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Full Paper

High productivity of immunostimulatory membrane vesicles of *Limosilactobacillus antri* using glycine

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Nanosized membrane vesicles (MVs) released by bacteria play important roles in both bacteria–bacteria and bacteria–host interactions. Some gram-positive lactic acid bacteria produce MVs exhibiting immunoregulatory activity in the host. We found that both bacterial cells and MVs of *Limosilactobacillus antri* JCM 15950, isolated from the human stomach mucosa, enhance immunoglobulin A production by murine Peyer's patch cells. However, the thick cell walls of gram-positive bacteria resulted in low MV production, limiting experiments and applications using MVs. In this study, we evaluated the effects of glycine, which inhibits cell wall synthesis, on the immunostimulatory MV productivity of *L. antri*. Glycine inhibited bacterial growth while increasing MV production, with 20 g/L glycine increasing MV production approximately 12-fold. Glycine was most effective at increasing MV production when added in the early exponential phase, which indicated that cell division in the presence of glycine increased MV production. Finally, glycine increased MV productivity approximately 16-fold. Furthermore, glycine-induced MVs promoted interleukin-6 production by macrophage-like J774.1 cells, and the immunostimulatory activity was comparable to that of spontaneously produced MVs. Our results indicate that glycine is an effective agent for improving the production of MVs with immunostimulatory activity in gram-positive lactic acid bacteria, which can be applied as mucosal adjuvants and functional foods.

Key words: lactic acid bacteria, membrane vesicle, glycine, immunostimulatory activity

INTRODUCTION

Most bacteria release membrane vesicles (MVs) that are 20–400 nm in diameter and covered with a phospholipid bilayer [1, 2]. MVs contain nucleic acids, proteins, cell wall components, and signaling molecules and are associated with bacteria–bacteria interactions, such as quorum sensing [3], horizontal gene transfer [4], and biofilm formation [5]. They also protect their parent bacteria by acting as decoys for antibiotics and bacteriophages [6]. Recently, MVs were found to be involved in bacteria–host interactions, such as host immunomodulation [7] and the delivery of toxins and pathogenic components to the host [8].

Outer membrane vesicles (OMVs) derived from an outer membrane of gram-negative bacteria have long been studied [3, 8]. OMVs including pathogenic components as well as membrane proteins and lipopolysaccharide (LPS) have been applied as vaccines owing to their unique components and biological functions [9, 10]. On the other hand, it has been thought that gram-positive bacteria do not to release MVs due to the presence of thick cell walls; however, pathogenic bacteria such as *Staphylococcus aureus* [11] and *Clostridium perfringens* [12] have been reported to produce MVs. Recently, probiotic gram-positive bacteria, including lactic acid bacteria and *Bifidobacterium*, have been shown to produce MVs, with the MVs being involved in the delivery of antimicrobial peptides, bacteriocins [13], and biofilm formation [14]. Furthermore, it has been reported that MVs derived from lactic acid bacteria and *Bifidobacterium* exhibit host immunomodulatory activities [15–17].

We previously reported that MVs derived from *Latilactobacillus sakei* subsp. *sakei* (previously *Lactobacillus sakei* subsp. *sakei*) NBRC 15893 promoted immunoglobulin (Ig) A production by

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murine Peyer's patch (PP) cells. Secretory IgA plays an important role in defense against infections in the intestinal immune system and maintenance of the composition of the gut microbiota [18]. In this process of enhancement of IgA production, MVs stimulate dendritic cells via Toll-like receptor (TLR) 2, which recognizes conserved structures of cell wall components of gram-positive bacteria [19] and induces the production of factors involved in IgA class switch recombination and B cell differentiation into plasma cells [20]. In addition, MVs derived from *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*) enhance IgA production by PP cells via TLR2 [16]. These MVs, which activate the mucosal immune system, are expected to be applicable as safe adjuvants for noninvasive mucosal vaccines that induce antigen-specific IgA on the mucosa, such as nasal mucosal vaccines and sublingual vaccines [21]; however, gram-positive bacteria produce fewer MVs than gram-negative bacteria, which limits the research and application of MVs.

To enhance MV production by gram-positive lactic acid bacteria, it is crucial to understand the mechanisms of MV formation. In gram-negative bacteria, a lack of lipoproteins and peptidoglycans breaks crosslinks between the outer and inner membranes, resulting in the curvature of the outer membrane and the formation of OMVs [22]. OMVs are also generated by the accumulation of misfolded proteins in the periplasm or the loss of peptidoglycan [22], as well as by the reassembly of membrane fragments after the induction of cell lysis by endolysin, a phage-derived cell wall-hydrolyzing enzyme [23]. Conversely, the thick cell wall that comprises the outermost layer of gram-positive bacteria encloses the plasma membrane. Endolysins form pores in the cell wall of gram-positive bacteria as well, through which parts of the plasma membrane protrude as MVs [24], indicating that MV production can be promoted by introducing distortions or nanosized pores in the cell wall to release parts of the plasma membrane as MVs. In addition, for lactic acid bacteria to produce immunostimulatory MVs, the released plasma membrane should retain the cell wall components, such as lipoteichoic acid and lipoproteins, which are ligands for TLR2 [19]. Therefore, disrupting the rigid structure of the peptidoglycan layer would facilitate both the formation of pores in the cell wall and the translocation of these components into MVs.

The chemical structure of the peptidoglycan layer of lactic acid bacteria consists of sugar chains of alternately linked *N*-acetylglucosamine and *N*-acetylmuramic acid and peptide chains containing D- and L-alanine residues [25]. Antibiotic glycine is known to replace D- and L-alanine residues in the peptide chain of peptidoglycan in the bacterial cell wall, resulting in the inhibition of normal peptidoglycan synthesis [26]. Glycine enhances immunostimulatory OMV production by probiotic *Escherichia coli* Nissle 1917 [21]; however, the immunostimulatory molecule is LPS in the outer membrane, and the effects of glycine on MV production and its immunostimulatory activity in gram-positive lactic acid bacteria remain unknown.

We previously screened lactic acid bacteria with immunostimulatory activity and found that in addition to *L. sakei* NBRC 15893 [18], *Limosilactobacillus antri* (previously *Lactobacillus antri*) JCM 15950^T, which was isolated from the human gastric mucosa, promotes IgA production from murine PP cells. In the present study, we attempted to enhance the production of MVs with immunostimulatory activity in gram-positive lactic acid bacteria using glycine, with *L. antri* JCM 15950^T as a model strain. Furthermore, the immunostimulatory activities of glycine-induced MVs and spontaneously produced MVs were examined to verify whether MV production could be increased while maintaining immunostimulatory activity.

MATERIALS AND METHODS

Bacterial strain and culture conditions

L. antri JCM 15950^T was purchased from the Japan Collection of Microorganisms (JCM) of the RIKEN BioResource Research Center (Ibaraki, Japan). *L. antri* was anaerobically cultured at 37°C in de Man, Rogosa, and Sharpe (MRS) medium with an AnaeropPack (Mitsubishi Gas Chemical Company, Tokyo, Japan) or Culture-tech (As One, Osaka, Japan) system. Cell concentration was measured as turbidity at 660 nm using a U-5100 spectrophotometer (Hitachi, Tokyo, Japan). Glycine was added to the culture broth during cultivation at a volume ratio of 3:47 (glycine solution:culture broth).

Ethanol-killed bacteria were prepared by incubating the bacterial cells in 70% (v/v) ethanol at room temperature (18–25°C) for 5 min and subsequently drying at reduced pressure.

Measurement of cell viability

Bacterial cells were stained with 1 µg/mL 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Dojindo Laboratories, Kumamoto, Japan) for 5 min at room temperature. The cells were then stained with 6 µg/mL propidium iodide (PI; Fujifilm Wako Pure Chemical, Osaka, Japan) for 5 min at room temperature. DAPI and PI were detected with an EVOS DAPI light cube (excitation, 357/44 nm; emission, 447/60 nm) and EVOS Qdot 705 light cube (excitation, 445/45 nm; emission, 710/40 nm), respectively, using an EVOS M5000 fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA). Cells stained only with DAPI were classified as viable cells, and those stained with both DAPI and PI were classified as dead cells. Cell viability was defined as the percentage of viable cells relative to the total number of cells.

Purification of MVs

The culture broth was centrifuged (8,500 × g, 5 min, 4°C), and the supernatant was filtered through a 0.22-µm polyethersulfone (PES) membrane filter unit (Thermo Fisher Scientific). The precipitate was collected by ultracentrifugation (150,000 × g, 2 hr, 4°C) and washed with 10 mM HEPES containing 0.85% NaCl (pH 6.8; referred to as HEPES-NaCl). The washed precipitate collected by ultracentrifugation (150,000 × g, 2 hr, 4°C) was suspended in HEPES-NaCl and served as the crude MV fraction.

MVs were purified via density gradient ultracentrifugation or gel filtration chromatography. Density gradient ultracentrifugation was performed as previously described [18]. For gel filtration chromatography, Sephacryl S-500 HR (Cytiva, Tokyo, Japan) packed in a column (ø 1.5 × 10 cm) was equilibrated with HEPES-NaCl, and the crude MV fraction was added to the column. MVs were eluted with HEPES-NaCl, and the eluate was collected as fractions of 500 µL each. The purified MV suspension was concentrated using a Vivaspin Turbo 15 centrifugal concentrator (MWCO 30,000, PES membrane; Sartorius, Goettingen, Germany), if needed.

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Quantification of MVs

The lipid concentration was used as the MV concentration. It was quantified by staining the samples with 5 µM 4-[6-[4-(diethylamino) phenyl]-1,3,5-hexatrien-1-yl]-1-[3-(triethylammonio) propyl] pyridinium dibromide (FM4-64; Biotium, Fremont, CA, USA), which stains lipid membranes [27], at 37°C for 10 min. Fluorescence intensity was measured at an excitation wavelength of 515 nm and a fluorescence wavelength of 640 nm using a Beacon 2000 fluorescence polarization spectrometer (Thermo Fisher Scientific). Protein concentrations were quantified using a Qubit Protein Assay Kit (Thermo Fisher Scientific) and Qubit 4 fluorometer (Thermo Fisher Scientific) according to the manufacturer's instructions.

Quantification of phospholipids

Lipids were extracted from bacterial cells using the chloroform-methanol extraction method [28]. The bacterial cells were washed with saline, resuspended in chloroform-methanol (2:1, v/v), and homogenized on ice for 30 sec using a Handy Sonic UR-21P ultrasonic disrupter (Tomy Seiko, Tokyo, Japan). The supernatant was obtained by centrifugation (2,000 × g, 5 min, 4°C), water (1/5 volume) was added to it, and the supernatant was vortexed for 1 min. The chloroform layer was collected after centrifugation (2,000 × g, 5 min, 4°C). Phospholipids were determined using the Stewart method [29, 30]. Briefly, 20 µL of MV suspension, 700 µL of ammonium ferrothiocyanate solution (27.0 g/L iron(III) chloride hexahydrate, 30.4 g/L ammonium thiocyanate), and 700 µL of chloroform were vortexed for 5 min, and the absorbance of the chloroform layer at 488 nm was measured. L-α-phosphatidylethanolamine was used as a standard.

Transmission electron microscopy (TEM)

MVs were observed using TEM. A 150-mesh formvar support film (PVF-C15 STEM Cu150 Grid, Okenshoji, Tokyo, Japan) was hydrophilized for 60 sec using a hydrophilic processor (DII-29020HD, JEOL, Tokyo, Japan). MVs on the film were stained with 2% uranyl acetate or an EM Stainer (Nissin EM, Tokyo, Japan) for 5 min and observed using a transmission electron microscope (H7500, Hitachi, or JEM-1400, JEOL) at an acceleration voltage of 80 kV.

Scanning electron microscopy (SEM)

Bacterial cells were observed using SEM. They were pre-fixed in 0.1 M sodium phosphate buffer (pH 7.2) containing 2% (w/v) glutaraldehyde at 4°C for 2 hr. The washed cells were post-fixed in a phosphate buffer containing 1% osmium tetroxide at 4°C for 1 hr. After washing, the cells were sequentially dehydrated in 60, 70, 90, 95, and 99% (v/v) ethanol. The samples were suspended in isoamyl acetate and dried under reduced pressure for 4 hr. Cells were coated with 10-nm gold using a non-equilibrium material preparation apparatus (MUE-201-HC3, ULVAC, Kanagawa, Japan) and observed using a scanning electron microscope (JSM-7500F, JEOL) at an acceleration voltage of 5.0 kV.

Dynamic light scattering (DLS) analysis

The particle size distributions and particle numbers of the MV suspensions were analyzed at 25°C in a 12-mm square polystyrene cell (DTS0012, Malvern Instruments, Malvern, UK) using a Zetasizer Nano ZS particle analyzer (Malvern Instruments) and the Zetasizer software (Malvern Instruments). The mode diameter (particle diameter with the maximum frequency distribution) and average diameter (mean particle diameter) were analyzed.

Evaluation of immunostimulatory activity

BALB/c mice (female, 7–12 weeks old; Japan SLC, Shizuoka, Japan) were bred, and murine PP cells were prepared and cultured as previously described [18]. The experimental animal protocols were approved by the Animal Ethics Committee of Kansai University (Approval No. 1715, 1912) and were conducted in accordance with the ARRIVE guidelines (https://arriveguidelines.org/).

PP cells were suspended in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), 55 µM 2-mercaptoethanol, 100 µg/mL streptomycin, and 100 U/mL penicillin. The cells were inoculated into a 96-well plate at a density of 1.0×10^5 cells/well and then cultured with or without samples for 4 days at 37°C in the presence of 5% CO_2. For neutralization of TLR2, PP cells were preincubated with 0.5 µg/mL anti-TLR2 antibody (purified anti-mouse/human CD282, clone T2.5, BioLegend, San Diego, CA, USA) or an isotype antibody (purified mouse IgG1, clone MOPC-21, BioLegend) for 30 min and then cultured with or without samples for 4 days at 37°C.

Macrophage-like J774A.1 cells derived from BALB/c mice were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB) of the National Institute of Biomedical Innovation, Health, and Nutrition. J774A.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) containing 10% (v/v) fetal bovine serum (Biowest, Nuaillé, France), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in the presence of 5% CO_2. The cells were inoculated into a 96-well plate (Thermo Scientific) at a density of 1.0×10^5 cells/well and pre-cultured for 3 hr. After addition of the samples, the cells were cultured for 24 hr. Pam2CSK4 (1 µg/mL; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), a synthetic ligand for TLR2/6, was used as the positive control. The concentrations of IgA and IL-6 in the culture supernatant were determined by ELISA as described previously [18, 20].

Statistical analysis

All data are expressed as the mean ± standard error (SE). $P<0.05$ was considered statistically significant based on the Dunnett's test or Tukey's test.

RESULTS

Production and immunostimulatory activities of MVs

The ability of *L. antri* to activate the mucosal immune system was examined using murine PP cells. Ethanol-killed bacterial cells promoted IgA production by PP cells in a concentration-dependent manner (Fig. 1a). To confirm whether *L. antri* can produce MVs, the precipitate obtained via ultracentrifugation of a 24-hr culture broth supernatant was fractionated via density gradient ultracentrifugation. TEM observations revealed that MVs with typical spherical structures, with diameters ranging between approximately 30 and 200 nm in the 1.14 g/cm^3 fraction, were present (Fig. 1b). The purified MVs enhanced IgA production by PP cells as well as ethanol-killed bacterial cells (Fig. 1c). Furthermore, we investigated the involvement of TLR2

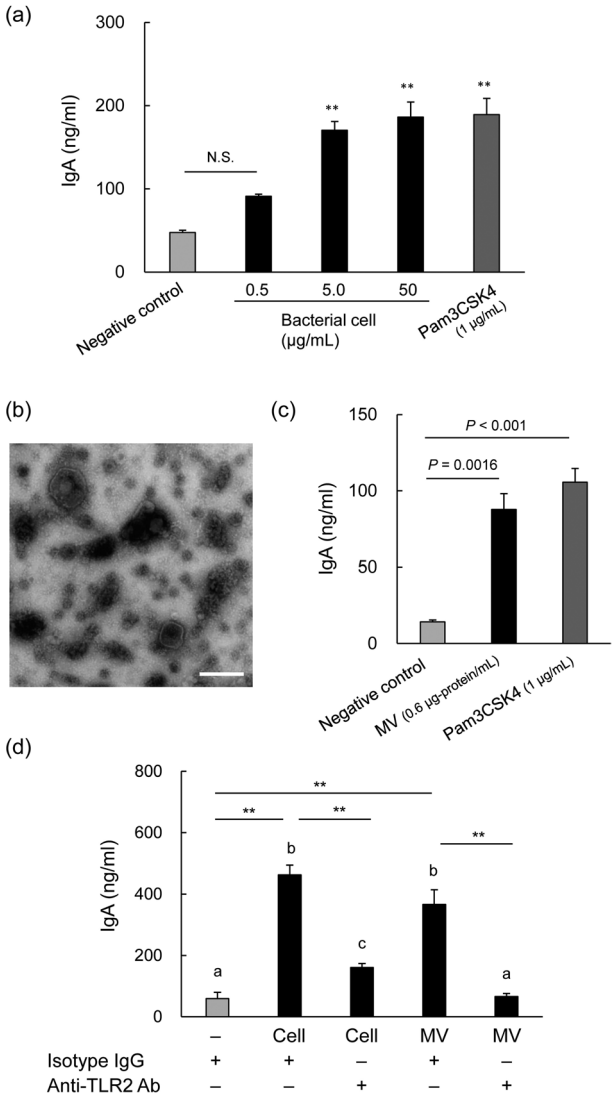

Fig. 1. IgA-enhancing activity of bacterial cells and MVs of *L. antri*. (a) Enhancement of IgA production by Peyer's patch cells induced by ethanol-killed *L. antri*. Mean ± SE (n=3). **p<0.001 vs. negative control based on Dunnett's test. (b) TEM image of MVs derived from *L. antri* stained with 2% uranyl acetate. The scale bar indicates 200 nm. (c) Enhancement of IgA production by MVs derived from *L. antri*. Mean ± SE (n=3). Statistical analysis was performed using a Dunnett's test. (d) Role of TLR2 in immunostimulatory effects of bacterial cells (5 μg dry cells/mL) and MVs (1.0 μg protein/mL) of *L. antri*. Bars with the same letters are not significantly different from each other (p>0.05), as assessed via Tukey's test. **p<0.001. IgA: immunoglobulin A; MV: membrane vesicles; TEM: transmission electron microscopy; SE: standard error; TLR2: toll-like receptor-2.

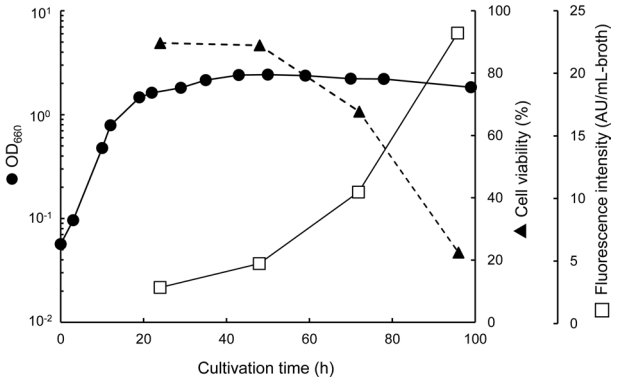

Fig. 2. Time course of proliferation and MV production in *L. antri*. The MV concentration was evaluated in crude MV fractions as the fluorescence intensity of FM4-64. Cell viability was determined via DAPI and PI staining. Experiments were performed at least twice, and representative results are shown. MV: membrane vesicles; OD: optical density.

in the IgA-enhancing effects of MVs and the bacterial cells. TLR2 forms heterodimers with TLR1 or TLR6 and recognizes gram-positive bacterial cell wall components such as lipoteichoic acid, lipoproteins, and peptidoglycan [19]. IgA production enhanced by MVs and the bacterial cells were eliminated upon neutralization of TLR2 with anti-TLR2 antibodies, indicating that immunostimulatory components and the properties of enhanced IgA production were conferred to MVs from the parent cells (Fig. 1d). The IgA-enhancing activities of lactic acid bacterial cells differed by strain (data not shown), which suggested that the immunostimulatory activities of MVs depend on the properties of cell wall components of the parent cells.

Next, the relationship between cell growth and MV production was investigated to understand the MV-producing ability of *L. antri* (Fig. 2). The growth of *L. antri* reached the stationary phase after 20 hr of culture, and cell viability decreased after 48 hr. The specific growth rate was 0.22 hr^{-1}, and the doubling time was 3.1 hr in the exponential phase. The MV concentrations of crude MV fractions (200 μL) prepared from culture broth (25 mL) at 24 (early stationary phase), 48 (mid stationary phase), 72 (early death phase), and 96 hr (death phase) were determined by staining with FM4-64. After 48 hr, the amount of MVs increased as cell viability decreased, although the turbidity did not change significantly. This suggested that the cells died but that the shape of the cell walls remained intact and that MV production was associated with cell death accompanied by damage to the plasma membrane.

Effects of glycine on bacterial growth and MV production

In gram-positive bacteria, MVs are formed by the extrusion of plasma membranes through pores in the cell wall [24]. Here, we investigated whether glycine, which inhibits cell wall peptidoglycan synthesis [26], can enhance MV production by *L. antri*.

When *L. antri* was cultured in MRS medium supplemented with glycine at 0−25 g/L, glycine concentrations >15 g/L inhibited growth, and almost no growth was observed in the medium supplemented with 25 g/L of glycine (Fig. 3a). To investigate the effects of glycine on MV production, crude MV fractions (300 μL) were prepared from each culture broth (25 mL), and the lipid concentration was determined using FM4-64. Lipid concentrations in the crude MV fractions increased in a glycine concentration-dependent manner (Fig. 3b). At 20 g/L, glycine inhibited bacterial growth and increased the MV concentration 12-fold, which indicated that glycine enhanced MV production in lactic acid bacteria despite growth inhibition (Fig. 3b). Cell viability in cultures without glycine was 93.0 ± 0.8%, and it decreased to 87.1 ± 1.1% in the presence of 20 g/L glycine. However, the decrease was much smaller than that due

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to extended cultivation, suggesting that factors other than cell death are responsible for the increase in MV production upon the addition of glycine.

Next, we investigated whether the addition of glycine after bacterial growth can increase MV production. Glycine (20 g/L) was added at the beginning of culture (0 hr) and at the early (4 hr), mid (8 hr), and late exponential (16 hr) phases (Fig. 2); *L. antri* was cultured for a total of 24 hr. MV production per culture broth was highest when glycine was added in the early exponential phase (Fig. 4). MV production was only slightly enhanced by glycine addition after the late exponential phase, which indicated that multiple divisions in the presence of glycine were effective for increasing MV production. Therefore, in subsequent experiments, glycine was added at a concentration of 20 g/L after 4 hr of culture, that is, in the early exponential phase.

Structural characteristics of glycine-induced MVs

To confirm the effects of glycine on cell wall structure, we investigated the morphologies and surface structures of bacterial cells cultured with the addition of glycine (20 g/L; added after 4 hr during 24 hr of culture). SEM observations revealed that the morphologies of the bacterial cells cultured in the absence of glycine were uniformly rod shaped (Fig. 5a), whereas the long diameters of the cells in the presence of glycine were generally shorter, and some cells were distorted and had partial concave areas (Fig. 5b). Additionally, the surfaces of the bacterial cells cultured in the absence of glycine were smooth (Fig. 5a), whereas those of the cells cultured in the presence of glycine were uneven and rough, and MV-like structures adhered to them (Fig. 5b).

TEM observations confirmed the presence of MVs with diameters ranging from 30–300 nm in crude MV fractions obtained from the culture supernatants both in the presence and absence of glycine (Fig. 5c, 5d). No morphological differences were observed between the glycine-induced and spontaneously produced MVs. In the presence of glycine, larger MVs with diameters exceeding approximately 100 nm were more abundant (Fig. 5d). DLS analysis showed that the mode diameter and average diameter of MVs were 27.9 ± 5.6 and 71.8 ± 0.1 nm in the absence of glycine and 41.7 ± 11.9 and 151.4 ± 1.9 nm in the presence of glycine, respectively (Fig. 5e). Although some of the smaller diameter (<30 nm) structures could have contained culture medium-derived material, these results indicated that MVs induced by glycine have larger diameters than those produced spontaneously.

Immunostimulatory activity of glycine-induced MVs

We previously reported that MVs derived from *L. sakei* NBRC 15893 induce the production of the proinflammatory cytokine IL-6 from $CD11c^+$ cells, including dendritic cells and macrophages in PP cells, resulting in the enhancement of IgA production [20]. To evaluate the immunostimulatory activity of glycine-induced and spontaneously produced MVs of *L. antri*, their ability to induce IL-6 production was investigated using macrophage-like J774A.1 cells. When these MVs were added to J774A.1 cells at various phospholipid concentrations, both types induced IL-6 production in a concentration-dependent manner (Fig. 6). There was no difference in the immunostimulatory activities of glycine-induced and spontaneously produced MVs.

Effects of glycine on MV productivity

The MVs of gram-positive bacteria are composed of the plasma membranes of the parent cells. To evaluate the effects of glycine on MV production in *L. antri*, bacteria and purified MVs were obtained from the culture broth (300 mL), and the percentage of plasma membrane released as MVs by the bacterial cells was calculated. In the absence of glycine, 2.2% of the total

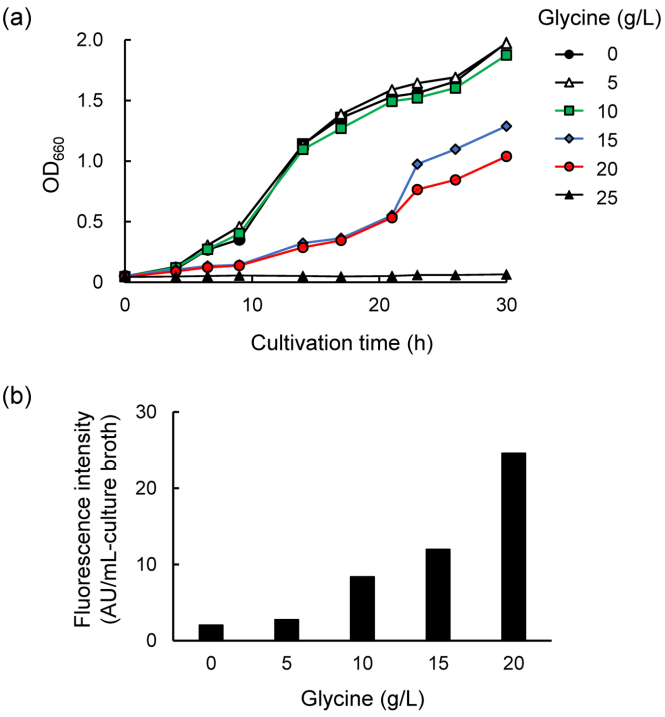

Fig. 3. Effects of glycine on the growth and MV production of *L. antri*. (a) Effects of glycine on bacterial growth. Glycine was added to the MRS medium beforehand. (b) Effects of glycine on MV production. The MV concentration was evaluated as the fluorescence intensity of FM4-64. Experiments were performed at least twice, and representative results are shown. MV: membrane vesicles; OD: optical density.

Fig. 4. Effects of the timing of glycine addition on MV production in *L. antri*. The MV concentration was evaluated as the fluorescence intensity of FM4-64. Experiments were performed at least twice, and representative results are shown. MV: membrane vesicles.

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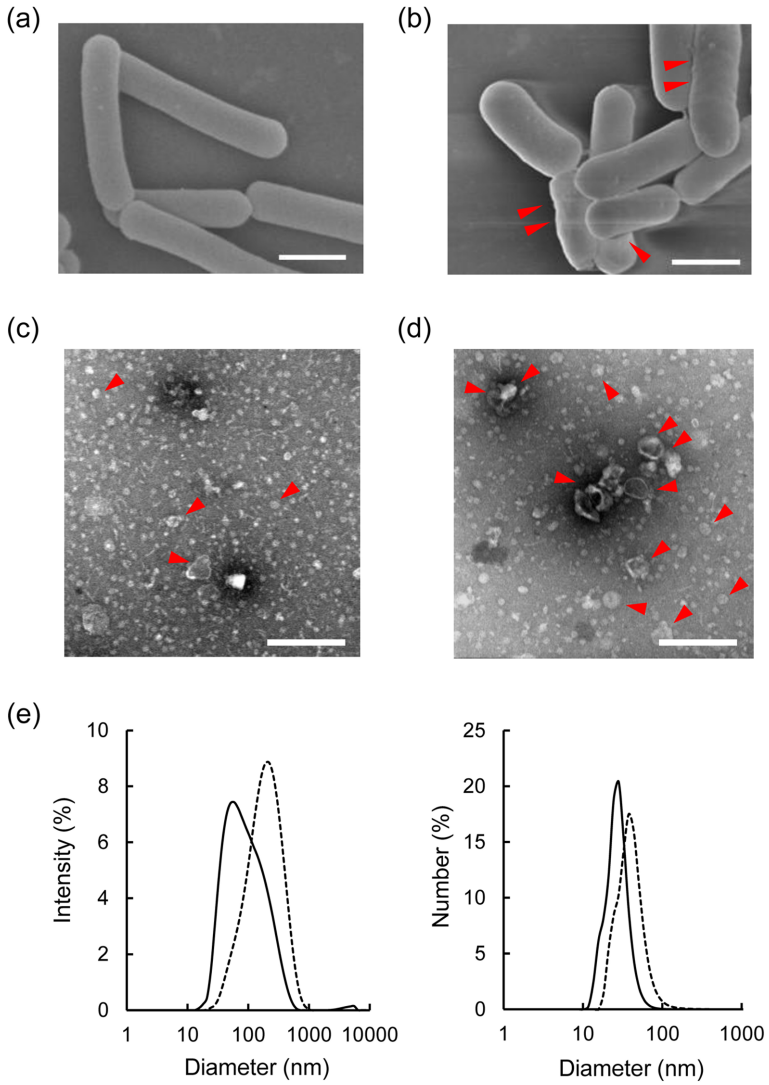

Fig. 5. Characteristics of spontaneously produced and glycine-induced MVs of *L. antri*. SEM images of bacterial cells grown (a) in the absence of glycine and (b) in the presence of glycine. Red arrows indicate distortion of cell morphology. Scale bars show 500 µm. TEM images of (c) spontaneously produced MVs and (d) glycine-induced MVs stained with an EM Stainer. The red arrows indicate typical MVs with larger diameters. Scale bars show 500 nm. (e) Size distribution of spontaneously produced MVs (solid line) and glycine-induced MVs (dotted line). MV: membrane vesicles; SEM: scanning electron microscopy; TEM: transmission electron microscopy.

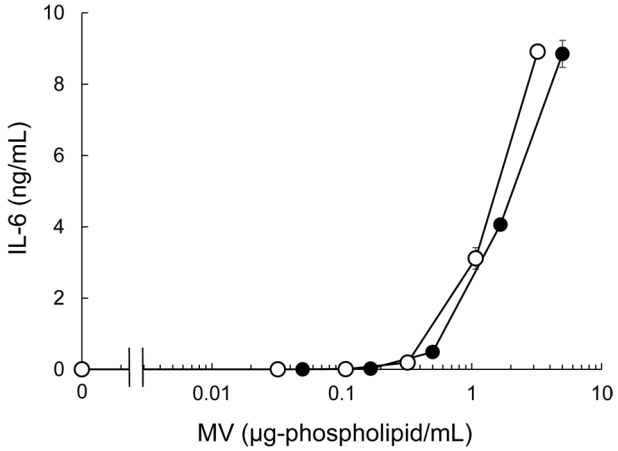

Fig. 6. Immunostimulatory activities of spontaneously produced and glycine-induced MVs of *L. antri*. Immunostimulatory activities of spontaneously produced MVs (open circles) and glycine-induced MVs (closed circles) were evaluated based on their ability to induce IL-6 production in J774.1A cells. Mean ± SE (n=3). MV: membrane vesicles.

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phospholipids in the bacteria were released as MVs after 24 hr of culture, whereas the addition of glycine increased this percentage to 19% (Table 1).

In the presence of glycine, MV concentrations increased rapidly during the first 24 hr of incubation. In the absence of glycine, MV concentrations gradually increased until 96 hr. The amount of MVs obtained in the absence of glycine after 96 hr of incubation was approximately 40% of that obtained in the presence of glycine (Fig. 7a). MV production per culture broth and per unit time was approximately 16 times higher after glycine addition (Fig. 7b).

DISCUSSION

The physiological functions of MVs of gram-positive lactic acid bacteria have been discovered and studied; however, the low productivity of MVs has been a problem in research and applications. As no general method has yet been established to increase MV production in gram-positive bacteria, in the present study, we attempted to enhance the productivity of immunostimulatory MVs of *L. antri* JCM 15950 using glycine as an antimicrobial agent.

Several MV-formation mechanisms, including blebbing [22] and explosive cell lysis [23], have been proposed for gram-negative bacteria. Moreover, some of the mechanisms in gram-positive bacteria have also become clearer in the past few years, and the bubbling cell death model, in which endolysin partially degrades the cell wall and the plasma membrane is extruded through pores as MVs, has been proposed [24]. Since the origin of MVs in gram-positive bacteria is the plasma membrane within the cell wall, MVs would not be released if the cell wall was robust, without any pores or distortions.

We found that only 2.2% of the total bacterial phospholipids were spontaneously released as MVs in *L. antri* after 24 hr of culture. For *Bacillus subtilis*, a gram-positive bacterium, it has also been reported that only a low proportion of total cells release MVs under normal culture conditions [24], which indicates that the majority of the bacterial cell population does not spontaneously produce MVs. It was further reported that gram-positive bacteria produced MVs via cell lysis and that endolysin released from dead cells induced MV production in the surrounding cells [24]. In the present study, extended cultivation may have increased MV production as a result of cell lysis after cell death. In contrast, the addition of glycine increased MV production without decreasing cell viability. Thus, the mechanism underlying this increase appeared to be different from that observed with extended cultivation. These results suggest that in gram-positive bacteria, the formation of small pores in the cell wall may be a more important event related to MV production than cell death.

Finally, we demonstrated that glycine increased MV productivity of *L. antri* up to 16-fold, indicating that cell wall weakening by glycine is much more effective for increasing MV production than long-term cultivation. In gram-negative *E. coli* Nissle 1917, it was reported that glycine increased OMV production 51-fold [21]. This high rate of increase suggests that *E. coli* may release OMVs while propagating at a large growth rate. In addition to glycine, penicillin G, a β-lactam antibiotic that inhibits cell wall synthesis, has also been reported to increase MV production by *Corynebacterium glutamicum* [31]. These findings suggest that an abnormal cell wall structure may trigger MV formation in gram-positive bacteria. It has also been reported that the peptidoglycan layer originally has pores that are a few nanometers in diameter and that cell lysis is induced when pores that are 15–24 nm in diameter or larger are formed in gram-

Table 1. Percentage of phospholipids released as MVs by bacterial cells

Glycine (g/L)	OD_{660}	Phospholipid (μg/mL-broth)		MVs to total phospholipids (%)
		Cell	MV	
0	1.7	4.4 ± 0.21	0.098 ± 0.073	2.2
20	0.81	2.1 ± 0.045	0.48 ± 0.15	19

MV: membrane vesicles; OD: optical density.

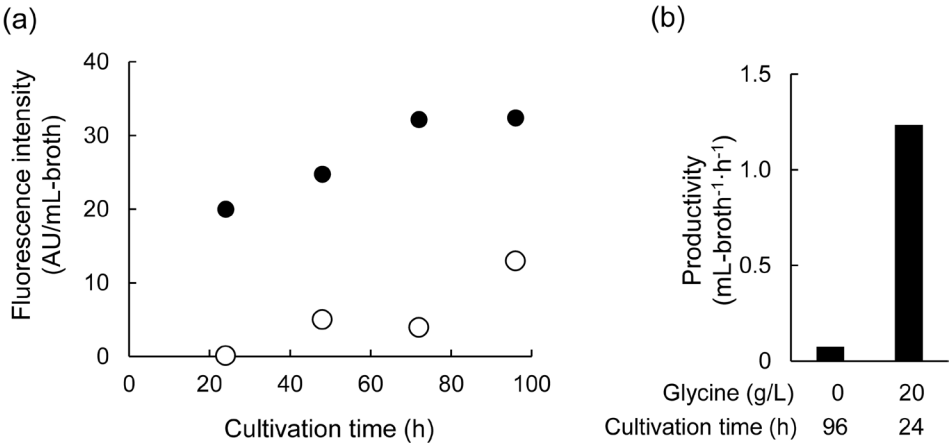

Fig. 7. Effects of glycine on MV productivity in *L. antri*. (a) Time course of MV production in the absence (open circles) and presence of glycine (closed circles). (b) MV productivity of *L. antri* in the absence and presence of glycine. MV: membrane vesicles.

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positive bacteria [32]. It was also shown that as the diameters of the pores increased, the diameters of the spherical bulges in the plasma membrane extruding from them increased as well [32]. Furthermore, peptidoglycan layers of gram-positive bacteria are reported to have a porous mesh structure with a low areal density [33]. We showed that the diameters of glycine-induced MVs were larger than those of spontaneously produced MVs, possibly due to the formation of larger pores in the cell wall by glycine. The inhibition of cell wall synthesis by glycine may have further roughened the mesh structure and facilitated the localized formation of large holes, which may have caused the plasma membrane to be released as MVs.

Here, we showed that *L. antri* MVs enhance IgA production via TLR2 and induce IL-6 production from macrophages, indicating that MVs can activate both innate and acquired immune systems, which results in antibody secretion. In addition, glycine-induced MVs also have an immunostimulatory activity comparable to that of spontaneously produced MVs. MVs derived from other lactic acid bacteria, such as *L. sakei* MVs [20] and *L. plantarum* MVs [16] also enhance IgA production by inducing cytokine production via TLR2. TLR2 is considerably involved in immune activation by lactic acid bacterial cells [34, 35] and recognizes lipoteichoic acid and lipoprotein, forming heterodimers with TLR1 or TLR6 [36]. It has been reported that lipoteichoic acid is present on the surface layer of *Lactobacillus gasseri* MVs [37] and that the lipoproteins are present in L. plantarum MVs [16]. Since these molecules anchor to the plasma membrane in bacterial cells, it is presumed that some of these molecules that stimulate TLR2 are retained in the membrane of *L. antri* MVs after passing through pores in the cell wall and that glycine has no effect on this process. On the other hand, in *E. coli*, glycine-induced MVs have been reported to have almost the same level of immunostimulatory activity as non-induced MVs, although the endotoxin activity of glycine-induced MVs is reduced compared with that of non-induced MVs [21]. It is necessary to understand the further immunostimulatory mechanisms of MV in *L. antri*, especially to identify the molecules in MVs that are involved in the activation of TLR2.

In summary, we demonstrated that *L. antri*, a gram-positive lactic acid bacterium, produces MVs that promote mucosal IgA production and that glycine is extremely effective in enhancing the production of MVs while maintaining their immunostimulatory activity. This finding is expected to make significant contributions to MV research in gram-positive bacteria and the application of MVs as a mucosal adjuvant and functional food that regulates the intestinal immune system. However, the source of MVs in gram-positive bacteria is the plasma membrane, and the upper limit of MV production per bacterial cell is the total lipid content of the cell. Therefore, to further increase the amount of MVs, it is necessary to either increase the density of bacterial cells by controlling the culture conditions or to develop methods that artificially form pores in the cell wall and then lower the osmotic pressure to release the cell membrane.

AUTHOR CONTRIBUTIONS

S.Y-Y. designed the study, conducted the experiments, analyzed the data, and wrote and edited the manuscript. Y.S., K.N., A.S., and T.I. conducted the experiments and collected and analyzed the data. T.I., J.K., and Y.K. contributed to the manuscript review and interpreted data. All authors analyzed the data, discussed the results, and approved the final version of the manuscript for publication.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available from the corresponding author upon reasonable request.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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