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Characterization of outer membrane vesicles of *Acetobacter pasteurianus* NBRC3283

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***Acetobacter pasteurianus* is characterized as a fermenting bacterium of kurozu, which is a common traditional Japanese black vinegar. Recently, we separated acid-resistant and low Toll-like receptor 4 (TLR4)-stimulatory lipopolysaccharides (LPS) from *A. pasteurianus*. We also showed that their lipid A parts possessed a novel sugar backbone that is responsible for the low TLR4-stimulatory and acid−resistant properties of the LPS. Outer membrane vesicles (OMVs) are nano-sized spherical structures secreted from many gram-negative bacteria. OMVs contain LPS and act as immunomodulants such as vaccines. In this study, we investigated OMVs secreted from *A. pasteurianus*. OMV secretion from *A. pasteurianus* NBRC 3283 cells was observed after 2 days in culture by transmission electron microscopy imaging. Thus OMVs were separated from the culture supernatants by ultracentrifugation and then purified by OptiPrep density gradient centrifugation. The OMVs contained several proteins including outer membrane proteins, and several sugars as components of LPS. The OMVs weakly stimulated TLR4 in accordance with the activity of *A. pasteurianus* LPS. Additionally, the TLR2-stimulating activity of the OMVs was significantly potent, indicating the existence of lipoproteins. Furthermore OMV-like spherical particles were observed in kurozu. Some of these particles are probably derived from *A. pasteurianus*. These data suggest that *A. pasteurianus* produce OMVs that contain LPS and probably lipoproteins, and can modulate the innate immune system.**

[**Key words:** Acetic acid bacteria; Lipopolysaccharide; Lipoprotein; Outer membrane vesicles; Toll-like receptor]

Acetic acid bacteria have been shown to be widely distributed in the environment, including in fruits, flowers, insect guts, and sometime in soil, and several of them are used as fermented food and for functional molecule production (1). Among them, the genus *Acetobacter* is the most popular for fermentation of vinegar around the world. *Acetobacter pasteurianus* is characterized as the fermenting bacterium of a black vinegar, kurozu, which is a common traditional Japanese vinegar with a characteristic dark amber color and is made from unpolished rice (2,3).

Kurozu is believed to have beneficial health effects, e.g., it is considered to relieve hypertension, prevent cancer, and improve allergic symptoms, and it has recently been used as a health drink in Japan. Previously, we found that the kurozu hydrophobic fraction (FK-OS2), which was separated from kurozu by hydrophobic interaction chromatography, possessed Toll-like receptor 2 (TLR2)- and weak TLR4-stimulating activity (4). We also showed that the fraction contained lipopolysaccharides (LPS)-like and lipoprotein-like components. LPS is an outer membrane component of gram-negative bacteria and is known to stimulate TLR4 (5,6). Since oral administration of LPS has been reported to modulate immune responses (7), the LPS-like components in kurozu may be responsible for the beneficial health effects of kurozu. Thus we further investigated the LPS-like components.

Recently, we separated LPS from *A. pasteurianus* (Ap-LPS) and found that they possessed weak TLR4-stimulating activity (8). Since the Ap-LPS showed a similar SDS-PAGE profile to kurozu-derived LPS-like components, kurozu appeared to contain Ap-LPS. LPS are a heterogeneous mixture of macromolecular amphiphilic glycoconjugates consisting of a polysaccharide part and a glycolipid anchor, called lipid A (5). Lipid A is known to be responsible for the activities of LPS. We thus analyzed the structure of lipid A in Ap-LPS and identified it as a novel sugar backbone with longer acyl chains (8). The structure was quite different from the structure of the potent TLR4-stimulating *Escherichia coli*-type lipid A, which may result in its weak TLR4-stimulatory property.

The LPS-like components in kurozu are acid-resistant over the 2-year fermentation and aging process in kurozu production. We further showed that they were resistant to mild acid hydrolysis in 1% acetic acid at 105 °C for 2.5 h, which is usually used for degradation of LPS (4). We also demonstrated that Ap-LPS are resistant to mild acid hydrolysis (8). Typical LPS is acid labile because a sugar component, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), which links the polysaccharide part and lipid A, is readily hydrolyzed by a mild acidic condition. We recently found that Ap-LPS contained D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) residues instead of Kdo (8). The substitution of Ko for Kdo is considered to be responsible for

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the acid–stable properties of LPS. Therefore, Ap-LPS are found to be intact in kurozu after long-time exposure to an acidic condition. These observations suggest that Ap-LPS can pass through gastric fluid after eating and interact with intestinal epithelial cells to modulate the host immune system.

Outer membrane vesicles (OMVs) are nano-sized spherical structures ranging from between 20 and 300 nm in diameter (9). OMVs are secreted during the growth of many gram-negative bacteria, and the vesicles are composed of bacterial components, including phospholipids, LPS, peptidoglycan, and outer membrane proteins. OMVs also hold periplasmic and cytoplasmic proteins, DNA, RNA, and enzymes. OMVs transport their components, which include biological functional molecules of the parent bacterium, to distant locations, enabling bacterial communication, the transfer of virulence factors, and the maintenance of bacterial communities. In the host, OMVs can enter into host cells and release their components, inducing virulence, inside the cells, contributing to pathogenesis of the parent bacteria (10). In addition, OMVs can act as immunomodulants against host immune systems by interaction with OMV components and host immune receptors. Some OMV components act as pathogen-associated molecular patterns (PAMPs) and stimulate innate immune receptors, such as Toll-like receptors (TLR) and Nod-like receptors (NLR), to modulate innate immune responses. Thus OMVs have been recently utilized as vaccines against infectious disease, such as meningococcal disease, since the PAMPs act as adjuvants (11).

Since OMVs have been separated and characterized in many gram-negative bacteria, we hypothesized that acetic acid bacteria would also secrete LPS-containing OMVs during their growth. In addition, OMVs are hypothesized to be produced in the kurozu fermentation period and exist in kurozu. In the present study, we investigated OMVs secreted from *A. pasteurianus* and their immunostimulatory activities.

MATERIALS AND METHODS

Preparation of bacterial culture supernatants *A. pasteurianus* NBRC 3283 was obtained from the National Institute of Technology and Evaluation Biological Resource Center (NBRC), Chiba, Japan. The bacteria were cultured in No. 804 broth, which consisted of 0.5% polypeptone, 0.5% yeast extract, 0.5% glucose, and 0.1% magnesium sulfate heptahydrate, at 25°C. The bacteria in frozen stock were streaked on an 804-agar plate and incubated for 4 days, and then precultured in the broth for 3 days with shaking. The preculture was diluted to 1/30 and incubated for the indicated days with shaking. The culture supernatants were obtained by centrifugation at 10,000×g for 20 min at 4°C, and then filtered through a 0.22-μm bottle top filter (Sartolab BT, Sartorius, Göttingen Germany).

Bacterial components The TLR2 ligand FSL-1, a synthetic diacylated lipopeptide, was purchased from EMC microcollections (Tübingen, Germany). *A. pasteurianus* LPS fraction (Ap-LPS) was prepared by phenol-hot water extraction followed by hydrophobic interaction chromatography, as previously described (8).

Black vinegar kurozu that had been produced by traditional static fermentation in ceramic pods was obtained from Shigehisa Moriichi Su-Jozojo, a traditional vinegar brewery in Fukuyama, Kagoshima, Japan. The Fukuyama kurozu, which was fermented for 6 months and then aged for 1–2 years, was filtered through diatomite and pasteurized at 90–95°C for 15 s using a plate heat exchanger. The kurozu was further filtered through a 0.22-μm bottle top filter immediately prior to use.

Isolation of OMVs OMVs were obtained by an ultracentrifugation method (12). The culture supernatants (2.4 L) or Fukuyama kurozu (2.4 L) were subjected to ultracentrifugation at 145,000×g for 3 h at 4°C. The pellets were washed with 50 mM HEPES buffer (pH 7.5) and resuspended in 400 μl of HEPES buffer to obtain crude OMV suspensions. The OMVs were then purified by flotation through OptiPrep density gradient centrifugation (13). The crude OMV suspensions were combined with OptiPrep (60% iodioxanol) in a ratio of 1:3 to adjust them to a 45% iodioxanol mixture, and 1 ml of the mixture was loaded into a 5-ml ultracentrifuge tube. They were overlaid successively with 0.75 ml 35%, 1 ml 30%, 0.5 ml 25%, 0.5 ml 20%, 0.5 ml 15%, and 0.25 ml 10% iodioxanol-HEPES buffer. The

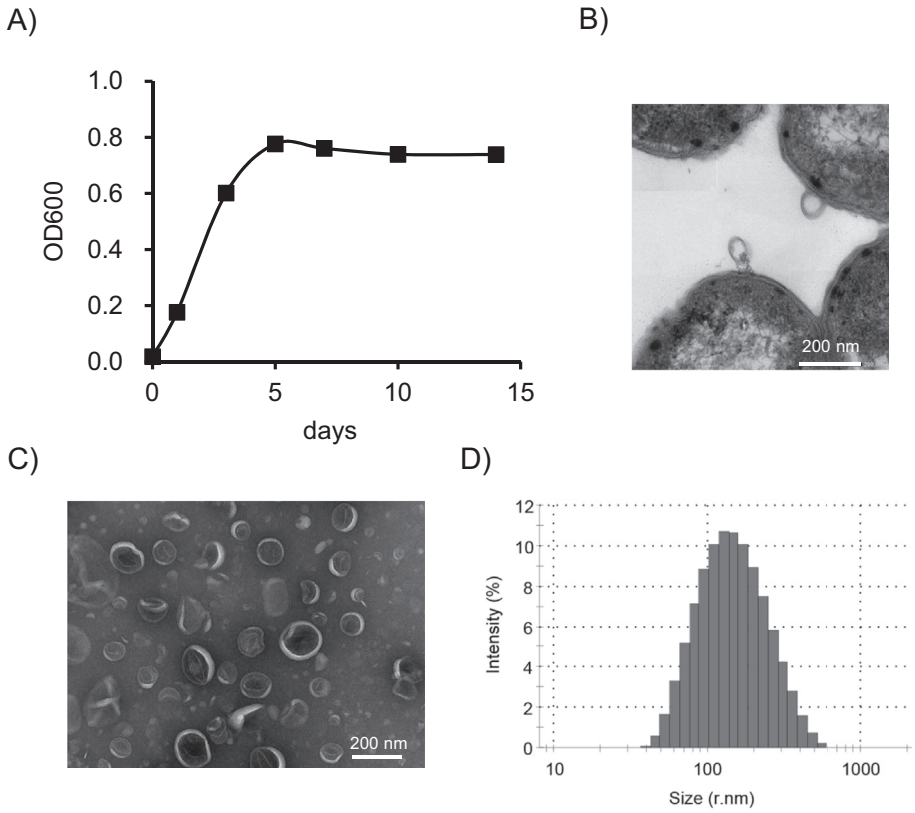

FIG. 1. Growth of *A. pasteurianus* NBRC 3283 and its OMVs secretion. (A) A typical growth curve of the cells in the No. 804 broth at 25 °C. Growth was measured by determining the optical density at 600 nm. (B) A TEM image of the cells secreting OMVs. The cells were obtained after 2 days in culture. (C) A typical TEM image of the crude OMVs obtained at day 3. (D) A DLS histogram of the crude OMVs obtained at day 3.