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Title	Histo-Blood Group Antigen-Like Substances of Human Enteric Bacteria as Specific Adsorbents for Human Noroviruses
Author(s)	Miura, Takayuki; Sano, Daisuke; Suenaga, Atsushi et al.
Citation	Journal of Virology, 87(17), 9441-9451 https://doi.org/10.1128/JVI.01060-13
Issue Date	2013-06-26
Doc URL	https://hdl.handle.net/2115/54031
Type	journal article
File Information	Miura2013JVIRevisedv2.pdf



JVI01060 Revised manuscript

Title: Histo-Blood Group Antigen-Like Substances of Human Enteric Bacteria as Specific Adsorbents for Human Noroviruses

Running title: Bacterial HBGAs Specifically Bind to Human Norovirus

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Abstract word count: 245

Text word count: 6884

23 **Abstract**

24 Histo-blood group antigens (HBGAs) have been suggested to be receptors or co-receptors
25 for human noroviruses (HuNoVs) expressed on the intestinal epithelium. We isolated an enteric
26 bacterium strain (SENG-6), closely related to *Enterobacter cloacae*, bearing HBGA-like substances
27 from a fecal sample of a healthy individual using a biopanning technique with anti-HBGA
28 antibodies. The binding capacities of four genotypes of norovirus-like particles (NoVLPs) to
29 *Enterobacter* sp. SENG-6 cells were confirmed by enzyme-linked immunosorbent assay (ELISA).
30 Transmission electron microscopy visualized that NoVLPs bound mainly to extracellular polymeric
31 substances (EPS) of *Enterobacter* sp. SENG-6, where the HBGA-like substances were localized.
32 EPS that contained HBGA-like substances extracted from *Enterobacter* sp. SENG-6 was shown by
33 ELISA to be capable of binding to NoVLPs of GI.1 wild-type strain (8fIIa) and GII.6 that can
34 recognize A antigen, but not to the NoVLP GI.1 mutant strain (W375A) that loses the binding
35 capability to A antigen. Enzymatic cleavage of terminal N-acetylgalactosamine residues in the
36 bacterial EPS weakened bacterial EPS binding to the GI.1 wild strain (8fIIa). These results indicate
37 that A-like substances in the bacterial EPS play a key role in binding to NoVLPs. Since the specific
38 binding of HuNoVs to HBGA-positive enteric bacteria is likely to affect the transmission and
39 infection processes of HuNoVs in their hosts and in the environments, further studies of human
40 enteric bacteria and their binding capacity to HuNoVs will provide a new scientific platform for
41 understanding interactions between two types of microbes that were previously regarded as
42 biologically unrelated.

43

44 **Introduction**

45 Human noroviruses (HuNoVs) are major causative agents of nonbacterial acute
46 gastroenteritis in humans, which constitute a substantial disease burden worldwide (1). Noroviruses
47 belong to the genus *Norovirus* in the family *Caliciviridae*. The genus *Norovirus* is divided into 5
48 genogroups, i.e., GI, GII, GIII, GIV, and GV, and the strains in each genogroup can be further
49 divided into genotypes (2). GI, GII, and GIV NoVs, which include at least 15 genotypes, 18
50 genotypes, and 1 genotype, respectively (3), infect humans of all age groups, causing symptoms
51 such as nausea, vomiting, diarrhea, abdominal cramps, headache, and fever (4). Human-to-human
52 infection is the main transmission route of HuNoVs, but contaminated water and sea products such
53 as oysters are reported to be sources or vehicles of infection (5, 6) because of environmental
54 contamination with domestic wastewater (7, 8).

55 The lack of tissue cells for replicating HuNoVs has impeded the study of the life cycle of
56 this important human pathogen. One major finding related to productive infections with HuNoVs is
57 the interaction with histo-blood group antigens (HBGAs), which have been proposed to be
58 receptors or co-receptors of human small intestinal epithelial cells for HuNoVs (9, 10). HBGA
59 comprise ABH and Lewis antigens, which are structurally related oligosaccharides, and each
60 HuNoV genotype or strain has its own HBGA recognition pattern profile (11-13). For example,
61 virus-like particles (VLPs) of Norwalk virus (NV/68), a genotype 1 strain in genogroup I (GI.1) and
62 the prototype strain of norovirus, bind to HBGA in saliva from secretor-positive individuals and
63 preferentially bind to H type 1, Le^b, and type A carbohydrates (11, 14). Furthermore, VLPs of GII.4
64 (r104) can recognize a broader range of blood group carbohydrates than other genotypes (12),
65 although the ligand binding patterns have changed over time (15). The importance of the HBGA

66 recognition pattern for HuNoV infections has been emphasized because GII.4 strains are the most
67 prevalent etiological agents of infectious diseases caused by norovirus, probably because of their
68 broad HBGA recognition profile.

69 In this study, we focused on a group of human enteric bacteria that produce
70 HBGA-positive extracellular polymeric substances (EPS). EPS comprise organic macromolecules
71 such as polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds located on
72 or outside the cell surface (16). Humans possess immunoglobulin M (IgM) antibodies against
73 non-self HBGA in the blood, which is attributable to the presence of enteric bacteria with blood
74 group activity (17). This led us to speculate that human enteric bacteria may capture HuNoV
75 particles via specific interactions with HBGA-like bacterial substances. To elucidate the specific
76 interaction between HuNoV particles and HBGA-like bacterial substances, we screened blood
77 group-active human enteric bacteria from human feces using a biopanning technique with
78 anti-HBGA antibodies. We tested the binding capacity of four genotypes to norovirus-like particles
79 (NoVLPs) for bacterial cells using enzyme-linked immunosorbent assay (ELISA). NoVLP binding
80 to bacterial cells was observed by transmission electron microscopy (TEM), and the localization of
81 HBGA-like bacterial substances was analyzed by immuno-TEM. EPS, surface-retained organic
82 matter (SOM), and lipopolysaccharide (LPS) were extracted from bacterial cells, and the
83 interactions between HBGA-like substances in the extracted bacterial polymers and NoVLPs were
84 examined by ELISA. The specific interactions between HBGA-like substances and NoVLPs were
85 evaluated further by the quartz crystal microbalance (QCM) method.

86

87 **Materials and Methods**

Isolation of human enteric bacteria bearing HBGA-like substances. We screened enteric bacteria bearing HBGA-like substances from human feces using anti-HBGA antibodies. Fifty microliters of anti-blood group A, B, or O(H) mouse monoclonal antibodies (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA) were added to each well of an ELISA plate and kept at room temperature (RT) for 1 h to coat the well. The wells were washed two times with 0.1 M phosphate-buffered saline (PBS), and a diluted human fecal suspension derived from a healthy adult was added to the wells. After incubation at RT for 1 h, the wells were washed two times with PBS and 2% brilliant green bile broth (Kanto Chemical Co., Inc., Japan), *E. coli* broth (Nihon Pharmaceutical Co., Ltd., Japan), Enterobacteriaceae enrichment mannitol broth (Nihon Pharmaceutical Co., Ltd.), or enterobacteria enrichment broth-Mossel (Nihon Pharmaceutical Co., Ltd.) was added to each well. After overnight incubation at 37°C under microaerobic conditions, the enriched cells were cultured further by streaking onto an agar plate containing the same medium, and several colonies were selected randomly. The selected colonies were cultured again by streaking onto the same agar plates, and a single colony was selected. The selected cells were cultured in the same culture broth, collected by centrifugation, and suspended in PBS. Tenfold dilution series were prepared with PBS, and the OD₆₀₀ values of the diluted cell suspensions were measured using a spectrophotometer. The presence of HBGA-like substances on the cell surface in the diluted cell suspensions were detected on the basis of their aggregation activities with anti-blood group A, B, or O(H) antibody-coated beads from a blood typing kit (ABO sphia; Kamakura Techno-Science Inc., Japan). The names of the antibodies used in the blood typing kit were not disclosed, but it could detect at least type 1 and type 2 HBGAs because human blood, saliva, and sperm could be used as samples. The minimum OD₆₀₀ in the tenfold dilution series that gave a

positive result was determined for each isolated strain. The isolated bacterial strains were identified by 16S rRNA gene sequencing by targeting its 1466 nucleotides using the primers 27F, 530F, 907R, and 1492R (18).

Assay for HBGA binding activity of NoVLPs. NoVLP genotypes GI.7 (AB758449), GII.3 (AB758450), GII.4 (AB668028), and GII.6 (AB758451) were prepared according to a published method (19). These genotypes were selected because GII.3, GII.4, and GII.6 are the most prevalent GII noroviruses (20-22), while GI.7 is also detected frequently in epidemiological studies (23). The HBGA binding profile of each genotype (Table 1) was tested as follow. Fifty microliters of NoVLP suspension were added to each well of an ELISA plate and kept at 4°C for 24 h to coat the well. Triplicate wells were used for each sample. The wells were washed two times with PBS and blocked with 5% bovine serum albumin (BSA) in PBS. After incubation at RT for 2 h, the wells were washed two times with PBS, and 50 µL of biotin-conjugated type 1 A- (GLT 01-084, GlycoTech, MD, USA), B- (GLT 01-085, GlycoTech), H- (GLT 01-037, GlycoTech), or Lewis b-carbohydrate (GLT 01-042, GlycoTech) (diluted to 1:10 with PBS containing 5% BSA; Eve Bio-Science Co., Ltd., Japan) were added to the wells. Plates were incubated at RT for 2 h. The wells were washed two times with PBS and 50 µL of horseradish peroxidase (HRP)-conjugated streptavidin (518-59941, Thermo Scientific Japan) diluted to 1:10 with PBS containing 5% BSA was inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with PBS, and the bound HRP-conjugated streptavidin was measured by coloring with o-phenylenediamine dihydrochloride (P-7288; Sigma-Aldrich, Japan) and H₂O₂ in citrate-phosphate buffer for 30 min. The coloring reaction was stopped with 2 M H₂SO₄. The absorbance at 490 nm was measured using a plate reader (ARVO MX; Perkin Elmer, Japan).

Assay to measure the binding activity of NoVLP to bacterial cells. NoVLP binding to

isolated bacterial cells was investigated by ELISA and TEM. The isolated bacterial strain *Enterobacter* sp. SENG-6 was incubated in Luria–Bertani medium at 37°C for 18 h, and the number of cells suspended in PBS (pH 6.5) was adjusted to 10^9 cells/mL. *Staphylococcus epidermidis* (ATCC 35984), an HBGA-negative strain, was incubated in Reasoner’s 2A medium at 37°C for 18 h, and the cell number was adjusted to 10^9 cells/mL. One milliliter of the cell suspension was centrifuged at $3000 \times g$ for 5 min, re-suspended with 400 μ L of NoVLPs (GI.7, GII.3, GII.4, or GII.6) in PBS (10^{9-10} particles/mL), and incubated at RT for 1 h. NoVLPs suspended in PBS (without bacterial cells) were used as controls. The mixture was filtered using a mixed cellulose acetate membrane filter (0.20- μ m pore size and 13-mm diameter; 13CP020AN; Advantec, Toyo Roshi Kaisha Ltd., Japan) to recover unbound NoVLPs. The recovered NoVLPs were detected using ELISA as follows. Fifty microliters of NoVLP suspension were added to each well of an ELISA plate and kept at RT for 1 h to coat the well. Triplicate wells were used for each sample. The wells were then washed two times with PBS and blocked with 5% BSA in PBS. After incubation at RT for 2 h, the wells were washed two times with PBS, and 50 μ L of anti-NoVLP rabbit antibody (diluted to 1:100 with PBS containing 5% BSA; Eve Bio-Science Co., Ltd., Japan) was added to the wells (the anti-NoVLP rabbit antibodies were purified using protein A from the antisera of rabbits immunized separately with each genotype). The plates were incubated at RT for 1 h. The wells were washed two times with PBS, and 50 μ L of HRP-conjugated anti-rabbit IgG antibody (SAB-300; Stressgen Bioreagents, Belgium) diluted to 1:500 with PBS containing 5% BSA was inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with PBS, and the bound HRP-conjugated antibodies were measured as described previously.

TEM imaging of NoVLP binding to bacterial cells was performed as follows.

Enterobacter sp. SENG-6 was incubated at 37°C for 18 h, as described previously, and the number of cells suspended in M9 broth was adjusted to 10^9 cells/mL. One milliliter of the cell suspension was centrifuged at $3000 \times g$ for 5 min, and the resulting cell pellet was re-suspended in M9 broth (a washing step). The washing step was repeated two times. The cell suspension (10^9 cells/mL) was centrifuged at $3000 \times g$ for 5 min, resuspended with 100 μ L of GII.6 NoVLPs in PBS (10^{11-12} particles/mL), and incubated at 4°C for 1 h. The washing step with M9 broth was repeated two times, and the washed cells were placed on collodion membrane-coated copper grids, negatively stained with 1% phosphotungstic acid, and observed using a transmission electron microscope (JEM-1400; JEOL Ltd., Japan).

Immuno-TEM observation for localizing HBGA-like substances. The localizations of

HBGA-like substances were analyzed by immuno-TEM. *Enterobacter* sp. SENG-6 was incubated at 37°C for 18 h, as described previously. Two milliliters of cell suspension were centrifuged at $3000 \times g$ for 5 min, re-suspended in 2 mL of PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde, and incubated at 4°C for 2 h. The fixed cells were collected by centrifugation at $12,000 \times g$ for 5 min. The resulting cell pellet was washed by soaking in 2 mL of PBS at 4°C for 10 min. This washing step was repeated two times. The cell pellet was dehydrated by soaking in 2 mL of increasing concentrations of ethanol (70, 80, 90, and 95%) and then mixed gently using a rotator at 4°C for 15 min. The following dehydration and infiltration reactions were also conducted on a rotator at 4°C. For complete dehydration, the pellet was soaked in 2 mL of 100% ethanol for 20 min. This final dehydration step was repeated two times. Next, the cell pellet was infiltrated with 2 mL of LR white resin (medium grade; London Resin Company Ltd., England) and 100% ethanol (1:2)

176 mixture for 12 h, followed by 2 mL of LR white resin and 100% ethanol (2:1) mixture for 12 h. The
 177 solution was replaced with 2 mL of pure LR white resin for 1 h, followed by another 2 mL of pure
 178 LR white resin for 12 h. The cell pellet in the pure LR white resin were polymerized using an
 179 ultraviolet polymerizer (TUV-100; Dosaka EM Co., Ltd., Japan) at 4°C for 3 days. Ultrathin
 180 sections (70-nm thick) were prepared from the embedded cell pellet using a diamond knife on an
 181 ultramicrotome (RMC MTXL; Boeckeler Instruments Inc., USA) and then placed on nickel grids.
 182 The ultrathin sections on grids were washed by floating the grid upside-down on a drop of PBS at
 183 RT for 1 min. The following incubations were also conducted by floating the grid upside-down on
 184 drops of reagent at RT. The sections were blocked with 4% BSA in PBS for 5 min and incubated
 185 with anti-blood group A, B, or O(H) mouse monoclonal antibody (sc-69951, sc-69952, and
 186 sc-52372, respectively; Santa Cruz Biotechnology Inc.) diluted to 1:30 with PBS containing 1%
 187 BSA for 2 h, followed by washing with PBS (six washes, 1 min each). In a competitive adsorption
 188 test, an ultrathin section was incubated with 100 µg/mL of *Soybean* agglutinin prior to incubation
 189 with anti-blood group A antibody (sc-69951, Santa Cruz Biotechnology Inc.). The bound primary
 190 antibodies were localized by incubating the sections on anti-mouse IgM antibody gold conjugate
 191 (10 nm, ab39613; Abcam, Japan) diluted to 1:10 with PBS containing 1% BSA for 1 h, followed by
 192 washing with PBS (six washes, 1 min each). The sections were fixed with 2% glutaraldehyde in
 193 PBS for 15 min, followed by washing with deionized distilled water (DDW; seven washes, 1 min
 194 each). Finally, the sections were stained with 5% uranyl acetate, followed by washing with 50%
 195 ethanol for 1 min and DDW (four washes, 1 min each). Stained sections were observed by TEM. A
 196 grid was also prepared without the incubation with primary antibodies, which was used as a control.

197 ***Assay of NoVLP binding to HBGA-like substances in bacterial polymeric substances.***

198 Based on a hypothesis that HBGA-like substances were polysaccharides in EPS or LPS, we
 199 extracted EPS and LPS from *Enterobacter* sp. SENG-6. Bacterial EPS was also extracted from *S.*
 200 *epidermidis* ATCC 35984. EPS was extracted according to Liu and Fang (24) with some
 201 modifications. This procedure was used because Pan et al. found that the application of this method
 202 to algae–bacteria biofilm produced a higher yield of carbohydrate in EPS, whereas the protein yield
 203 was lower than that with other extraction methods (25). The bacterial strains were incubated at
 204 37°C for 18–20 h in 400 mL of each medium, as described previously. The cell suspension (10^{10-11}
 205 cells/mL) was transferred to 2 × 225-mL centrifuge tubes and centrifuged at 3000 ×*g* for 5 min. The
 206 resulting cell pellet was re-suspended in 40 mL of PBS using a vortex mixer for 2 min to displace
 207 EPS from the cells. No chemicals were used in this first step of EPS recovery. Therefore, the
 208 destruction of bacterial cells and the recovery of cellular proteins and genomic DNA components
 209 were minimized. Eighty milliliters of the cell suspension were transferred to 2 × 50-mL centrifuge
 210 tubes and centrifuged at 9000 ×*g* for 5 min. The supernatant containing EPS was collected and
 211 filtered using a mixed cellulose ester membrane filter (0.20-μm pore size and 25-mm diameter;
 212 25AS020AS; Advantec, Toyo Roshi Kaisha Ltd.) to remove bacterial cells. Eighty milliliters of the
 213 filtrate were purified with mixed cellulose dialysis tubing (MWCO of 3500–5000 Da; 131 204;
 214 Spectrum Laboratories Inc., USA) in DDW at 4°C for 24 h. The purified solution was lyophilized at
 215 –50°C for 48 h, and the resulting pellet was dissolved in 4 mL of DDW.

216 The remaining bacterial cells in the EPS extract from *Enterobacter* sp. SENG-6 were used
 217 to extract SOM, which is mainly composed of LPS according to Takaara et al. (26). The cell pellet
 218 was lyophilized using a vacuum freeze dryer, and the lyophilized cells were suspended in 4 mL of
 219 autoclaved distilled water at 65°C. Next, 4 mL of 90% phenol (65°C) was added. After vigorous

220 stirring with a vortex mixer, the samples were stirred for 15 min at 65°C and left for 15 min on ice.
221 The cooled samples were centrifuged at 4000 $\times g$ for 30 min at 2°C. Components of the
222 cell-associated hydrophilic substances, which comprised mainly LPS and cellular RNA (27), were
223 fractionated in the aqueous phase. The aqueous phase (4 mL) was then mixed with 4 mL of 90%
224 phenol at 65°C. The samples were cooled on ice and centrifuged at 4000 $\times g$ for 30 min at 2°C. The
225 aqueous phase was collected and treated with 100 mg/mL RNase A (Sigma-Aldrich) for 6 h at 37°C
226 to eliminate RNA components. Next, RNase A molecules were removed by phenol chloroform
227 precipitation, and organic substances in the supernatant were purified further by ethanol
228 precipitation. The degradation of RNA molecules by the RNase A treatment was confirmed visibly
229 by agarose gel (1.5%) electrophoresis, ethidium bromide staining, and UV light excitation. The
230 pellet collected after ethanol precipitation was suspended in DDW and stored as an SOM fraction at
231 -80°C until further analysis. In addition to SOM, LPS of *Enterobacter* sp. SENG-6 was extracted
232 separately using a LPS Extraction Kit (17141; Cosmo Bio Co., Ltd., Tokyo, Japan). The endotoxin
233 units of SOM and LPS were quantified using an EndoLISA Endotoxin Detection Kit (609033; J.K.
234 International, Tokyo, Japan) to confirm the presence of lipid A in the extracted bacterial substances.

235 NoVLP binding to the extracted EPS, SOM, and LPS was analyzed by ELISA. Fifty
236 microliters of NoVLP GII.6 suspension in PBS (10^{9-10} particles/mL) was added to each well of an
237 ELISA plate and maintained at RT for 1 h to coat the well. Triplicate wells were used for each
238 sample. The wells were washed two times with PBS and blocked with 5% BSA in PBS. After
239 incubation at RT for 2 h, the wells were washed two times with PBS and 50 μL of the extracted EPS,
240 SOM, or LPS was applied to the wells. After incubation at RT for 1 h, the wells were washed two
241 times with PBS and 50 μL of anti-blood group A, B, or O(H) mouse monoclonal antibodies

(sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA) diluted to 1:100 with PBS containing 5% BSA was applied to the wells. After incubation at RT for 1 h, the wells were washed two times with PBS and 50 μ L of HRP-conjugated anti-mouse IgM antibody (A90-101P; Bethyl Laboratories, USA) diluted to 1:500 with PBS containing 5% BSA was inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with PBS and the bound HRP-conjugated antibodies were measured as described previously. Wells without addition of EPS were used as controls.

In order to prove the specific binding of NoVLPs to the bacterial EPS, GI.1 NoVLPs (Norwalk virus, strain 8fIIa, GenBank accession number: M87661) and its mutant strain (W375A), kindly provided by Dr. Mary K. Estes, Baylor College of Medicine, were tested against the EPS extracted from *Enterobacter* sp. SENG-6. The mutant strain (W375A) does not recognize A and O(H) antigens (28). Furthermore, the extracted EPS treated with α -N-acetyl-galactosaminidase, which specifically catalyzes the hydrolysis of α -linked D-N-acetyl-galactosamine residues from oligosaccharides, was also used in ELISA for detecting A antigen. The enzyme treatment was performed as follows. One hundred micro-liters of reaction mixture contained 88 μ L of extracted EPS, 10 μ L of 10X G7 Reaction Buffer, 1 μ L of α -N-acetyl-galactosaminidase to yield a final concentration of 0.2 U/mL (New England Biolabs, Inc., Japan), and 1 μ L of 10 mg/mL BSA. One micro-liter of DDW was added instead of α -N-acetyl-galactosaminidase to control EPS. The tubes were incubated at 37 °C for 24 hr.

Specific interaction between NoVLPs and HBGA-like substances. The QCM method was used to evaluate the specific interaction between GII.6 NoVLPs and HBGA-like substances in the extracted EPS. The principle of QCM is the same as an electric scale where the frequency of an

energized quartz crystal changes relative to the amount of substances accumulated on the quartz crystal surface. Therefore, the specific interaction between ligand and receptor molecules is detected when the ligand molecules are inoculated to the quartz crystal surface where the receptor molecules are immobilized. Fifty microliters of NoVLP suspension in PBS at a concentration of 20 μg total protein/mL was placed on a sensor chip and incubated at RT for 1 h. The sensor chip was blocked with 0.1% BSA in PBS. After incubation at RT for 1 h, the chip was washed with DDW and installed in the reactor of a QCM instrument (Single-Q0500; BioLab; AsOne Co., Ltd., Japan). The reactor was filled with 500 μL of PBS and stirred at 300 rpm during measurements. When the frequency of the chip stabilized, 3- μL aliquot of 1 mg TOC/mL of the extracted EPS in PBS was inoculated into the reactor consecutively to produce a total of 24 μL , and the change in the frequency of the chip (ΔF) was recorded. This adsorption experiment was performed three times, and the adsorption isotherms were obtained. As controls, adsorption isotherms were obtained without NoVLPs immobilized on the sensor chip. The difference between the ΔF values of the test and control conditions ($\Delta\Delta F$) was used to calculate the equilibrium binding constant. The molecular weight of the HBGA-like EPS from *Enterobacter* sp. SENG-6 was estimated to calculate the apparent equilibrium binding constant of the HBGA-like EPS from *Enterobacter* sp. SENG-6 and GII.6 NoVLP according to the Langmuir adsorption isotherm. In brief, the extracted EPS was fractionated using an ultrafiltration device (Nanosep Centrifugal Device; MWCO of 300 kDa; Pall Corporation, USA), and the filtrate was collected. The amount of A-like substances in the filtrate was measured by ELISA using anti-blood group A antibody, as described above.

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the partial 16S rRNA gene sequence of *Enterobacter* sp. SENG-6 is AB758448. The accession

numbers for the capsid genes of the HuNoV genotypes of GI.7, GII.3, GII.4, and GII.6 are AB758449, AB758450, AB668028, and AB758451, respectively.

Results

Isolation of human enteric bacteria bearing HBGA-like substances. We screened blood group-active enteric bacteria from human feces using a biopanning technique with anti-HBGA antibodies. Of 35 isolated strains, 21 screened bacterial strains had blood group activity with A, B, or O(H) (Table 2). The phylogenetic relationships of the 16S rRNA gene sequences of the 21 isolated strains and related enteric bacteria are shown in Fig. 1. These enteric bacteria were gram-negative and were genetically related to *Enterobacter cloacae* (ATCC 13047^T), *Shigella flexneri* (ATCC 29903^T), or *Escherichia fergusonii* (ATCC 35469^T). From these, we isolated a bacterium (designated strain SENG-6) that was closely related to *Enterobacter cloacae* (ATCC 13047^T) with a 16S rRNA gene sequence similarity of 99.9%. Strain SENG-6 had A, B, and O(H) blood group activities. Twelve of 15 strains that were closely related to *S. flexneri* (ATCC 29903^T), sharing 99.3–99.9% of its 16S rRNA gene sequence, had both A and O(H) blood group activities (e.g., NMCA1-7 and NMC20B1-4), whereas two other strains (MURA1-1 and MUR18B1-4) with similarity of 99.5 and 99.6%, respectively, had only A blood group activity, while NMC20B1-6 with a similarity of 99.5% had only O(H) blood activity. All 5 strains that were closely related to *E. fergusonii* (ATCC 35469^T), sharing 99.4–99.8% of its 16S rRNA gene sequence, had both A and O(H) blood group activities.

Strains SENG-6 and NMC18B1-6 produced HBGA-positive results at the minimum optical density (OD₆₀₀) of 0.068 and 0.058, respectively (Table 2), which showed that these strains

308 carried a high abundance of HBGA-like substances. SENG-6 possessed A, B, and O(H) blood
 309 group activities; therefore, this strain was used in further experiments. *Staphylococcus epidermidis*
 310 (ATCC 35984) was used as the HBGA-negative strain because it had an HBGA-negative result,
 311 even at an OD₆₀₀ of 1.357 (Table 2).

312 **NoVLP binding to isolated bacterial cells.** The blood group activity test suggested that
 313 HBGA-like substances were present in the bacterial outer components, including LPS of
 314 gram-negative bacteria and/or EPS, including the capsule. The binding of NoVLP genotypes GI.7,
 315 GII.3, GII.4, and GII.6 to *Enterobacter* sp. SENG-6 and *S. epidermidis* ATCC 35984 were tested.
 316 Each NoVLP genotype was mixed with each strain, and unbound NoVLPs were detected by ELISA
 317 (Fig. 2). Each NoVLP genotype was also mixed with PBS (without bacterial cells), which was used
 318 as a negative control. All NoVLP genotypes tested bound significantly to *Enterobacter* sp. SENG-6
 319 ($p < 0.01$). While GII.4 NoVLP bound to *S. epidermidis* ATCC 35984 (Fig. 2A), it was previously
 320 shown that this genotype recognizes a broad range of ligands other than HBGAs, such as those
 321 containing sialic acid (29-31).

322 The direct visualization with TEM confirmed that NoVLP genotypes GI.7 and GII.6
 323 bound to *Enterobacter* sp. SENG-6 (Fig. 3A and 3B). The majority of these NoVLPs were observed
 324 to bind mainly to the EPS of *Enterobacter* sp. SENG-6 (Fig. 3A and 3B), whereas a few NoVLPs
 325 were observed in the region adjacent to bacterial cells. However, it was not determined by this
 326 technique whether NoVLP bound to LPS of *Enterobacter* sp. SENG-6 because most LPS
 327 components were covered by EPS, which would hamper the interaction between NoVLPs and LPS.

328 **Localization of HBGA-like substances in bacterial cell components.** Ultrathin sections
 329 of *Enterobacter* sp. SENG-6 were examined by immuno-TEM to determine the localization of

330 HBGA-like substances. *Enterobacter* sp. SENG-6 was labeled with gold nanoparticles carrying
 331 anti-mouse IgM after reacting with anti-blood group A, B, or O(H) antibody (used as the primary
 332 antibody). A number of gold nanoparticles were observed in the extracellular spaces of
 333 *Enterobacter* sp. SENG-6 (Fig. 3C), which had reacted with anti-A antibody. The scattered
 334 occurrence of A-like substances outside bacterial cells suggests that these antigens were present at
 335 the terminals of each extracellular polymer, although this needs to be confirmed by the structural
 336 analysis of EPS. Fig. 3D shows that the number of gold nanoparticles in the ultrathin section was
 337 reduced dramatically by pretreatment of the ultrathin section with *Soybean* agglutinin, which is a
 338 lectin specific to N-acetylgalactosamine. This showed that anti-A antibodies labeled with gold
 339 nanoparticles in the ultrathin section in Fig. 3C specifically bound to A-like substances that
 340 contained N-acetylgalactosamine. In contrast, few B- and O(H)-like substances were observed in
 341 *Enterobacter* sp. SENG-6 (Fig. 3E and 3F, respectively), although the B and O(H) blood group
 342 activities of this bacterial strain were confirmed using the blood group typing kit (Table 2). The
 343 failure to detect B- and O(H)-like substances in the immuno-TEM images may be attributable to a
 344 subtle difference in the recognition of the antibodies used in the blood group typing kit and
 345 immuno-TEM or insufficient amounts of B- and O(H)-like substances in immuno-TEM, although it
 346 is difficult to compare the relative amounts of each HBGA-like substance. Gold nanoparticles did
 347 not accumulate at the edges of the cell surfaces (Fig. 3C), which showed that only limited amounts
 348 of HBGA-like substances were present in LPS of *Enterobacter* sp. SENG-6, even if blood
 349 type-active substances were present.

350 ***NoVLP binding to HBGA-like substances in bacterial EPS, SOM, and LPS.*** TEM
 351 observations showed that NoVLPs were captured mainly in the bacterial EPS where the A-like

352 substances were present, whereas LPS had none or a low level of specific binding sites for NoVLPs
 353 (Fig. 3). To further elucidate the localization of HBGA-like substances, EPS, SOM, and LPS were
 354 extracted separately from *Enterobacter* sp. SENG-6 and tested to detect HBGA-like substances by
 355 ELISA using GII.6 NoVLP. GII.6 is one of the best genotypes for the binding assay because this
 356 genotype was shown to bind to A, B, and O(H) antigens (Table 1). The bacterial EPS were also
 357 extracted from *S. epidermidis* ATCC 35984 and used in the same ELISA. SOM and LPS of *S.*
 358 *epidermidis* ATCC 35984 were not extracted because *S. epidermidis* ATCC 35984 is gram-positive.

359 Fig. 4A shows that A- and B-like substances were detected in the extracted EPS of
 360 *Enterobacter* sp. SENG-6, which were trapped by GII.6 NoVLP immobilized in ELISA. B-like
 361 substances were not detected in immuno-TEM (Fig. 3E), but the extraction and concentration of
 362 EPS allowed them to be detected by ELISA. However, no significant signals for A-, B-, and
 363 O(H)-like substances were detected in SOM and LPS of *Enterobacter* sp. SENG-6. Likewise, no
 364 signals for A-, B-, and O(H)-like substances were detected in EPS of *S. epidermidis* ATCC 35984
 365 (Fig. 4A). *Enterobacter* sp. SENG-6 was positive for all A, B, and O(H) blood group antigens
 366 (Table 2), whereas O(H)-like substances were not detected by ELISA, which may be caused by the
 367 difference in the recognition of anti-O(H) antibodies used in the blood typing kit and ELISA.

368 The binding between GII.6 NoVLP and EPS of *Enterobacter* sp. SENG-6 containing
 369 A-like substances is shown clearly in Fig. 4A, although it is not clear whether the A-like substances
 370 in EPS of *Enterobacter* sp. SENG-6 are the main binding sites for GII.6 NoVLP. To test the
 371 function of A-like substances in EPS as the binding site for NoVLP, we performed ELISA using the
 372 NoVLPs of GI.1 wild-type (8fIIa) and mutant (W375A) strains. The GI.1 wild-type strain (8fIIa)
 373 can recognize A antigen, whereas the mutant strain (W375A) has lost its ability for binding to A

antigen because of a point mutation from tryptophan to alanine at the 375th position (28). Fig. 4B shows that A-like substances in EPS of *Enterobacter* sp. SENG-6 were detected when NoVLP of the GI.1 wild-type strain (8fIIa) was immobilized in ELISA, but they were not detected when NoVLP of the GI.1 mutant strain (W375A) was immobilized. Furthermore, the enzymatic treatment of EPS with N-acetylgalactosaminidase, which can release the terminal N-acetylgalactosamine from A antigen, weakened the interaction between EPS and NoVLP of the GI.1 wild-type strain (8fIIa) (Fig. 4B). These results show that A-like substances in EPS of *Enterobacter* sp. SENG-6 are the binding sites of NoVLPs. The A antigen has also been found in HBGA-like substances in the digestive tissues of oysters (32), and the critical role of the terminal N-acetylgalactosamine residue in the strain-dependent accumulation of HuNoVs in oysters has been discussed previously (29, 30). Bacterial A-like substances can also affect the genotype-dependent fate of HuNoVs in the environment because each HuNoVs genotype has its own HBGA binding profile (12).

The adsorption isotherms of the extracted EPS were determined for GII.6 NoVLP, which was immobilized on a sensor chip (Fig. 5A and 5B). The ΔF values for EPS of *Enterobacter* sp. SENG-6 with GII.6 NoVLP were higher than those under the NoVLP-negative condition, and this difference was statistically significant according to a Mann–Whitney U test performed for each EPS concentration (Fig. 5A). In contrast, the ΔF values for the EPS extracted from *S. epidermidis* ATCC 35984 were not different from those under the NoVLP-negative condition (Fig. 5B). These results indicated that the EPS extracted from *Enterobacter* sp. SENG-6 can interact specifically with NoVLP. In order to estimate the molecular weight of HBGA-positive EPS from *Enterobacter* sp. SENG-6, the amount of A-like substances in the EPS extracted from *Enterobacter* sp. SENG-6 was determined before and after ultrafiltration with a molecular weight cut off (MWCO) of 300 kDa. As

396 a result, the amount of A-like substances was significantly reduced by ultrafiltration (Fig. 5C),
 397 which suggested that the majority of A-like substances existed in EPS with a molecular weight
 398 >300 kDa. If we assume that A-like antigens dominated EPS and that EPS carrying the HBGA-like
 399 substances had a molecular weight of 300 kDa, the reciprocal plots of $\Delta\Delta F$ (the difference between
 400 ΔF with NoVLPs and that without NoVLPs in Fig. 5A) and EPS concentration is obtained (Fig. 5D),
 401 which gives the apparent equilibrium binding constant of $3.1 \times 10^6 \text{ M}^{-1}$ according to the Langmuir
 402 adsorption isotherm. Since the molecular weight of HBGA-positive EPS is expected to be larger
 403 than 300kDa (Fig. 5C), the apparent equilibrium binding constant for the HBGA-positive EPS of
 404 *Enterobacter* sp. SENG-6 and GII.6 NoVLP must be $>3.1 \times 10^6 \text{ M}^{-1}$.
 405

406 Discussion

407 **Blood group activities of human enteric bacteria.** The presence of blood group-active enteric
 408 bacteria was reported many years ago. Springer et al. tested 282 gram-negative bacterial strains
 409 from genera such as *Escherichia*, *Salmonella*, and *Klebsiella*, which were isolated from clinical
 410 specimens, and approximately 50% of the strains inhibited anti-blood group A, B, and/or O(H)
 411 agglutinins. Of the tested strains belonging to *Escherichia*, some strains had a single aggregation
 412 activity (A, B, or O(H)), whereas others had multiple aggregation activities (ABO(H), AO(H), AB,
 413 or BO(H)) (17). In the present study, 21 blood group-active strains were isolated from human feces
 414 using a biopanning technique with anti-HBGA antibodies (Table 2). The isolated bacterial strains
 415 must represent only a small fraction of the actual population of HBGA-positive bacteria, because
 416 we only analyzed culturable strains in a fecal sample from a single individual under microaerobic
 417 conditions in this study, while it is recognized that the majority of enteric bacteria in human feces

418 are likely to be dead, viable but not culturable (33), or obligate anaerobes.

419 The blood group activities of various widely distributed gram-negative bacteria were
 420 reported initially by Springer et al. (17), as described above, and these blood group activities of
 421 enteric bacteria were attributed to the presence of HBGA-like “O antigen” in LPS. The structural
 422 basis of the HBGA-like O antigen was clarified recently. For example, Anderson et al. showed that
 423 the O-antigen polysaccharide of *E. coli* O86 contained a B-type trisaccharide where alpha-L-fucose
 424 and beta-D-galactose formed part of the O-antigen backbone, while alpha-D-galactose was
 425 conjugated to beta-D-galactose (34). *Salmonella milwaukee* O:43 (group U) also has human blood
 426 group B activity with the O-polysaccharide (35). Therefore, we initially postulated that HBGA-like
 427 substances were present in LPS of the isolated strains. However, immuno-TEM imaging (Fig. 3)
 428 and ELISA (Fig. 4) clearly showed that HBGA-like substances were localized mainly in the
 429 bacterial EPS. Only *Klebsiella* sp. was suggested to have blood group active substances in its
 430 capsular polysaccharide, although this was not proved (17). This is the first study to show that
 431 human enteric bacteria possess HBGA-like substances in their EPS and that they can capture
 432 HuNoVLPs.

433 ***Interaction between bacterial substances and NoVLPs.*** Four genotypes of NoVLP (GI.7,
 434 GII.3, GII.4 and GII.6) were used in the binding assay to HBGA-positive (*Enterobacter* sp.
 435 SENG-6) and HBGA-negative (*S. epidermidis* ATCC35984) cells, and only GII.4 bound to both
 436 strains (Fig. 2A). It has been shown that NoV GII.4 may bind to multiple carbohydrate moieties,
 437 including alpha-D-Gal, alpha-D-Man/alpha-D-Glc and alpha-L-Fuc, in addition to GalNAc,
 438 GlcNAc and sialic acid (30). Although extracellular polymeric substances of planktonic *S.*
 439 *epidermidis* cells have not been well analyzed so far, the broad binding capacity of NoV GII.4 is

likely to contribute to the binding to HBGA-negative cells of *S. epidermidis* ATCC35984 observed in the present study. This binding property of NoV GII.4 did not allow us to use this genotype in the following binding assays indicated in Fig. 3 to 5, because it was necessary to analyze the specific interaction between bacterial HBGAs and NoVLPs. In that context, NoV GII.6 was available for those binding assays in the present study, because this genotype can recognize all HBGAs tested (Table 1) but seemed not to have a binding spectrum as broad as GII.4, judged by the binding assay shown in Fig. 2. Further analysis on the binding spectrum of multiple genotypes of HuNoVs other than GII.4 may provide a clue for better understanding the genotype-specific lifestyle of HuNoVs.

Significance of the blood group-active enteric bacteria: a hypothesis. The binding is likely to affect at least two aspects of the ecology of HuNoVs, particularly the fate of HuNoVs in their hosts after HuNoVs enter the human intestine and encounter large numbers of enteric bacteria. In this regard, there is increasing understanding of the roles played by the intestinal microbiota in viral infections. Kane et al. showed that bacterial LPS plays a key role in the infection of mouse mammary tumor virus (MMTV) using antibiotic-treated and germ-free mice (36). MMTV-bound bacterial LPS was recognized by Toll-like receptor 4, and it affected the induction of the inhibitory cytokine interleukin-10. However, this enhanced viral titers and MMTV overcame the immune response. Kuss et al. found that antibiotic-treated mice were less susceptible to poliovirus infection and demonstrated that exposure to bacteria or their N-acetylglucosamine-containing surface polysaccharides, including LPS and peptidoglycan, enhanced the infectivity of poliovirus (37). These results suggest that antibiotic-mediated microbiota depletion reduces virus infections and that viruses may exploit intestinal microbes for replication and transmission. Therefore, it is likely that the commensal microbiota in the human intestine will also affect the likelihood of infection with

462 human enteric viruses, including HuNoVs, in the intestine. A higher level of blood group-active
463 EPS in the mucosa of the intestine (jejunum and ileum) probably provides HuNoVs with a greater
464 opportunity for infecting intestinal cells because norovirus particles may be retained in the intestine
465 provided that norovirus-binding bacteria are present, although it is also possible that bacterial
466 HBGA will be an inhibitor of HuNoV binding to human HBGA on intestinal cells. The abundance
467 of blood group-active EPS could also affect rotavirus infection, another major gastroenteritis virus,
468 because spike protein VP8 of human rotavirus recognizes HBGAs (38, 39). Thus, enteric virus
469 infections may be characterized on the basis of the bacterial community composition of the human
470 intestine, which varies considerably with age, lifestyle, and season. Understanding the role of the
471 intestinal microbiota in viral transmission and pathogenesis may lead to the development of new
472 antiviral strategies, although the role of bacterial HBGAs in the infections of intestinal mucosal
473 cells with HuNoVs remains largely speculative at this moment.

474 Another important implication concerning HBGA-positive bacterial EPS for the HuNoVs
475 life cycle is the transport of viral particles from acute gastroenteritis patients to aquatic
476 environments. Enteric viruses are known to be associated mainly with particles in environmental
477 waters (40). Various environmental materials can capture human enteric viruses, including activated
478 sludge (41), estuarine sediment (41, 42), river sediment (43), and soils (41, 44). da Silva et al.
479 showed that the GI and GII noroviruses found in wastewater samples were associated mainly with
480 particles measuring $<180\text{ }\mu\text{m}$ (45). Sano et al. demonstrated that indigenous noroviruses were not
481 present in the permeates of mixed liquor and treated wastewater filtered using a microfiltration
482 membrane (pore size, $0.1\text{ }\mu\text{m}$) (46). Gentry et al. showed that an unexpectedly large number of
483 HuNoVs were present in the fraction collected using 63- and 200- μm -mesh plankton nets, which

suggested the binding of HuNoV particles to phytoplankton and zooplankton (47). Viral particles associated with environmental materials are physically more stable than freely moving viral particles (48). Viruses associated with organic or inorganic particles are protected from inactivation by chlorine (49), UV (40), and salinity (osmotic shock) (50) because these exogenous stresses are impeded by the entities that capture the viral particles. Furthermore, enteric viruses associated with environmental suspended solids may be transported in a different manner from freely moving virions (51, 52). Thus, HuNoV particles associated with HBGA-positive EPS that is attached to or disengaged from enteric bacterial cells must have different fates from free viral particles. Since the proteolytic virucidal activity of some bacterial species has been reported (53), the association of HuNoV particles with HBGA-positive EPS detached from bacterial cells may have an advantage in terms of its environmental persistency. These hypotheses about the impact of bacterial HBGAs on the environmental persistency of HuNoVs largely remain to be proved, and are challenging issues in further studies.

Avidity of the blood group-active bacterial EPS with HuNoV particles. Thus, the effect of the blood group-active bacterial EPS on the fate of HuNoVs is totally dependent on the binding avidity of EPS to HuNoV particles. If we assume that all the components in the extracted EPS are blood group-active and that they have a molecular weight >300 kDa (Fig. 5C), we can estimate the apparent equilibrium binding constant according to the Langmuir adsorption isotherm as $>3.1 \times 10^6 \text{ M}^{-1}$. This value is comparable with that obtained for the interactions between β -galactose specific lectins and lactose-carrying glycopolystylenes, which have binding constants of 10^6 to 10^7 M^{-1} (54). The interactions between enteric viruses and environmental adsorbents were previously shown to have no binding specificity (41-45, 47), whereas HuNoV binding to bacterial HBGAs is regarded as

506 specific, as illustrated by human HBGAs and HuNoVs (12). In addition to enthalpy-driven
507 adsorption via an array of hydrogen binding routes (28), the entropy effect caused by multiple
508 binding sites also contributes to the increased binding avidity, although structural analyses of
509 bacterial EPS are essential to obtain an insight into the true value of the binding constant. The
510 specific binding capacity of blood group-active enteric bacteria with HuNoVs provides a new
511 scientific platform for understanding the interactions between two types of microbes that were
512 previously regarded as biologically unrelated.

513

514 **Conclusions**

515 *Enterobacter* sp. SENG-6, an isolated strain from a fecal sample of a healthy person,
516 exhibited blood group activity. We observed A-like substances in its extracellular region. EPS
517 extracted from *Enterobacter* sp. SENG-6 captured norovirus particles, specifically via A-like
518 substances, with an avidity comparable to those between multi-glycoconjugate polymers and lectins.
519 Further studies of human enteric bacteria capable of binding to HuNoVs are warranted to gain new
520 insights into the biological role of HBGA-positive bacterial EPS in the transmission and infection
521 of HuNoVs in the environment, and are likely to provide a clue for the development of novel
522 prevention strategies.

523

524 **Acknowledgments**

525 We are very grateful to Dr. Mary Estes, Baylor College of Medicine, USA for providing
526 the NoVLP GI.1 wild-type strain (8fIIa) and the mutant strain (W375A). We also thank Ms.
527 Natsumi Ushijima, Support Section for Education and Research, Graduate School of Dental

528 Medicine, Hokkaido University, for her technical assistance during TEM observations. We thank
529 Ms. Rie Nomachi, Ms. Kanae Kawamura, and Ms. Megumi Tazawa, Division of Environmental
530 Engineering, Faculty of Engineering, Hokkaido University, for their technical support during
531 NoVLPs production and bacterial cultivation. This study was supported by the Japan Science and
532 Technology Agency through Core Research for Evolutionary Science and Technology, and the
533 Japan Society for the Promotion of Science through Grant-in-Aid for Young Scientist (A)
534 (22686049), Challenging Exploratory Research (21659157), and Research Fellowships for Young
535 Scientists (23-4434).

536

537

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686 Figure Legends

687 **FIG. 1.** Phylogenetic relationships of 16S rRNA gene sequences of isolated bacterial strains and
 688 related enteric bacteria. The phylogenetic tree was constructed by the neighbor-joining method with
 689 1,000 bootstrap replicates using ClustalX (version 2.1). Bold type indicates the bacterial strains
 690 isolated in this study, and *Salmonella enterica* serovar Typhimurium SL1344 was used as an
 691 outgroup. The scale bar represents the number of substitutions per site.

692 **FIG. 2.** NoVLP binding to bacterial cells. (A) *Enterobacter* sp. SENG-6 and *Staphylococcus*
 693 *epidermidis* (ATCC 35984) were mixed with each NoVLP genotype (GI.7, GII.3, GII.4, and GII.6)
 694 in PBS. Unbound NoVLPs were recovered by filtration and detected by ELISA. Suspended
 695 NoVLPs in PBS that were not mixed with bacterial cells were filtered and used as controls. The
 696 results were expressed as the absorbance relative to the control; therefore, values statistically <1
 697 (dashed line) indicated the significant binding of NoVLPs to bacterial cells. The error bars represent
 698 the standard deviations of triplicate independent measurements. ** $p < 0.01$ (t -test). (B) GI.7
 699 NoVLPs. (C) GII.3 NoVLPs. (D) GII.4 NoVLPs. (E) GII.6 NoVLPs. Black dots in the viewing
 700 field in (B)–(E) are avidin-conjugated gold nanoparticles (average diameter = 5nm), which were
 701 added to estimate the diameter of NoVLPs.

702 **FIG. 3.** Binding of NoVLPs to bacterial cells and localization of HBGA-like substances of
 703 *Enterobacter* sp. SENG-6. *Enterobacter* sp. SENG-6 was reacted with GI.7 (A) or GII.6 (B)
 704 NoVLPs in PBS. NoVLPs are indicated by arrows in (A) and (B). For the localization of
 705 HBGA-like substance, ultrathin sections of *Enterobacter* sp. SENG-6 were labeled with
 706 immuno-gold nanoparticles after reacting with anti-blood group A, B, and O(H) antibodies (primary
 707 antibodies) and observed by TEM. Ultrathin sections without primary antibodies were used as

708 controls. (C) A-like substances. (D) A-like substances after pretreatment with *Soybean* agglutinin, a
 709 lectin specific to N-acetylgalactosamine. (E) B-like substances. (F) O(H)-like substances. (G)
 710 Primary antibody-negative control.

711 **FIG. 4.** Detection of HBGA-like substances in extracted bacterial polymers. (A) The wells on an
 712 ELISA plate were coated with GII.6 NoVLPs, and EPS, SOM, and LPS extracted from
 713 *Enterobacter* sp. SENG-6, and EPS extracted from *Staphylococcus epidermidis* (ATCC 35984)
 714 were inoculated into the wells. We used ELISA to measure A-, B-, and O(H)-like substances in the
 715 EPS, SOM, or LPS which bound to NoVLPs. A well without the incubation of bacterial polymeric
 716 substances was used as a control. (B) The wells were coated with GI.1 NoVLPs (8fIIa or W375A),
 717 and the EPS extracted from *Enterobacter* sp. SENG-6 or the EPS treated with
 718 α -N-acetyl-galactosaminidase was reacted. A-like substances which bound to the NoVLPs were
 719 detected by ELISA. All results in (A) and (B) were expressed as the absorbance relative to the
 720 control; therefore, values statistically >1 (dashed line) indicated the presence of A-, B-, or O(H)-like
 721 substances. The error bars represent the standard deviations of triplicate independent measurements.
 722 * $p < 0.05$; ** $p < 0.01$ (t -test).

723 **FIG. 5.** Binding avidity between NoVLPs and bacterial EPS. Adsorption isotherm between GII.6
 724 NoVLPs and EPS from *Enterobacter* sp. SENG-6 (A) and *Staphylococcus epidermidis* (ATCC
 725 35984) (B), measured using the QCM method. GII.6 NoVLPs were immobilized on a sensor chip
 726 and various amounts of extracted EPS were inoculated. The change in frequency of the sensor chip
 727 was measured, which depended on the amount of EPS bound to GII.6 NoVLPs. The error bars
 728 represent the standard deviations of triplicate independent measurements. Black dots (diamonds in
 729 A and triangles in B) are ΔF values with NoVLPs, while white dots are those without NoVLPs. (C)

730 Detection of A-like substances in EPS extracted from *Enterobacter* sp. SENG-6 before and after
731 ultrafiltration. The wells of an ELISA plate were coated with GII.6 NoVLPs, and the wells were
732 inoculated with EPS extracted from *Enterobacter* sp. SENG-6 before or after ultrafiltration with a
733 MWCO of 300 kDa. A well without EPS was used as a control. The results were expressed as the
734 absorbance relative to the control. The error bars represent the standard deviations. $*p < 0.05$ (t -test).
735 (D) Reciprocal plots of $\Delta\Delta F$ and the concentration of EPS. $\Delta\Delta F$ is the difference between the ΔF
736 value with NoVLPs and that without NoVLPs.

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739 Table 1. Binding profiles of norovirus-like particles prepared in this study

Genotype (GenBank accession number)	Histo-blood group antigens			
	A (Type 1)	B (Type 1)	H (Type 1)	Lewis b (Type 1)
GI.7 (AB758449)	+	—	—	—
GII.3 (AB758450)	+	—	—	—
GII.4 (AB668028)	+	+	+	+
GII.6 (AB758451)	+	+	+	+

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742 Table 2. Blood group activities of isolated and reference bacterial strains used in this study

Strain	Growth medium	Blood group activities			Min. OD ₆₀₀ with HBGA positive	Genetically similar strain based on 16S rRNA gene analysis (strain, similarity)
		A	B	O(H)		
SENG-6	BGLB ^b	+	+	+	0.068	<i>Enterobacter cloacae</i> (ATCC 13047 ^T , 99.9%)
NMCA1-7	BGLB	+	–	+	0.920	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
NMCA1-8	BGLB	+	–	+	0.082	<i>Escherichia fergusonii</i> (ATCC 35469 ^T , 99.8%)
NMC18B1-3	BGLB	+	–	+	0.091	<i>Escherichia fergusonii</i> (ATCC 35469 ^T , 99.4%)
NMC18B1-6	BGLB	+	–	+	0.058	<i>Escherichia fergusonii</i> (ATCC 35469 ^T , 99.5%)
NMC20B1-4	BGLB	+	–	+	0.821	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.5%)
NMC20B1-6	BGLB	–	–	+	0.302	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.5%)
KKA1-1	EC ^c	+	–	+	0.465	<i>Escherichia fergusonii</i> (ATCC 35469 ^T , 99.8%)
KKB1-1	EC	+	–	+	0.502	<i>Escherichia fergusonii</i> (ATCC 35469 ^T , 99.5%)
TZWH1-1	EEM ^d	+	–	+	0.502	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
TZWH1-2	EEM	+	–	+	0.089	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
TZWH2-2	EEM	+	–	+	0.515	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.3%)
TZWH2-3	EEM	+	–	+	0.570	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
TZWH2-4	EEM	+	–	+	0.568	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.4%)
TZWA1-2	EEM	+	–	+	0.532	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
TZWA1-3	EEM	+	–	+	0.504	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
TZWA1-4	EEM	+	–	+	0.257	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.4%)
MURA1-1	Mossel ^e	+	–	–	0.407	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.5%)
MURA1-6	Mossel	+	–	+	0.428	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.9%)
MUR18B1-4	Mossel	+	–	–	0.367	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.6%)
MUR20B1-4	Mossel	+	–	+	0.263	<i>Shigella flexneri</i> (ATCC 29903 ^T , 99.7%)
ATCC 35984 ^a	R2A ^f	–	–	–	–	–

743 a, *Staphylococcus epidermidis* ATCC35984 was used as the HBGA-negative strain, and it gave an
744 HBGA-negative result, even at an OD₆₀₀ of 1.357; b, 2% Brilliant green bile broth; c, *Escherichia*
745 *coli* broth; d, Enterobacteriaceae enrichment mannitol broth; e, Enterobacteria enrichment
746 broth-Mossel; f, Reasoner's 2A.