety of important virulence factors involved in the colonization, invasion, and proliferation of bacteria that give it advantages during the establishment of infection and the potential to damage and destroy tissue and evade host immune responses [2]. The virulence factors of *Aeromonas* can be classified as extracellular and structural, among these are secretion systems II, III, and VI [3,4].

Currently, particles known as outer membrane vesicles (OMVs) are considered a zero-secretion system produced by Gram-negative bacteria [5]. The outer membrane vesicles are spherical particles, 20 to 250 nm in diameter. They are composed of a double membrane of phospholipids, lipopoly saccharide, and outer membrane proteins. The lumen may contain DNA, RNA, periplasmic space components, and other components associated with virulence [6-8].

OMVs facilitate secretion of insoluble or hydrophobic materials such as membrane proteins and signaling molecules. Moreover, unlike other secretion systems, OMVs may be a means by which some soluble proteins and other compounds susceptible to enzymatic decomposition, such as toxins and nucleic acids, may be encapsulated to be released in a protective structure. The latter allows them to reach the destination without any alteration [9]; therefore, it is believed that OMVs play an important role in pathogenicity.

In the genus *Aeromonas*, only one piece of evidence exists that suggests that OMVs are involved in pathogenicity. Longa., *et al*. in 2008 [10] conducted a study to evaluate the pathogenic mechanisms of *Aeromonas* strains in intestinal tissue of the mouse, using transmission electron microscopy (TEM). In the micrographs obtained by these authors, ultra structural changes were observed that occurred subsequent to the interaction of the strains with host tissue. One of the most important findings was the production of OMVs by *Aeromonas* and, subsequently, their adherence to the intestinal epithelium followed by their destruction, thus relating the production of OMVs with the pathogenicity of the studied strains. For this reason, it is of vital importance to continue exploring the role of OMVs in the pathogenicity of this bacterial genus.

Materials and Methods

Biological material and growth conditions

The strain *A. hydrophila* F-0050 (kindly provided by Patricia Arzate-Barbosa, BSc) was used. It was of clinical origin, isolated from the diarrheal stool of a pediatric patient; the strain was genetically characterized and identified by RFLP-PCR. The strain was grown in tryptone soy agar (TSA) and incubated at 37°C for 24h. An isolated colony was used to obtain OMVs by inoculation into 5 ml of tryptone soy broth (TSB) and incubation at 37°C overnight. One hundred plates with Craig agar (30 g/l of CAS-amino acids, 4 g/l yeast extract, 15 g/l bacteriological agar, 0.4 g/l of potassium phosphate dihydrate) were inoculated with the bacterial culture obtained and incubated under the same conditions to obtain sufficient biomass for the isolation and purification of OMVs.

Isolation and purification of OMVs

The biomass obtained was harvested in 50 ml of filtered phosphate buffered saline (PBS). The acquired suspension was centrifuged at 10,000 × g for 20 min and the resulting supernatant was filtered through a 0.22 µm membrane. Subsequently, the sample was ultracentrifuged at 100,000 × g for 1h at 4°C and the obtained pellet was resuspended in 500 µl of PBS. Purification of OMVs was performed by ultracentrifugation in an iodixanol concentration gradient [11].

Transmission Electron Microscopy (TEM)

For bacteria microscopy, we worked with bacterial colonies grown on a plate with Craig agar, to which a thin layer of molten agar was added and allowed to solidify; subsequently, a small block of bacterial growth was cut and processed for TEM. Furthermore, 20 µl of the sample obtained from the OMV purification technique was taken and placed on a formvar-coated grid, where it was allowed to adsorb for 1 min and the excess was eliminated. Later, negative staining for TEM contrasted with 1% phosphor tungstic acid was performed. Both samples were analyzed with a JEOL Model 1010 transmission electron microscope at an accelerating voltage of 60 Kv.

Results and Discussion

In a significant number of pathogenic bacteria, OMVs have been shown to play an important role in pathogenicity; for example, in the transport and release of cytotoxic toxins in antigenic mimicry and in host tissue destruction [12,13]. Consequently, in pathogenic *Aeromonas*, bacterial pathogenesis may be actively involved in colonization, transmission of virulence factors in host cells, and/or modulation of the host immune response [14].

Since *Aeromonas* is a ubiquitous bacterial genus whose many species are known to adapt to stressful conditions, they are able to produce OMVs in clinical and environmental strains, thus *Aeromonas* may contribute to bacterial survival by reducing levels of toxic compounds or neutralizing environmental agents such as antimicrobial peptides. OMVs may also mediate cell aggregation to form bacterial communities such as biofilm [15].

Cross-sectional TEM images of rod-shaped bacterium were obtained. These TEM images clearly show the inner membrane and cell wall, the periplasmic space, and the outer membrane.

Protrusions can be seen around the outer membrane of the bacterium that form a type of septum and that separate as OMVs. In some images, the vesicles are about to separate from the outer membrane (Figure 1A), whereas, in others, separated vesicles near the site of the outer membrane from where they originated can be seen; it can also be seen that the site is intact (Figure 1B).

The images obtained allow us to demonstrate the release of OMVs in a clinical origin strain of *A. hydrophila* during growth on a solid medium. In other pathogenic and non-pathogenic Gram-negative bacteria, this phenomenon has been demonstrated with similar techniques that allow detailed observation of the outer membrane releasing OMVs [16].

OMV formation generally occurs in three stages: the first is a protrusion of the outer membrane; the second is self-strangulation of the protrusion; and finally, its release in the form of a vesicle [17]. Some images show events that could represent these steps of OMV formation in *Aeromonas* since protrusions are observed on the membrane, as well as a protrusion with a septum that separates the outer membrane and the fully detached vesicles. However, detailed molecular analysis is needed to accurately describe the mechanism by which these vesicles are formed.

It is known that OMVs are not a product of cell death and that their release does not alter the structure of the outer membrane [17,18]. This is evident in the figure where vesicles are seen close to the site that produced them without observing any changes in the membrane (Figure 1B).

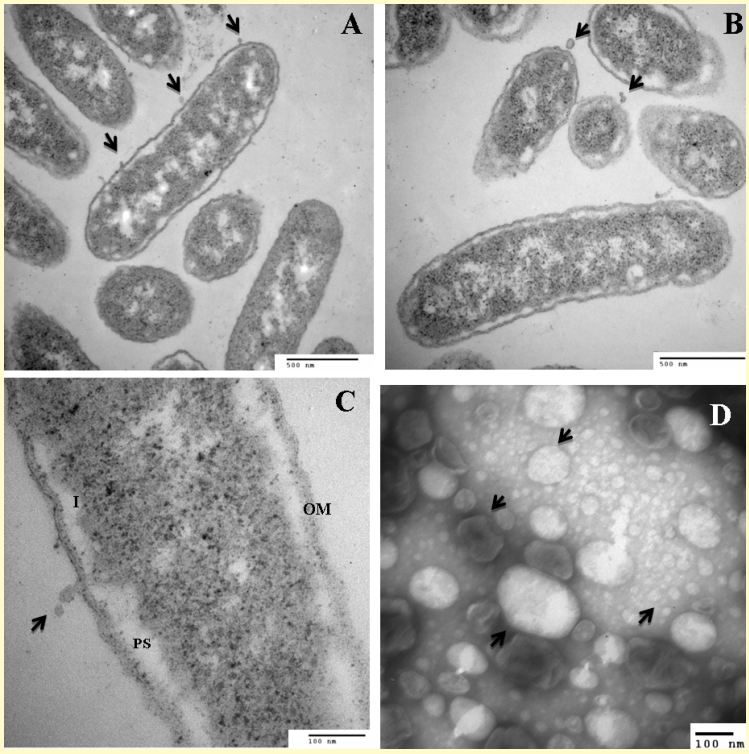

Figure 1: *Transmission electron microscopy images of A. hydrophila releasing outer membrane vesicles.*

A. Rod-shaped bacterium with bulges around its envelope.

B. Vesicles released near the site of origination.

C. A chain of vesicles linked to the cell membrane.

D. Purified outer membrane vesicles.

IN: Inner Membrane; OM: Outer Membrane; PS: Periplasmic Space; Scale bar = 100 nm-500 nm.

In other images, chains of vesicles linked to the outer membrane are observed. These chains were composed of up to four vesicles; vesicles that were not linked to the outer membrane were also observed (Figure 1C). Longa., *et al*. [10] in 2008, described the release of OMVs by *Aeromonas* when infecting the mouse intestine; in this work, bacterium releasing OMVs are also observed, but during their growth *in vitro*.

Once it was demonstrated that *Aeromonas* produce OMVs during growth on a solid medium, OMVs were isolated and purified, and characterized by TEM. The images show abundant spherical or hemispherical particles bound by a double membrane. The observed particles depictvarious sizes ranging from 20 to 200 nm in diameter (Figure 1D).

Conclusion

Like other Gram-negative bacteria *Aeromonas* can produce OMVs during growth in vitro and in vivo. In addition the purification technique described allows obtaining vesicles with the quality and purity required for studies aimed at elucidating the involvement of OMVs in the metabolism and pathogenicity of these bacteria.

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Conflict of interest

The author(s) declare that they have no competing interests.

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