ART & TECHNOLOGY PUBLISHING PTE. LTD.

Gene Science and Engineering

2023 Volume 3, Issue 1

ISSN: 2529-7651

Production of Outer Membrane Vesicles From Acetic Acid Bacteria and Their Properties

Masahito Hashimoto*

Department of Chemistry, Biotechnology, and Chemical Engineering, Kagoshima University. Kagoshima, Kagoshima 890-0065, Japan

***Corresponding author:** Masahito Hashimoto, hassy@eng.kagoshima-u.ac.jp

Abstract

Outer membrane vesicles (OMVs) are mainly composed of lipopolysaccharides (LPS), phospholipids, outer membranes, and periplasmic proteins. Recently OMV vaccines have been developed in which LPS acts as an adjuvant. However, attenuation of the toxicity of typical LPS is necessary to reduce the adverse effects of the OMV vaccine. We have previously found that the acetic acid bacterium *Acetobacter pasteurianus* produces LPS with low immunostimulatory activity. In this study, we isolated OMVs from *Acetobacter pasteurianus* and analyzed its immunostimulatory activity. *A. pasteurianus* NBRC 3283 was cultured at 27°C in 804 broth and vesicle secretion from the cells was observed by TEM imaging after 2 days of culture. Vesicles were separated from the culture medium after 7 days of culture by ultracentrifugation. The results showed that the precipitate contained vesicles that could be purified by OptiPrep density gradient centrifugation. As the vesicles were composed of LPS and outer membrane proteins, we determined that they were OMVs and named them Ap-OMVs. Furthermore, we found that Ap-OMV stimulated toll-like receptor 2 (TLR2) and weakly stimulated TLR4 in TLR-expressing cells and J774A.1 cells. Furthermore, OMV-like vesicles were detected in Japanese black vinegar. These data suggest that *A. pasteurianus* produces OMVs containing LPS and stimulates the immune system.

Keywords

Acetobacter
Lipopolysaccharides
Outer membrane vesicles
Toll-like receptors
Black vinegar

1. Introduction

Bacterial outer membrane vesicles (OMVs) are vesicles of 20–300 nm produced by gram-negative bacteria and known to contain antigens such as lipopolysaccharide (LPS), lipoprotein (LP), peptidoglycan (PGN), and other microbial molecular patterns (MAMPs), outer membrane proteins (OMP) and polysaccharides [1]. In recent years, the functions of OMVs have attracted attention, and it is now known that OMVs are involved in the interaction between bacteria and host cells,

such as regulating the immune system by contacting and invading host cells. The application of OMVs as vaccines has also been investigated, and some have already been put to practical use, especially against meningococci. However, OMVs derived from general bacteria have disadvantages, such as the need for weakening due to the strong toxicity of LPS.

Acetic acid bacteria is a generic term that includes many genera of bacteria used not only for acetic acid fermentation but also for the production of sorbose and biocellulose. Among acetic acid bacteria, *Acetobacter pasteurianus* is a gram-negative bacterium that oxidizes ethanol to produce acetic acid and is known as one of the fermenting microorganisms in black vinegar, a specialty brewed vinegar of Kagoshima Prefecture. Recently, we have shown that the LPS of *A. pasteurianus* (Ap-LPS) is a weakly toxic bacterium that weakly activates human toll-like receptor 4 (TLR4) [2]. In this paper, we focus on acetic acid bacteria and describe their ability to produce OMVs and their immunological properties [3].

2. OMV production by acetic acid bacteria

OMVs are known to be released from bacteria by budding through the curvature of the membrane on the surface of the bacteria. We investigated whether OMV is produced by acetic acid bacteria using the bacteria and culture supernatant. *A. pasteurianus* NBRC3283 was used as the strain, which was grown on 804 medium (Hipolypepton-Yeast extract-Glucose-$MgSO_4$) at 27°C with shaking. The growth of the bacteria reached a stationary phase in about 5 days. When the bacteria were followed during culture using TEM, vesicle emergence from the surface of the bacteria was observed on day 2, suggesting the production of OMVs (**Figure 1A**). The supernatant was filtered through a membrane filter, ultracentrifuged and the precipitates were observed by TEM, and it was found that vesicles that appeared to be OMVs could be recovered after the third day of culture (**Figure 1B**). SDS-PAGE images of the precipitates showed that they contained LPS, so the number of precipitates recovered was compared based on the hexose content, and the highest amount was found on day 7 of the culture.

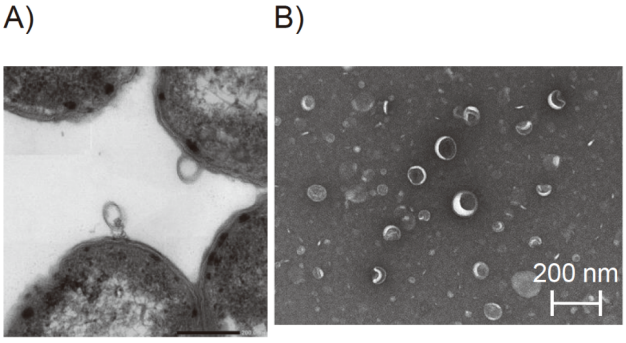

Figure 1. TEM images of (A) *A. pasteurianus* on day 2 of culture, and (B) vesicles from *A. pasteurianus* on day 7 of culture.

Therefore, vesicles were attempted to be separated by density gradient centrifugation using OptiPrep. The precipitate derived from the culture supernatant on day 7 was separated using an OptiPrep step density gradient of 10%–45%, and vesicles could be separated at the 20% and 25% boundaries. The vesicle fraction was recovered by ultracentrifugation, yielding around 1–2 mg (dry weight) of vesicles from 2.4 L of culture. DLS measurements showed that the vesicles were estimated to have a diameter of around 120 nm, whereas measurements from TEM images showed no major discrepancies, ranging from 80–120 nm.

After the separation of the vesicles by SDS-PAGE, visualization of the sugars by periodate-silver staining showed a band pattern similar to that of acetate-type LPS (**Figure 2A**). Monosaccharide determination by the alditol acetate method showed that the vesicles contained glucose and rhamnose as the major constituent sugars, with a small amount of galactose and mannose. This was similar to the constituent sugars of Ap-LPS, indicating that the vesicles contained LPS from acetic acid bacteria. The CBB-stained bands were also analyzed by mass spectrometry after trypsin digestion, and the outer membrane protein

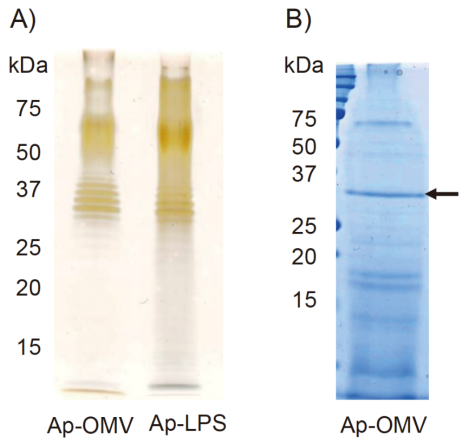

Figure 2. SDS-PAGE gel images of Ap-OMV and Ap-LPS. (A) Periodate-silver staining; (B) CBB staining, arrows indicate bands of OmpA.

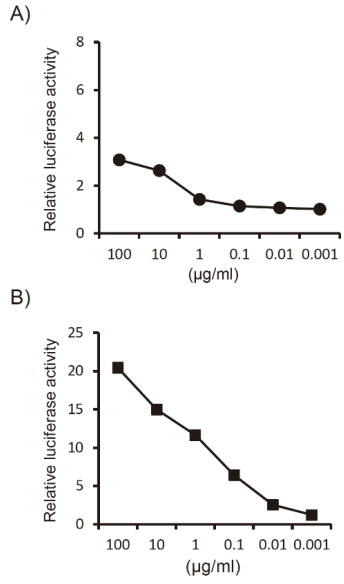

Figure 3. TLR activation potential of Ap-OMV. (A) Ba/mTLR4-MD2 cells; (B) Ba/mTLR2 cells. The activity was detected by NF-κB-dependent luciferase assay.

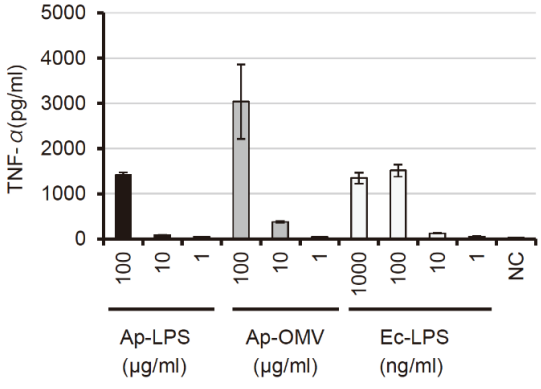

Figure 4. Ap-OMV's ability to induce TNF-α production against J774A.1

OmpA of *A. pasteurianus* was also detected (**Figure 2B**). These results indicate that the vesicles produced by *A. pasteurianus* are OMVs, and this fraction was designated Ap-OMV.

3. Immunological properties of OMVs produced by acetic acid bacteria

Since Ap-OMV has LPS, it is expected that it can activate innate immunity. Therefore, the activation of TLR2 and TLR4 was examined using forced-expression cells. The results showed that TLR4 was weakly activated (**Figure 3A**), consistent with the properties of Ap-LPS. TLR2 could also be activated (**Figure 3B**). This suggests that OMVs also contain lipoproteins, although unidentified.

The ability to induce cytokine production was also investigated. Using mouse macrophage-like cells J774A.1, it was found that Ap-OMV induced the production of TNF-α (**Figure 4**). This suggests the involvement of components other than LPS in the activity of Ap-OMV. The activity of Ap-OMV was 1/1,000-fold weaker than that of purified LPS (EC-LPS) from *E. coli*, indicating that Ap-OMV is an attenuated form of OMV.

4. OMV-like vesicles in black vinegar

Since acetic acid bacteria produce OMVs, the possibility that black vinegar, in which acetic acid bacteria are the fermenting microorganisms, also contains OMVs was investigated. The black vinegar used was brown rice black vinegar produced by static fermentation in Fukuyama Town, Kagoshima Prefecture. The black vinegar was filtered through a membrane filter and ultracentrifuged to obtain precipitates similar to *A. pasteurianus* culture supernatant, and TEM observation revealed the presence of vesicles similar to those of Ap-OMV (**Figure 5A**). Therefore, vesicles were separated in an OptiPrep step density gradient, similar to Ap-OMV. After the separation of the vesicles by SDS-

PAGE, the sugars were visualized by periodate-silver staining, which showed a band pattern similar to that of acetobacter-type LPS (**Figure 5B**). These results indicate that structures similar to Ap-OMV are present in black vinegar.

5. Conclusion

Until now, OMVs have been studied mainly in pathogenic bacteria for their pathogenic mechanisms and their application to vaccines. However, the toxicity of the LPS contained in OMVs has been a problem, and processing of OMVs for use in modulating the immune system has been time-consuming. On the other hand, OMVs produced by acetic acid bacteria, shown in this paper, can weakly activate the innate immune system because they have attenuated LPS. This indicates that the toxicity of LPS can be utilized as it is, without attenuation. Black vinegar with acetic acid bacteria as the fermenting bacterium also contained OMV-like structures. Since drinking black vinegar is believed to regulate the immune system, it is possible that this structure may be responsible for its activity. In the future, we would like to clarify the *in vivo* effects of Bacillus acetate OMV and the contribution of Bacillus acetate to immunomodulation.

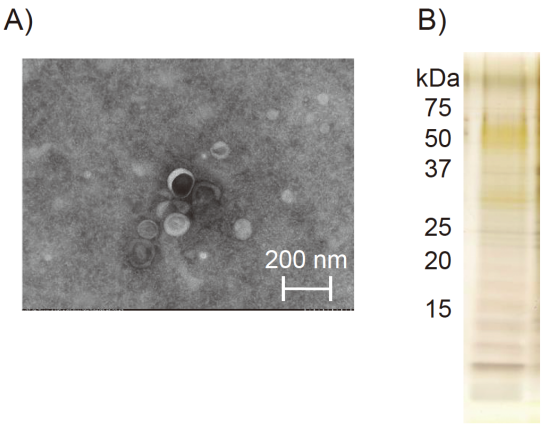

Figure 5. Results obtained from black vinegar. (A) TEM image of OMV-like structures from black vinegar; (B) SDS-page gel image with periodate-silver staining.

Disclosure statement

The author declares no conflict of interest.

References

[1] Pathirana RD, Kaparakis-Liaskos M, 2016, Bacterial Membrane Vesicles: Biogenesis, Immune Regulation and Pathogenesis. Cell Microbiol, 18: 1518–1524.

[2] Hashimoto M, Ozono M, Furuyashiki M, et al., 2016, Characterization of a Novel D-Glycero-D-Talo-Oct-2-Ulosonic Acid-Substituted Lipid A Moiety in the Lipopolysaccharide Produced by the Acetic Acid Bacterium *Acetobacter pasteurianus* NBRC 3283. J Biol Chem, 291: 21184–21194.

[3] Hashimoto M, Matsumoto T, Tamura-Nakano M, et al., 2018, Characterization of Outer Membrane Vesicles of *Acetobacter pasteurianus* NBRC3283. J Biosci Bioeng, 125: 425–431.