



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

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5 **Running title:** Bacterial HBGAs Specifically Bind to Human Norovirus

6  
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22

**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). HBGAs  
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 HBGAs in saliva from secretor-positive individuals and  
63 preferentially bind to H type 1, Le<sup>b</sup>, 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 HBGAs 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**

88 ***Isolation of human enteric bacteria bearing HBGA-like substances.*** We screened enteric bacteria  
89 bearing HBGA-like substances from human feces using anti-HBGA antibodies. Fifty microliters of  
90 anti-blood group A, B, or O(H) mouse monoclonal antibodies (sc-69951, sc-69952, and sc-52372,  
91 respectively; Santa Cruz Biotechnology Inc., USA) were added to each well of an ELISA plate and  
92 kept at room temperature (RT) for 1 h to coat the well. The wells were washed two times with 0.1  
93 M phosphate-buffered saline (PBS), and a diluted human fecal suspension derived from a healthy  
94 adult was added to the wells. After incubation at RT for 1 h, the wells were washed two times with  
95 PBS and 2% brilliant green bile broth (Kanto Chemical Co., Inc., Japan), *E. coli* broth (Nihon  
96 Pharmaceutical Co., Ltd., Japan), Enterobacteriaceae enrichment mannitol broth (Nihon  
97 Pharmaceutical Co., Ltd.), or enterobacteria enrichment broth-Mossel (Nihon Pharmaceutical Co.,  
98 Ltd.) was added to each well. After overnight incubation at 37°C under microaerobic conditions, the  
99 enriched cells were cultured further by streaking onto an agar plate containing the same medium,  
100 and several colonies were selected randomly. The selected colonies were cultured again by  
101 streaking onto the same agar plates, and a single colony was selected. The selected cells were  
102 cultured in the same culture broth, collected by centrifugation, and suspended in PBS. Tenfold  
103 dilution series were prepared with PBS, and the OD<sub>600</sub> values of the diluted cell suspensions were  
104 measured using a spectrophotometer. The presence of HBGA-like substances on the cell surface in  
105 the diluted cell suspensions were detected on the basis of their aggregation activities with anti-blood  
106 group A, B, or O(H) antibody-coated beads from a blood typing kit (ABO sphia; Kamakura  
107 Techno-Science Inc., Japan). The names of the antibodies used in the blood typing kit were not  
108 disclosed, but it could detect at least type 1 and type 2 HBGAs because human blood, saliva, and  
109 sperm could be used as samples. The minimum OD<sub>600</sub> in the tenfold dilution series that gave a

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

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

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



154 TEM imaging of NoVLP binding to bacterial cells was performed as follows.  
155 *Enterobacter* sp. SENG-6 was incubated at 37°C for 18 h, as described previously, and the number  
156 of cells suspended in M9 broth was adjusted to 10<sup>9</sup> cells/mL. One milliliter of the cell suspension  
157 was centrifuged at 3000 ×g for 5 min, and the resulting cell pellet was re-suspended in M9 broth (a  
158 washing step). The washing step was repeated two times. The cell suspension (10<sup>9</sup> cells/mL) was  
159 centrifuged at 3000 ×g for 5 min, resuspended with 100 μL of GII.6 NoVLPs in PBS (10<sup>11-12</sup>  
160 particles/mL), and incubated at 4°C for 1 h. The washing step with M9 broth was repeated two  
161 times, and the washed cells were placed on collodion membrane-coated copper grids, negatively  
162 stained with 1% phosphotungstic acid, and observed using a transmission electron microscope  
163 (JEM-1400; JEOL Ltd., Japan).

164 ***Immuno-TEM observation for localizing HBGA-like substances.*** The localizations of  
165 HBGA-like substances were analyzed by immuno-TEM. *Enterobacter* sp. SENG-6 was incubated  
166 at 37°C for 18 h, as described previously. Two milliliters of cell suspension were centrifuged at  
167 3000 ×g for 5 min, re-suspended in 2 mL of PBS containing 4% paraformaldehyde and 0.1%  
168 glutaraldehyde, and incubated at 4°C for 2 h. The fixed cells were collected by centrifugation at  
169 12,000 ×g for 5 min. The resulting cell pellet was washed by soaking in 2 mL of PBS at 4°C for 10  
170 min. This washing step was repeated two times. The cell pellet was dehydrated by soaking in 2 mL  
171 of increasing concentrations of ethanol (70, 80, 90, and 95%) and then mixed gently using a rotator  
172 at 4°C for 15 min. The following dehydration and infiltration reactions were also conducted on a  
173 rotator at 4°C. For complete dehydration, the pellet was soaked in 2 mL of 100% ethanol for 20 min.  
174 This final dehydration step was repeated two times. Next, the cell pellet was infiltrated with 2 mL  
175 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 No VLP 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- $\mu$ m 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 ×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 ×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

242 (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA) diluted to  
243 1:100 with PBS containing 5% BSA was applied to the wells. After incubation at RT for 1 h, the  
244 wells were washed two times with PBS and 50  $\mu$ L of HRP-conjugated anti-mouse IgM antibody  
245 (A90-101P; Bethyl Laboratories, USA) diluted to 1:500 with PBS containing 5% BSA was  
246 inoculated into each well. After incubation at RT for 1 h, the wells were washed four times with  
247 PBS and the bound HRP-conjugated antibodies were measured as described previously. Wells  
248 without addition of EPS were used as controls.

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

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

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

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

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

288

## 289 **Results**

290 ***Isolation of human enteric bacteria bearing HBGA-like substances.*** We screened blood  
291 group-active enteric bacteria from human feces using a biopanning technique with anti-HBGA  
292 antibodies. Of 35 isolated strains, 21 screened bacterial strains had blood group activity with A, B,  
293 or O(H) (Table 2). The phylogenetic relationships of the 16S rRNA gene sequences of the 21  
294 isolated strains and related enteric bacteria are shown in Fig. 1. These enteric bacteria were  
295 gram-negative and were genetically related to *Enterobacter cloacae* (ATCC 13047<sup>T</sup>), *Shigella*  
296 *flexneri* (ATCC 29903<sup>T</sup>), or *Escherichia fergusonii* (ATCC 35469<sup>T</sup>). From these, we isolated a  
297 bacterium (designated strain SENG-6) that was closely related to *Enterobacter cloacae* (ATCC  
298 13047<sup>T</sup>) with a 16S rRNA gene sequence similarity of 99.9%. Strain SENG-6 had A, B, and O(H)  
299 blood group activities. Twelve of 15 strains that were closely related to *S. flexneri* (ATCC 29903<sup>T</sup>),  
300 sharing 99.3–99.9% of its 16S rRNA gene sequence, had both A and O(H) blood group activities  
301 (e.g., NMCA1-7 and NMC20B1-4), whereas two other strains (MURA1-1 and MUR18B1-4) with  
302 similarity of 99.5 and 99.6%, respectively, had only A blood group activity, while NMC20B1-6 with  
303 a similarity of 99.5% had only O(H) blood activity. All 5 strains that were closely related to *E.*  
304 *fergusonii* (ATCC 35469<sup>T</sup>), sharing 99.4–99.8% of its 16S rRNA gene sequence, had both A and  
305 O(H) blood group activities.

306           Strains SENG-6 and NMC18B1-6 produced HBGA-positive results at the minimum  
307 optical density (OD<sub>600</sub>) 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 OD<sub>600</sub> 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

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

386         The adsorption isotherms of the extracted EPS were determined for GII.6 NoVLP, which  
387 was immobilized on a sensor chip (Fig. 5A and 5B). The  $\Delta F$  values for EPS of *Enterobacter* sp.  
388 SENG-6 with GII.6 NoVLP were higher than those under the NoVLP-negative condition, and this  
389 difference was statistically significant according to a Mann–Whitney U test performed for each EPS  
390 concentration (Fig. 5A). In contrast, the  $\Delta F$  values for the EPS extracted from *S. epidermidis* ATCC  
391 35984 were not different from those under the NoVLP-negative condition (Fig. 5B). These results  
392 indicated that the EPS extracted from *Enterobacter* sp. SENG-6 can interact specifically with  
393 NoVLP. In order to estimate the molecular weight of HBGA-positive EPS from *Enterobacter* sp.  
394 SENG-6, the amount of A-like substances in the EPS extracted from *Enterobacter* sp. SENG-6 was  
395 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  $\Delta F$  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

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

448 ***Significance of the blood group-active enteric bacteria: a hypothesis.*** The binding is  
449 likely to affect at least two aspects of the ecology of HuNoVs, particularly the fate of HuNoVs in  
450 their hosts after HuNoVs enter the human intestine and encounter large numbers of enteric bacteria.  
451 In this regard, there is increasing understanding of the roles played by the intestinal microbiota in  
452 viral infections. Kane et al. showed that bacterial LPS plays a key role in the infection of mouse  
453 mammary tumor virus (MMTV) using antibiotic-treated and germ-free mice (36). MMTV-bound  
454 bacterial LPS was recognized by Toll-like receptor 4, and it affected the induction of the inhibitory  
455 cytokine interleukin-10. However, this enhanced viral titers and MMTV overcame the immune  
456 response. Kuss et al. found that antibiotic-treated mice were less susceptible to poliovirus infection  
457 and demonstrated that exposure to bacteria or their N-acetylglucosamine-containing surface  
458 polysaccharides, including LPS and peptidoglycan, enhanced the infectivity of poliovirus (37).  
459 These results suggest that antibiotic-mediated microbiota depletion reduces virus infections and that  
460 viruses may exploit intestinal microbes for replication and transmission. Therefore, it is likely that  
461 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  $<180 \mu\text{m}$  (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,  $0.1 \mu\text{m}$ ) (46). Gentry et al. showed that an unexpectedly large number of  
483 HuNoVs were present in the fraction collected using 63- and 200- $\mu\text{m}$ -mesh plankton nets, which

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

497 *Avidity of the blood group-active bacterial EPS with HuNoV particles.* Thus, the effect  
498 of the blood group-active bacterial EPS on the fate of HuNoVs is totally dependent on the binding  
499 avidity of EPS to HuNoV particles. If we assume that all the components in the extracted EPS are  
500 blood group-active and that they have a molecular weight >300 kDa (Fig. 5C), we can estimate the  
501 apparent equilibrium binding constant according to the Langmuir adsorption isotherm as  $>3.1 \times 10^6$   
502  $M^{-1}$ . This value is comparable with that obtained for the interactions between  $\beta$ -galactose specific  
503 lectins and lactose-carrying glycopolystylenes, which have binding constants of  $10^6$  to  $10^7 M^{-1}$  (54).  
504 The interactions between enteric viruses and environmental adsorbents were previously shown to  
505 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

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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  $\alpha$ -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  $\Delta F$  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  $\Delta F$   
736 value with NoVLPs and that without NoVLPs.

737

738

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)	+	+	+	+

740

741

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

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

743 a, *Staphylococcus epidermidis* ATCC35984 was used as the HBGA-negative strain, and it gave an  
744 HBGA-negative result, even at an OD<sub>600</sub> 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.