Title: Bacterial histo-blood group antigens contributing to genotype-dependent removal of human noroviruses with a microfiltration membrane

Authors: Mohan Amarasiri[1], Satoshi Hashiba[1], Takayuki Miura[2], Toyoko Nakagomi[3], Osamu Nakagomi[3], Satoshi Ishii[4], Satoshi Okabe[1], and Daisuke Sano[*1]

[1]Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13, West 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

[2]New Industry Creation Hatchery Center (NICHe), Tohoku University, 6-6-04 Aramaki Aza Aoba, Aoba-ku, Sendai, Miyagi 980-8579, Japan

[3]Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

[4]Department of Soil, Water, and Climate, University of Minnesota, 258 Borlaug Hall, 1991 Upper Buford Circle, St. Paul, MN 55108, USA

*__Corresponding author__: Daisuke Sano, Ph.D., Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13, West 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

Tel: +81-11-706-7597, Fax: +81-11-706-7162

E-mail: dsano@eng.hokudai.ac.jp

Abbreviations:

DALY: disability-adjusted life years

ELISA: enzyme-linked immunosorbent assay

EPS: extracellular polymeric substance

HBGA: histo-blood group antigen

HuNoV: human norovirus

IgG: immunoglobulin G

IgM: immunoglobulin M

LB-EPS: loosely-bound extracellular polymeric substances

LPS: lipopolysaccharide

MF: microfiltration

MBR: membrane bioreactor

MLSS: mixed liquor suspended solids

NoVLP: norovirus-like particle

SMP: soluble microbial product

TB-EPS: tightly-bound extracellular polymeric substance

TEM: transmission electron microscopy

Abstract

We demonstrated the genotype-dependent removal of human norovirus particles with a microfiltration (MF) membrane in the presence of bacteria bearing histo-blood group antigens (HBGAs). Three genotypes (GII.3, GII.4, and GII.6) of norovirus-like particles (NoVLPs) were mixed with three bacterial strains (*Enterobacter* sp. SENG-6, *Escherichia coli* O86:K61:B7, and *Staphylococcus epidermidis*), respectively, and the mixture was filtered with an MF membrane having a nominal pore size of 0.45 μm. All NoVLP genotypes were rejected by the MF membrane in the presence of *Enterobacter* sp. SENG-6, which excreted HBGAs as extracellular polymeric substances (EPS). This MF membrane removal of NoVLPs was not significant when EPS was removed from cells of *Enterobacter* sp. SENG-6. GII.6 NoVLP was not rejected with the MF membrane in the presence of *E. coli* O86:K61:B7, but the removal of EPS of *E. coli* O86:K61:B7 increased the removal efficiency due to the interaction of NoVLPs with the exposed B-antigen in lipopolysaccharide (LPS) of *E. coli* O86:K61:B7. No MF membrane removal of all three genotypes was observed when *S. epidermidis*, an HBGA-negative strain, was mixed with NoVLPs. These results demonstrate that the location of HBGAs on bacterial cells is an important factor in determining the genotype-dependent removal efficiency of norovirus particles with the MF membrane. The presence of HBGAs in mixed liquor suspended solids from a membrane bioreactor (MBR) pilot plant was confirmed by immune-transmission electron microscopy, which implies that bacterial HBGAs can contribute to the genotype-dependent removal of human noroviruses with MBR using MF membrane.

Keywords: extracellular polymeric substance, histo-blood group antigen, human norovirus, lipopolysaccharide, microfiltration, membrane bioreactor

1. Introduction

Noroviruses are single-stranded, non-enveloped, positive-sense RNA viruses that belong to family *Caliciviridae* (Robilotti et al., 2015). Currently there are more than 40 genotypes, which are divided into 6 major genogroups (GI-GVI) with a proposed seventh (Eden et al., 2014; Vinjé, 2014). The GI, GII, and GIV strains infect humans (Eden et al., 2014). Human noroviruses (HuNoVs) are responsible for more than 90% of non-bacterial epidemic gastroenteritis cases and attributable to 18% of acute gastroenteritis patients worldwide with 15.1 million disability-adjusted life years (DALYs) (Ahmed et al., 2014; Kirk et al., 2015; Patel et al., 2008). Outbreaks of HuNoV infection can occur in various community settings such as hospital wards, cruise ships, and restaurants and can affect all age groups, with particularly higher infection risks to organ transplant and immune-compromised patients (Glass et al., 2009).

Wastewater is a major transmission source of HuNoVs (van den Berg et al., 2005; Victoria et al., 2010). In 2012, a large outbreak of HuNoV infection (more than 10,000 gastroenteritis patients) in eastern Germany was likely caused by frozen strawberries imported from China (Bernard et al., 2014). Multiple genotypes of HuNoVs were found from gastroenteritis patients, which supported the speculation that the imported strawberries were cultivated with untreated wastewater (Mäde et al., 2013). Other previous studies also reported that reclaimed wastewater used for irrigation (Brugha et al., 1999) and recreational activities (Hoebe et al., 2004) has caused HuNoV outbreaks. These cases exemplify the importance of the efficient removal of HuNoVs in wastewater treatment for reducing infection risks of HuNoVs through reclaimed wastewater.

The effects of the attachment of viral particles to a variety of wastewater solids, in which one of the main components is organic matter including bacterial cells, on the removal efficiency of enteric viruses from wastewater has been studied (Da Silva et al., 2008; Imai et al., 2011; Miura et al., 2015; Nordgren et al., 2009; Sano et al., 2004). Understanding the binding affinity and specificity of NoV particles to wastewater solids is thus crucial for achieving efficient removal of this human pathogen in wastewater treatment processes (Sano et al., 2010). However, the wide variety of wastewater solids makes it difficult to characterize the attachment of HuNoVs in wastewater, and the factors that affect the binding state of HuNoVs in wastewater have not been very well identified.

In our previous study, *Enterobacter* sp. SENG-6 bearing histo-blood group antigens (HBGAs) was isolated and proven to specifically capture noroviral particles (Miura et al., 2013). Human HBGAs are carbohydrates determining ABO and Lewis blood types. They have been suggested to be important factors for HuNoV infection of intestinal epithelial cells (Shirato et al., 2008; Tan and Jiang, 2005). Human HBGAs are found on mucosal epithelial cells and excreted in body fluids, including the saliva of secretor-positive (Se+) individuals (Tan and Jiang, 2005). The association between human HBGAs and HuNoV infection has been extensively studied using in-vitro binding assays, human volunteer challenge studies, and outbreak investigations (de Rougemont et al., 2011; Marionneau et al., 2002; Tan et al., 2008). The binding of HuNoV particles with non-human HBGAs has been also investigated, and it has been reported that the genotype-dependent bioaccumulation of HuNoVs in oyster is caused by the attachment to HBGA-like substances expressed on oyster tissue surface (Le Guyader et al., 2012, 2006). Since *Enterobacter* sp. SENG-6 excretes HBGAs as extracellular polymeric substances

(EPS) (Miura et al., 2013), the binding of HuNoVs to bacterial HBGAs may affect the persistency of HuNoVs in environments. As an example of the effect of HBGA-positive bacteria on the persistent property of HuNoVs, Li et al. (2015) reported that HBGA-expressing *Escherichia coli* maintained the antigen integrity and mucin-binding ability of NoVLPs after heat treatment (Li et al., 2015). However, the effect of bacterial HBGAs on the life cycle of HuNoVs, including the environmental persistency and removal properties in water and wastewater treatment processes, has not been fully investigated.

In this study, we tested the removal efficiency of norovirus-like particles (NoVLPs) associated with HBGA-positive enteric bacteria using a microfiltration (MF) membrane. Two HBGA-positive bacteria and one HBGA-negative bacterium were separately mixed with three genotypes of NoVLPs and filtered by an MF membrane installed in a spin column. The reduction of NoVLP in the filtrate was detected with enzyme-linked immunosorbent assay (ELISA). The presence of HBGAs in mixed liquor suspended solids (MLSS) from a membrane bioreactor (MBR) pilot plant was analyzed by immuno-transmission electron microscopy (TEM), and the effect of the localization of bacterial HBGAs on the genotype-dependent removal of noroviral particles with an MF membrane was discussed.

2. Materials and Methods

2.1 Bacterial strains and norovirus genotypes

Three bacterial strains (*Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7, and *Staphylococcus epidermidis*) and three norovirus genotypes (GII.3, GII.4 and GII.6) were used in this study (Table S1). *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7 (ATCC

12701) have HBGAs in EPS and lipopolysaccharides (LPS), respectively (Miura et al., 2013; Yi et al., 2006). *S. epidermidis* (ATCC 35984) was used as an HBGA-negative control strain (Miura et al., 2013). Luria-Bertani (LB) agar and broth were prepared according to the manufacturer's instruction manual (Zimbro et al., 2009) for the cultivation of *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, whereas Reasoner's 2A (R2A) agar and broth were used for the cultivation of *S. epidermidis* (Nihon Pharmaceuticals, Japan). HBGA activity of these bacterial strains was tested with the ABOsphia (Kamakura Techno Science Inc., Japan) blood typing kit according to the manufacturer's instructions. NoVLPs of genotypes GII.3, GII.4, and GII.6 were prepared as described previously (Kitamoto et al., 2002). These genotypes are the prevalent genotypes among recent NoV GII outbreaks (Han et al., 2011; Iritani et al., 2010; Zeng et al., 2012).

2.2. Removal of EPS and LPS from bacterial cells

The removal of EPS from bacterial cells was performed in two steps: the separate removal of loosely-bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) (Zhang et al., 2009). Bacterial cells of *Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7 and *S. epidermidis* were cultivated in relevant broth overnight. On the following day, 25 ml of the bacterial cell suspension including the growth medium were centrifuged at 6000 × g for 5 min to separate the supernatant and cell pellet. The supernatant includes soluble microbial products (SMP). The cell pellet was re-suspended in 5 ml of 0.1 M PBS (pH 6.5) and mixed for 2 min by a vortex machine. Then the cell suspension was shaken using a multi shaker for 10 min at 37 ˚C and again mixed for 2 min by a vortex machine. Finally, the cell suspension was centrifuged at 8000 × g for 10 min, and the supernatant including

LB-EPS was removed. The cell pellet was re-suspended in 5 ml of 0.1 M PBS and mixed for 3 min by a vortex machine. The cell suspension was then heated in a water bath at 80 ˚C for 30 min, and the final centrifugation at 12000 × g for 20 min was performed to remove TB-EPS in the supernatant. The heat treatment at 80˚C does not lead to the loss of antigen integrity and the leaching of bacterial intracellular components (Li et al., 2015). Cells after the removal of LB- and TB-EPS were used for the filtration experiment.

A fraction of the cell pellet remained after the TB-EPS removal was used for LPS extraction. LPS extraction was performed in four steps, namely lysis, LPS purification, washing, and elution using the LPS extraction kit (Intron Biotechnology, Inc., Korea) according to the manufacturer's instructions. Briefly, 1 ml of lysis buffer was added and thoroughly mixed using a vortex machine until the cell clump disappeared. Then 200 µl of chloroform was added and mixed vigorously for 10-20 sec using a vortex machine. The mixture was then incubated at room temperature for 5 min and again centrifuged at 18700 × g for 15 min at 4 ˚C using a Hitachi Rx series centrifuge with T15A33 rotor (Hitachi Corporation, Japan). Next, 400 µl of the supernatant was transferred to a new 1.5 ml centrifugation tube by carefully avoiding the transfer of white sediment, and 800 µl of purification buffer was added. The mixture was incubated at −20˚C for 10 min. The incubated mixture was then centrifuged at 18700 × g for 15 min at 4˚C using the T15A33 rotor. An LPS pellet was obtained by removing the supernatant. The pellet was then washed using 1 ml of 70% ethanol, and the mixture was centrifuged at 18700 × g for 3 min at 4˚C using the T15A33 rotor. The supernatant was removed, and 60 µl of 10 mM Tris-HCl buffer (pH 7.9) was added to the tube. This mixture was then boiled for 2 min

179 until the LPS pellet was completely dissolved. LPS samples were stored at −80˚C until
180 further experiments.
181
182 **2.3 ELISA for the detection of bacterial HBGAs**
183 ELISA using a GII.6 NoVLP-immobilized microplate was performed to confirm
184 the presence of bacterial HBGAs in EPS and LPS of *E. coli* O86:K61:B7. GII.6 NoVLP
185 was used as an adsorbent because this genotype can recognize HBGAs (Shirato et al.,
186 2008) and is less adhesive to non-HBGA substances (Miura et al., 2013). Firstly, GII.6
187 NoVLP was serially diluted 100 times with PBS (0.1 M, pH = 6.5) and added in triplicate
188 to the relevant wells of a Microlon 96 well flat bottom, high-binding ELISA plate (Greiner
189 Bio-One, Germany) and kept overnight at 4 ˚C until the NoVLPs were attached to the wells.
190 On the following day, the NoVLP suspension was removed, and the wells were washed
191 twice using 200 µl of PBS (pH=6.5) per well. The well surface was blocked using a 5%
192 bovine serum albumin (BSA) (Wako Chemicals, Japan) in PBS for 2 h at room
193 temperature and washed twice with PBS. After the blocking and washing, 50 µl of LPS
194 solution was added to the well and incubated at room temperature for 1 h. The wells were
195 washed twice with PBS and 50 µl each of anti-A, B, and H mouse monoclonal antibodies
196 (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA)
197 serially diluted up to 100-fold with 5% BSA in PBS added to relevant wells. The plate was
198 incubated for 1 h at room temperature, and washed twice with PBS. Then, 50 µl of goat
199 anti-mouse immunoglobulin M (IgM) HRP-conjugated antibody (A90-101P; Bethyl
200 Laboratories, USA) diluted 500-fold with 5% BSA in PBS were added to each well. The
201 plate was incubated at room temperature for 1 h, and washed four times with PBS.

O-phenylenediamine (OPD) solution was prepared by dissolving 0.52 g of citric acid, 0.71 g of disodium hydrogen phosphate (Na_2HPO_4), one OPD tablet (P-7288; Sigma-Aldrich, Japan), and 30 µl of hydrogen peroxide in 100 ml of MilliQ water. One hundred microliters of the OPD solution was added to each well, and the plate was incubated for 30 min at room temperature. Then 50 µl of 2 M H_2SO_4 was added to stop the coloring reaction. Absorbance at 490 nm in each well was measured for 0.1 sec using a multi-label counter (ARVO MX; Perkin Elmer Inc., Japan). The signal/noise (S/N ratio) was calculated by dividing the absorbance value from GII.6 NoVLP-immobilized well by that from GII.6 NoVLP-negative well. Student's t test was performed using Microsoft Excel 2010 to compare the S/N ratio values between samples.

2.4 Membrane filtration of NoVLPs

All bacteria grown overnight were centrifuged at 3000 × g for 15 min to obtain the cell pellet. The supernatant was removed, 10 ml of 0.1 M PBS was added to the tubes, and the cells were suspended by a vortex machine. Optical density was measured using a spectrophotometer (Bio-Rad Laboratories, USA) at 600 nm, and the value was adjusted to around 1.5 by careful addition of PBS (7.6×10^8–7.9×10^8 cells/ml). NoVLPs of human norovirus GII.3, GII.4, and GII.6 genotypes were prepared by the dilution of the original VLP suspension with PBS. Final particle numbers per milliliter were 2.0×10^{11} particles for GII.3, 1.2×10^{11} particles for GII.4, and 6.0×10^{11} particles for GII.6. Equal volumes of VLP and bacteria solutions were mixed for 15 min at 4 ˚C using a mechanical shaker. Then, 500 µL of the mixture of cells and NoVLP were filtered using NANOSEP MF GHP 0.45 µm filter with the effective filtration area of 0.28 cm^2 (PALL Corporation, Ann Arbor, MI,

USA) at 8,000 × g until the total volume passed through the membrane. The filtrate was collected and stored at 4 ˚C for further analysis.

2.5. ELISA for the detection of NoVLPs

The mixture of bacterial cells and NoVLP (before filtration) or NoVLP only and its filtrate obtained in the membrane filtration test were added to relevant wells in triplicate and kept overnight at 4 ˚C. Wells without any samples are considered as negative. On the following day, the wells were washed twice with PBS. The well surface was blocked using a 5% BSA in PBS for 2 h at room temperature. Wells were washed twice with PBS, and 50 µl of anti-NoVLP serum from rabbit diluted with 5% BSA in PBS was added to relevant wells. The plate was incubated for 1 h and washed twice with PBS. Goat Anti-Rabbit immunoglobulin G (IgG) pAb HRP conjugate (ADI-SAB-300-J; Enzo Life Sciences Inc., USA) was used as the secondary antibody. The secondary antibody was diluted with 5% BSA in PBS, and 50 µl was added to each well. The plate was incubated for 1 h and washed 4 times with PBS. The coloring reaction was performed using the OPD solution as described above, 50 µl of 2M H_2SO_4 was added to stop the coloring reaction, and absorbance at 490 nm (A490) in each well was measured for 0.1 sec using a multi-label counter (ARVO MX; Perkin Elmer Inc., Japan). The filtration experiment and the ELISA quantification is schematically explained in Figure S1 (supporting information). The Mixture/Filtrate ratio was then calculated as the A490 of mixture/A490 of filtrate. The major focus of this study is the retention of NoVLPs on the MF membrane due to the interaction with bacterial HBGAs, which can be reflected by the larger values of Mixture/Filtrate ratio. All tests were performed in triplicates and the average data were

presented. Student's t-test was performed using Microsoft Excel 2010 to compare the mixture/filtrate ratio values.

2.6. Immuno-TEM observation of the localization of HBGAs in MLSS from MBR

The localization of HBGAs in MBR MLSS was analyzed by immuno-TEM. Mixed liquor was taken from an MBR pilot plant (Miura et al., 2015). One hundred microliters of the mixed liquor were placed in a 2-mL tube for 30 min and resulting supernatant was removed. Sedimented MLSS were re-suspended in 2 mL of PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde, and incubated at 4 ˚C for 2 h. The fixed MLSS was collected by sedimentation. The sedimented MLSS were washed by soaking in 2 mL of PBS at 4 ˚C for 10 min. This washing step was repeated twice. The MLSS were 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 MLSS were soaked in 2 mL of 100% ethanol for 20 min. This final dehydration step was repeated twice. Next, the MLSS were infiltrated with 2 mL of LR white resin (Medium grade; London Resin Company Ltd., England) and 100% ethanol (1:2) mixture for 12 h, followed by 2 mL of LR white resin and 100% ethanol (2:1) mixture for 12 h. The solution was replaced with 2 mL of pure LR white resin for 1 h, followed by another 2 mL of pure LR white resin for 12 h. The MLSS and pure LR white resin were polymerized using an ultraviolet polymerizer (TUV-100; Dosaka EM Co., Ltd., Japan) at 4 ˚C for 3 days. Ultrathin sections measuring 70 nm in thickness were prepared

from the embedded MLSS using a diamond knife on an ultramicrotome (RMC MTXL; Boeckeler Instruments, Inc., USA). The sections were then placed on nickel grids.

The ultrathin sections on grids were washed by floating the grid upside down on a drop of PBS at room temperature for 1 min. The following incubations were also conducted by floating the grid upside down on drops of reagent at room temperature. The sections were blocked with 4% BSA in PBS for 5 min and incubated with anti-blood group A, B, or O(H) antibody diluted 1:30 in PBS containing 1% BSA for 2 h, followed by washing with PBS (6 changes, 1 min each). The bound primary antibodies were localized by incubating the sections on anti-mouse IgM antibody gold conjugate (10 nm, ab39613; Abcam, Japan) diluted 1:10 in PBS containing 1% BSA for 1 h, followed by washing with PBS (6 changes, 1 min each). The sections were fixed with 2% glutaraldehyde in PBS for 15 min, followed by washing with deionized distilled water (DDW; 7 changes, 1 min each). Finally, the sections were stained with 5% uranyl acetate, followed by washing with 50% ethanol for 1 min and DDW (4 changes, 1 min each). Stained sections were observed under TEM. Ultrathin sections without the primary antibodies were used as controls.

3. Results

3.1 Availability of bacterial HBGAs for NoVLP attachment

HBGAs associated with bacterial cells were detected by the blood typing kit. This kit detects A, B, and O(H) antigens on cells by forming an aggregate of cells and anti-HBGA antibody-coated beads. *Enterobacter* sp. SENG-6 displayed the highest response for all A, B, and O(H) antigens, whereas *E. coli* O86:K61:B7 and *S. epidermidis* gave negative results for all tested cell densities (Table 1, Figure S1). These A, B, and

O(H) antigens of *Enterobacter* sp. SENG-6 were not detected when EPS was removed mechanically, which indicates that bacterial HBGAs of *Enterobacter* sp. SENG-6 were included mainly in EPS. On the other hand, the B antigen in LPS of *E. coli* O86:K61:B7 was detected after the EPS removal (Table 1, Figure S2), showing that the B antigen in LPS of *E. coli* O86:K61:B7 can be recognized by the anti-B antibody in the kit, but this antigen-antibody interaction is physically blocked by the presence of EPS covering cells of *E. coli* O86:K61:B7. We also confirmed that A and O(H) antigens of *E. coli* O86:K61:B7 were not detected even after the EPS removal (Table 1, Figure S2).

For confirming the interaction between LPS of *E. coli* O86:K61:B7 and NoV particles, ELISA using a GII.6 NoVLP-immobilized microplate was performed. Extracted LPS from *E. coli* O86:K61:B7 was applied to the GII.6 NoVLP-immobilized well, and bacterial HBGAs in the trapped LPS were detected by anti-HBGA antibodies. The negative control condition (without LPS) gave a signal/noise ratio value larger than 1.0 for the B antigen, but a significantly higher value of the signal/noise ratio was observed under the test condition (with LPS, $p < 0.01$, Figure 1). Although a signal/noise ratio value larger than 1.0 with LPS was also observed for the O(H) antigen, the statistically significant difference was not obtained between test and negative conditions. These results indicate that the B antigen is the most abundant in LPS of *E. coli* O86:K61:B7 and can bind the GII.6 NoVLP.

3.2 Membrane filtration of NoVLPs mixed with bacterial cells

NoVLP of GII.3, GII.4, or GII.6 was mixed with suspended cells of *Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7, or *S. epidermidis*. The mixture of NoVLPs

and bacterial cells was filtered using NANOSEP MF GHP 0.45 μm filter in a spin column. NoVLPs in the mixture with bacterial cells (before filtration) and those in filtrate (after filtration) were detected by ELISA. As a bacteria-negative control, only NoVLPs (without bacterial cells) were also filtered separately. The mixture/filtrate ratio of ELISA signal was around 1.0 (0.91-1.01) for all genotypes tested under the bacteria-negative control (Figure 2, VLP only), which explains that NoVLP (diameter: ca. 40 nm) has passed through the MF membrane pore (nominal pore size of 0.45 μm). The mixture/filtrate ratio values were always less than 1.0 when cells of *S. epidermidis* were mixed with NoVLPs (Figure 2), probably because of an inhibitory effect of *S. epidermidis* cells on the signal detection in ELISA. The presence of *S. epidermidis* did not affect the passing of the majority of NoVLPs through the MF membrane pore as there were no HBGAs to retain the NoVLPs.

When NoVLPs were mixed with cells of *Enterobacter* sp. SENG-6, the mixture/filtrate ratio of ELISA signal was significantly larger than 1.0 for all genotypes tested ($p < 0.01$), showing that a significant amount of NoVLPs were trapped by *Enterobacter* sp. SENG-6 cells and did not pass through the MF membrane (Figure 2). On the other hand, the behavior of NoVLPs was dependent on the genotype when mixed with cells of *E. coli* O86:K61:B7. The mixture/filtrate ratio of GII.4 NoVLP was significantly larger than 1.0 (Figure 2(B)) ($p < 0.01$), but those of GII.3 and GII.6 were not (Figure 2(A) and (C)), which means that GII.3 and GII.6 NoVLPs were not trapped by *E. coli* O86:K61:B7 cells very effectively.

3.3 Impact of the location of bacterial HBGAs on the cell attachment of noroviral particles

LB- and TB-EPS were removed from cells of *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, and the membrane filtration test was performed using NANOSEP MF GHP 0.45 μm filter in a spin column to analyze the effect of the localization of bacterial HBGAs on the removal property of noroviral particles. The mixture/filtrate ratio of ELISA signal was compared between bacterial cells before and after the EPS removal. As a result, the removal of EPS from *Enterobacter* sp. SENG-6 cells has been attributed to a significantly lower mixture/filtrate ratio ($p < 0.01$). The difference in the ratio values for *Enterobacter* sp. SENG-6 with EPS between Figure 2(C) and Figure 3 was caused by experimental random errors in both values of numerator (A490 of mixture) and denominator (A490 of filtrate) in the ratio calculation. A490 values of mixture in Figure 2(C) and Figure 3 were 1.50 ± 0.13 and 1.13 ± 0.01, respectively, and those of filtrate were 0.24 ± 0.04 and 0.84 ± 0.08. Although we used the same amount of NoVLP in these experiments, this level variation in ELISA signal can be observed. On the other hand, the EPS-removed *E. coli* O86:K61:B7 cells gave a significantly higher value of the mixture/filtrate ratio ($p < 0.01$) (Figure 3). These results demonstrate that the localization of bacterial HBGAs is an important factor for the norovirus removal with the MF membrane, and the HBGAs in LPS of *E. coli* O86:K61:B7 have to be exposed for the association with GII.6 NoVLP.

3.4 Immuno-TEM confirmation of the localization of bacterial HBGAs

Ultrathin sections of MBR MLSS labeled with gold nanoparticles bearing anti-mouse IgM after reacting with anti-blood group A, B, or O(H) antibody (used as the primary antibody) were examined by immuno-TEM to study the localization of HBGAs. A

number of gold nanoparticles were observed in the EPS region of MLSS (Figure 4(A)) that had reacted with anti-A antibody. In contrast, the B and O(H) antigens were not detected in the EPS region, but a few gold nanoparticles carrying anti-B and O(H) antibodies were observed inside bacterial cells (Figure 4(B) and (C)). These immuno-TEM observations indicate that at least the A antigens can be present in the EPS region of MBR MLSS.

4. Discussion

We demonstrated the role of bacterial HBGAs on the MF membrane separation of HuNoVs. Two HBGA-positive bacteria, *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, or one HBGA-negative bacteria, *S. epidermidis*, were mixed with each genotype of NoVLP GII.3, GII.4, or GII.6, and the reduction of NoVLPs in the filtrate with an MF membrane (nominal pore size of 0.45 μm) installed in a spin column was compared. All results indicated that bacterial HBGAs in EPS and LPS could capture noroviral particles and contribute to the MF membrane removal, but the attachment of NoVLPs to HBGA-positive LPS is physically hindered by EPS covering bacterial cells.

The membrane used in this study is an MF membrane with a nominal pore size of 0.45 μm. MF membranes with a nominal pore size of 0.1 to 0.45 μm are very frequently employed in MBR systems for wastewater treatment (Kimura et al., 2015). A membrane pore size larger than 0.1 μm is commonly preferable because the membrane fouling associated with pore plugging is one of the most crucial problems in MBR operation (Guo et al., 2012). Noroviral particles have a diameter of around 40 nm (Koho et al., 2012) and mixture/filtration ratios around 1.0 is obtained in the absence of bacterial cells with all 3 types of NoVLPs (Figure 2, VLP only), which explains the sieve mechanism does not

appear to work in the removal of NoVLPs, as observed in the membrane filtration test of noroviral particles indigenous in wastewater (Sano et al., 2006). However, the significant reduction of NoVLPs of all genotypes was observed in the presence of *Enterobacter* sp. SENG-6 (Figure 2). *Enterobacter* sp. SENG-6 produced HBGAs in EPS (Table 1), which contributed to the efficient removal of all norovirus genotypes of GII.3, GII.4, and GII.6 with the MF membrane. For the efficient removal of HuNoV in the MBR, the association of viral particles to mixed liquor suspended solids (MLSS) and/or the cake layer formed on the membrane surface is of primary importance (Miura et al., 2015; Simmons et al., 2011). MLSS and the cake layer are mainly composed of bacterial cells and EPS (Lin et al., 2014), and the immuno-TEM images show that the A antigen was present in the EPS region of MBR MLSS (Figure 4(A)). A thorough understanding about the prevalence and spatial/temporal (seasonal) variation of the expression level of these bacterial HBGAs and the binding property of noroviral particles to bacterial components including EPS is thus critical for discussing the contribution of bacterial HBGAs in MLSS on the removal of HuNoVs in MBR.

The removal efficiency of NoVLPs in the presence of *E. coli* O86:K61:B7 was genotype-dependent, in which GII.4 NoVLP was well removed but GII.3 and GII.6 were not (Figure 2). The NoV genotype GII.4 can recognize broad types of epitope compared to the other genotypes (Bally et al., 2012; Ruvoën-clouet et al., 2013; Tamura et al., 2004). It is likely that the interaction between the GII.4 NoVLPs and *E. coli* O86:K61:B7 (Figure 2(B)) is caused by the broad spectrum of HBGA-epitope recognition of this genotype. Several phenomena have been proposed as the determination factors of virus removal efficiency with membrane filtration, including the attachment of viruses to biosolids

(Chaudhry et al., 2015b; Miura et al., 2015), the virus retention by cake layer on membrane surface (Lu et al., 2013), the virus retention on membrane surface (Chaudhry et al., 2015b), the initial concentration level of virus in feed water (Lu et al., 2016), and the virus types (Chaudhry et al., 2015a). This study verified for the first time that the epitope-recognizing capability of viral particle is one of the factors determining virus removal efficiency with membrane filtration.

All results from the present study provide evidence that the removal efficiency of NoV particles with the MF membrane is dependent on the location of bacterial HBGAs and the norovirus genotype. Different norovirus genotypes have different HBGA-binding profiles (Donaldson et al., 2008; Lindesmith et al., 2008; Shirato et al., 2008), which means that norovirus genotypes other than those tested in the present study have different removal properties by the MF membrane and HBGA-positive bacteria. Since a recent report describes that the HBGA-binding profile can also vary within a genotype (Hoa-Tran et al., 2015), a strain-dependent removal property can be observed when multiple strains from an identical genotype are used to test the removal efficiency with the MF membrane. Particularly, the rapid evolution of NoV GII.4 should be paid attention, because new variants have appear every 2-3 years and increased relative affinity towards the A, B, O(H) and Lewis antigens over the evolution has been observed (Ruvoën-clouet et al., 2013). The GII.4 NoVLP used in this study is a variant strain of Den Haag 2006b (Imai et al., 2011), which can recognize all the A, B, O(H), and Lewis antigens (Kroneman et al., 2011) as well as the other GII.4 strains (Shirato et al., 2008). This means that the Den Haag 2006b strain can be representative among GII.4 strains in terms of the MF membrane removal with HBGA-positive bacteria. Recent studies have shown the emergence of NoV GII.17

strains and the replacement of the currently predominant GII.4 Sydney strain (De Graaf et al., 2015; Zhang et al., 2015). It is expected that these GII.17 strains can be effectively removed by MF membrane because of the broad HBGA binding spectrum (Zhang et al., 2015), but it is necessary to conduct the filtration test as in this study for coming to a conclusion about the removal efficiency of the emerging NoV strains with MF membarne.

Jones et al. reported that *Enterobacter cloacae* (ATCC 13047), a relative of *Enterobacter* sp. SENG-6 (99.9% identity in the 16s rRNA gene sequence), bound norovirus GII.4 Sydney strain through the bacterial O(H) antigen, which resulted in the infection of the Sydney strain to cultivated B cells (Jones et al., 2014). Jones et al. (2014) used whole cell components of *Enterobacter cloacae* and detected the H antigen by western blotting. From the bacteriological viewpoint, even the 100% identity of 16S rRNA gene sequence is not enough to identify bacterial species, and multiple phenotypes including substrate availability must be tested for the species identification. Since enteric bacteria are so diverse, it is highly likely that *Enterobacter* sp. SENG-6 is identified as a new species in genus *Enterobacter*, which is different from *Enterobacter cloacae*. The comparison of the HBGA expression profile among *Enterobacter* spp. and other enteric bacteria is necessary to discuss the ubiquity of HBGA-positive bacteria in nature. Further understanding of the prevalence of HBGA-positive bacteria in environments may lead to better insights about norovirus survival and persistence in environmental waters and removability in wastewater treatment processes, including MBR. A small-scale (500µl) test for the membrane filtration was performed in this study to investigate the effect of specific interaction between bacterial HBGAs and noroviral particles on the removal efficiency, and the observations at a larger scale must be validated in the further study.

5. Conclusions

- The interaction with HBGA-positive bacteria contributes to the removal of HuNoV particles with MF membrane.
- Cell-associated and HBGA-positive EPS is important for removing HuNoV particles with MF membrane.
- The removal efficiency of HuNoV particles with MF membrane and bacterial HBGA is genotype-dependent.
- HBGAs are expressed in mixed liquor suspended solids from MBR, which can be available for the removal of NoVs in wastewater.

Acknowledgments

This study was supported by the Japan Science and Technology Agency (JST) through Core Research for Evolutionary Science and Technology (CREST) and the Japan Society for the Promotion of Science through Grant-in-Aid for Scientific Research (A) (26249075).

References

Ahmed, S.M., Hall, A.J., Robinson, A.E., Verhoef, L., Premkumar, P., Parashar, U.D., Koopmans, M., Lopman, B.A., 2014. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. Lancet Infect. Dis. 14, 725–730. doi:10.1016/S1473-3099(14)70767-4

Bally, M., Rydell, G.E., Zahn, R., Nasir, W., Eggeling, C., Breimer, M.E., Svensson, L., Hççk, F., Larson, G., 2012. Norovirus GII.4 virus-like particles recognize Galactosylceramides in domains of planar supported lipid bilayers. Angew. Chemie Int. Ed. 51, 12020–12024. doi:10.1002/anie.201205972

480 Bernard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., Ducomble, T.,
481 Siffczyk, C., Merbecks, S.S., 2014. Large multistate outbreak of norovirus
482 gastroenteritis associated with frozen strawberries, Germany, 2012. Euro Surveill.
483 19(8), pii=20719. doi:10.2807/1560-7917.ES2014.19.8.20719

484 Brugha, R., Vipond, I.B., Evans, M.R., Sandifer, Q.D., Roberts, R.J., Salmon, R.L., Caul,
485 E.O., Mukerjee, A.K., 1999. A community outbreak of food-borne small
486 round-structured virus gastroenteritis caused by a contaminated water supply.
487 Epidemiol. Infect. 122, 145–154.

488 Chaudhry, R.M., Holloway, R.W., Cath, T.Y., Nelson, K.L., 2015a. Impact of virus
489 surface characteristics on removal mechanisms within membrane bioreactors. Water
490 Res. 84, 144–152. doi:10.1016/j.watres.2015.07.020

491 Chaudhry, R.M., Nelson, K.L., Drewes, J.E., 2015b. Mechanisms of Pathogenic Virus
492 Removal in a Full-Scale Membrane Bioreactor. Environ. Sci. Technol. 49, 2815–2822.
493 doi:10.1021/es505332n

494 Da Silva, A.K., Le Guyader, F.S., Le Saux, J.C., Pommepuy, M., Montgomery, M.A.,
495 Elimelech, M., 2008. Norovirus removal and particle association in a waste
496 stabilization pond. Environ. Sci. Technol. 42, 9151–9157. doi:10.1021/es802787v

497 De Graaf, M., Van Beek, J., Vennema, H., Podkolzin, A.T., Hewitt, J., Bucardo, F.,
498 Templeton, K., Mans, J., Nordgen, J., Reuter, G., Lynch, M., Rasmussen, L., Iritani,
499 N., Chan, M., Martella, V., Ambert-Balay, K., Vinje, J., White, P., Koopmans, M.,
500 2015. Emergence of a novel GII.17 norovirus – End of the GII.4 era? Euro
501 Surveillence 20, pii=21178. doi:10.2807/1560-7917.ES2015.20.26.21178

502 de Rougemont, A., Ruvoen-Clouet, N., Simon, B., Estienney, M., Elie-Caille, C., Aho, S.,
503 Pothier, P., Le Pendu, J., Boireau, W., Belliot, G., 2011. Qualitative and quantitative
504 analysis of the binding of GII.4 norovirus variants onto human blood group antigens.
505 J. Virol. 85, 4057–4070. doi:10.1128/JVI.02077-10

506 Donaldson, E.F., Lindesmith, L.C., Lobue, A.D., Baric, R.S., 2008. Norovirus
507 pathogenesis: mechanisms of persistence and immune evasion in human populations.
508 Immunol. Rev. 225, 190–211. doi:10.1111/j.1600-065X.2008.00680.x

509 Eden, J.-S., Hewitt, J., Lim, K.L., Boni, M.F., Merif, J., Greening, G., Ratcliff, R.M.,
510 Holmes, E.C., Tanaka, M.M., Rawlinson, W.D., White, P.A., 2014. The emergence
511 and evolution of the novel epidemic norovirus GII.4 variant Sydney 2012. Virology

450-451, 106–113. doi:10.1016/j.virol.2013.12.005

Glass, R., Parashar, U., Estes, M., 2009. Norovirus gastroenteritis. N. Engl. J. Med. 361, 1776–1785. doi:10.1056/NEJMra0804575

Guo, W., Ngo, H.-H., Li, J., 2012. A mini-review on membrane fouling. Bioresour. Technol. 122, 27–34. doi:10.1016/j.biortech.2012.04.089

Han, T.-H., Kim, C.-H., Chung, J.-Y., Park, S.-H., Hwang, E.-S., 2011. Emergence of norovirus GII-4/2008 variant and recombinant strains in Seoul, Korea. Arch. Virol. 156, 323–329. doi:10.1007/s00705-010-0844-4

Hoa-Tran, T.N., Nakagomi, T., Sano, D., Sherchand, J.B., Pandey, B.D., Cunliffe, N.A., Nakagomi, O., 2015. Molecular epidemiology of noroviruses detected in Nepalese children with acute diarrhea between 2005 and 2011: increase and predominance of minor genotype GII.13. Infect. Genet. Evol. 30, 27–36. doi:10.1016/j.meegid.2014.12.003

Hoebe, C.J.P.A., Vennema, H., de Roda Husman, A.M., van Duynhoven, Y.T.H.P., 2004. Norovirus outbreak among primary schoolchildren who had played in a recreational water fountain. J. Infect. Dis. 189, 699–705. doi:10.1086/381534

Imai, T., Sano, D., Miura, T., Okabe, S., Wada, K., Masago, Y., Omura, T., 2011. Adsorption characteristics of an enteric virus-binding protein to norovirus, rotavirus and poliovirus. BMC Biotechnol. 11, 123. doi:10.1186/1472-6750-11-123

Iritani, N., Kaida, A., Kubo, H., Abe, A., Goto, K., Ogura, H., Seto, Y., 2010. Molecular epidemiology of noroviruses detected in seasonal outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan, from 1996-1997 to 2008-2009. J. Med. Virol. 82, 2097–2105. doi:10.1002/jmv.21915

Jones, M.K., Watanabe, M., Zhu, S., Graves, C.L., Keyes, L.R., Grau, K.R., Gonzalez-Hernandez, M.B., Iovine, N.M., Wobus, C.E., Vinje, J., Tibbetts, S. a., Wallet, S.M., Karst, S.M., 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. Science (80-.). 346, 755–759. doi:10.1126/science.1257147

Kimura, K., Ogyu, R., Miyoshi, T., Watanabe, Y., 2015. Transition of major components in irreversible fouling of MBRs treating municipal wastewater. Sep. Purif. Technol. 142, 326–331. doi:10.1016/j.seppur.2014.12.030

Kirk, M., Pires, S., Black, R., Caipo, M., Crump, J., Devleesschauwer, B., Dopfer, D.,

544 Fazil, A., Fischer-Walker, C., Hald, T., Hall, A., Keddy, K., Lake, R., Fanata, C.,
545 Torgerson, P., Havelaar, A., Angulo, F., 2015. World Health Organization estimates
546 of the global and regional disease burden of 22 foodborne bacterial, protozoal, and
547 viral diseases, 2010: A data synthesis. PLOS Med. 12, e1001921.
548 doi:10.1371/journal.pmed.1001921

549 Kitamoto, N., Tanaka, T., Natori, K., Takeda, N., Nakata, S., Jiang, X., Estes, M.K., 2002.
550 Cross-reactivity among several recombinant calicivirus virus-like particles (VLPs)
551 with monoclonal antibodies obtained from mice immunized orally with one type of
552 VLP. J. Clin. Microbiol. 40, 2459–2465. doi:10.1128/JCM.40.7.2459-2465.2002

553 Koho, T., Mäntylä, T., Laurinmäki, P., Huhti, L., Butcher, S.J., Vesikari, T., Kulomaa,
554 M.S., Hytönen, V.P., 2012. Purification of norovirus-like particles (VLPs) by ion
555 exchange chromatography. J. Virol. Methods 181, 6–11.
556 doi:10.1016/j.jviromet.2012.01.003

557 Kroneman, A., Vennema, H., Deforche, K., Avoort, H. v. d., Peñaranda, S., Oberste, M.S.,
558 Vinjé, J., Koopmans, M., 2011. An automated genotyping tool for enteroviruses and
559 noroviruses. J. Clin. Virol. 51, 121–125. doi:10.1016/j.jcv.2011.03.006

560 Le Guyader, F.S., Atmar, R.L., Le Pendu, J., 2012. Transmission of viruses through
561 shellfish: when specific ligands come into play. Curr. Opin. Virol. 2, 103–110.
562 doi:10.1016/j.coviro.2011.10.029

563 Le Guyader, F.S., Loisy, F., Atmar, R.L., Hutson, A.M., Estes, M.K., Ruvoën-Clouet, N.,
564 Pommepuy, M., Le Pendu, J., 2006. Norwalk virus-specific binding to oyster
565 digestive tissues. Emerg. Infect. Dis. 12, 931–936. doi:10.3201/eid1206.051519

566 Li, D., Breiman, A., Le Pendu, J., Uyttendaele, M., 2015. Binding to histo-blood group
567 antigen-expressing bacteria protects human norovirus from acute heat stress. Front.
568 Microbiol. 6:659. doi:10.3389/fmicb.2015.00659

569 Lin, H., Zhang, M., Wang, F., Meng, F., Liao, B.-Q., Hong, H., Chen, J., Gao, W., 2014. A
570 critical review of extracellular polymeric substances (EPSs) in membrane bioreactors:
571 Characteristics, roles in membrane fouling and control strategies. J. Memb. Sci. 460,
572 110–125. doi:10.1016/j.memsci.2014.02.034

573 Lindesmith, L.C., Donaldson, E.F., Lobue, A.D., Cannon, J.L., Zheng, D.-P., Vinje, J.,
574 Baric, R.S., 2008. Mechanisms of GII.4 norovirus persistence in human populations.
575 PLoS Med. 5, e31. doi:10.1371/journal.pmed.0050031

576 Lu, R., Li, Q., Yin, Z., Xagoraraki, I., Tarabara, V. V., Nguyen, T.H., 2016. Effect of virus
577 influent concentration on its removal by microfiltration: The case of human
578 adenovirus 2. J. Memb. Sci. 497, 120–127. doi:10.1016/j.memsci.2015.08.065

579 Lu, R., Mosiman, D., Nguyen, T.H., 2013. Mechanisms of MS2 bacteriophage removal by
580 fouled ultrafiltration membrane subjected to different cleaning methods. Environ. Sci.
581 Technol. 47, 13422–13429. doi:10.1021/es403426t

582 Mäde, D., Trübner, K., Neubert, E., Höhne, M., Johne, R., 2013. Detection and typing of
583 norovirus from frozen strawberries involved in a large-scale gastroenteritis outbreak
584 in Germany. Food Environ. Virol. 5, 162–168. doi:10.1007/s12560-013-9118-0

585 Marionneau, S., Ruvoën, N., Le Moullac–Vaidye, B., Clement, M., Cailleau–Thomas, A.,
586 Ruiz–Palacois, G., Huang, P., Jiang, X., Le Pendu, J., 2002. Norwalk virus binds to
587 histo-blood group antigens present on gastroduodenal epithelial cells of secretor
588 individuals. Gastroenterology 122, 1967–1977. doi:10.1053/gast.2002.33661

589 Miura, T., Okabe, S., Nakahara, Y., Sano, D., 2015. Removal properties of human enteric
590 viruses in a pilot-scale membrane bioreactor (MBR) process. Water Res. 75, 282–291.
591 doi:10.1016/j.watres.2015.02.046

592 Miura, T., Sano, D., Suenaga, A., Yoshimura, T., Fuzawa, M., Nakagomi, T., Nakagomi,
593 O., Okabe, S., 2013. Histo-blood group antigen-like substances of human enteric
594 bacteria as specific adsorbents for human noroviruses. J. Virol. 87, 9441–9451.
595 doi:10.1128/JVI.01060-13

596 Nordgren, J., Matussek, A., Mattsson, A., Svensson, L., Lindgren, P.-E., 2009. Prevalence
597 of norovirus and factors influencing virus concentrations during one year in a
598 full-scale wastewater treatment plant. Water Res. 43, 1117–1125.
599 doi:10.1016/j.watres.2008.11.053

600 Patel, M.M., Widdowson, M., Glass, R.I., Akazawa, K., Vinjé, J., Parashar, U.D., 2008.
601 Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg.
602 Infect. Dis. 14, 1224–1231. doi:10.3201/eid1408.071114

603 Robilotti, E., Deresinski, S., Pinsky, B.A., 2015. Norovirus. Clin. Microbiol. Rev. 28, 134–
604 164. doi:10.1128/CMR.00075-14

605 Ruvoën-clouet, N., Belliot, G., Le Pendu, J., 2013. Noroviruses and histo-blood groups:
606 the impact of common host genetic polymorphisms on virus transmission and
607 evolution. Rev. Med. Virol. 23, 355–366. doi:10.1002/rmv.1757

608 Sano, D., Matsuo, T., Omura, T., 2004. Virus-Binding Proteins Recovered from Bacterial
609 Culture Derived from Activated Sludge by Affinity Chromatography Assay Using a
610 Viral Capsid Peptide Virus-Binding Proteins Recovered from Bacterial Culture
611 Derived from Activated Sludge by Affinity Chromatog. doi:10.1128/AEM.70.6.3434

612 Sano, D., Ueki, Y., Watanabe, T., Omura, T., 2006. Membrane separation of indigenous
613 noroviruses from sewage sludge and treated wastewater. Water Sci. Technol. 54, 77–
614 82. doi:10.2166/wst.2006.451

615 Sano, D., Wada, K., Imai, T., Masago, Y., Omura, T., 2010. Norovirus-binding proteins
616 recovered from activated sludge micro-organisms with an affinity to a noroviral
617 capsid peptide. J. Appl. Microbiol. 109, 1923–1928.
618 doi:10.1111/j.1365-2672.2010.04821.x

619 Shirato, H., Ogawa, S., Ito, H., Sato, T., Kameyama, A., Narimatsu, H., Xiaofan, Z.,
620 Miyamura, T., Wakita, T., Ishii, K., Takeda, N., 2008. Noroviruses distinguish
621 between type 1 and type 2 histo-blood group antigens for binding. J. Virol. 82,
622 10756–10767. doi:10.1128/JVI.00802-08

623 Simmons, F.J., Kuo, D.H.-W., Xagoraraki, I., 2011. Removal of human enteric viruses by
624 a full-scale membrane bioreactor during municipal wastewater processing. Water Res.
625 45, 2739–2750. doi:10.1016/j.watres.2011.02.001

626 Tamura, M., Natori, K., Kobayashi, M., Miyamura, T., Takeda, N., 2004. Genogroup II
627 noroviruses efficiently bind to Heparan Sulfate proteoglycan associated with the
628 cellular membrane. J. Virol. 78, 3817–3826. doi:10.1128/JVI.78.8.3817

629 Tan, M., Jiang, X., 2005. Norovirus and its histo-blood group antigen receptors: an answer
630 to a historical puzzle. Trends Microbiol. 13, 285–293. doi:10.1016/j.tim.2005.04.004

631 Tan, M., Jin, M., Xie, H., Duan, Z., Jiang, X., Fang, Z., 2008. Outbreak studies of a GII-3
632 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral
633 infection. J. Med. Virol. 80, 1296–1301. doi:10.1002/jmv.21200

634 van den Berg, H., Lodder, W., van der Poel, W., Vennema, H., de Roda Husman, A.M.,
635 2005. Genetic diversity of noroviruses in raw and treated sewage water. Res.
636 Microbiol. 156, 532–540. doi:10.1016/j.resmic.2005.01.008

637 Victoria, M., Guimaraes, F., Fumian, T., Ferreira, F., Vieira, C., Shubo, T., Leite, J.,
638 Miagostovich, M., 2010. One year monitoring of norovirus in a sewage treatment
639 plant in Rio de Janeiro, Brazil. J. Water Health 08, 158–165.

640 doi:10.2166/wh.2009.012

641 Vinjé, J., 2014. Advances in Laboratory Methods for Detection and Typing of Norovirus. J.
642 Clin. Microbiol. 53, 373–381. doi:10.1128/JCM.01535-14

643 Yi, W., Bystricky, P., Yao, Q., Guo, H., Zhu, L., Li, H., Shen, J., Li, M., Ganguly, S., Bush,
644 C.A., Wang, P.G., 2006. Two different O-polysaccharides from Escherichia coli O86
645 are produced by different polymerization of the same O-repeating unit. Carbohydr.
646 Res. 341, 100–108. doi:10.1016/j.carres.2005.11.001

647 Zeng, M., Xu, X., Zhu, C., Chen, J., Zhu, Q., Lin, S., Jie, Y., 2012. Clinical and molecular
648 epidemiology of norovirus infection in childhood diarrhea in China. J. Med. Virol. 84,
649 145–151. doi:10.1002/jmv.22248

650 Zhang, H., Xia, J., Yang, Y., Wang, Z., Yang, F., 2009. Mechanism of calcium mitigating
651 membrane fouling in submerged membrane bioreactors. J. Environ. Sci. 21, 1066–
652 1073. doi:10.1016/S1001-0742(08)62383-9

653 Zhang, X.-F., Huang, Q., Long, Y., Jiang, X., Zhang, T., Tan, M., Zhang, Q.-L., Huang,
654 Z.-Y., Li, Y.-H., Ding, Y.-Q., Hu, G.-F., Tang, S., Dai, Y.-C., 2015. An outbreak
655 caused by GII.17 norovirus with a wide spectrum of HBGA-associated susceptibility.
656 Sci. Rep. 5, 17687. doi:10.1038/srep17687

657 Zimbro, M.J., Power, D.A., Miller, S.M., Wilson, G.E., Johnson, J.A., 2009. Difco and
658 BBL Manual, Second Edi. ed. Becktor, Dickinson and Company, Maryland.

659

660

661 **Table 1.** Detection of bacterial histo-blood group antigens by a blood typing kit

	Enterobacter sp. SENG-6						*E. coli* O86:K61:B7 (ATCC 12701)						*Staphylococcus epidermidis* (ATCC 35984)		
	with EPS			w/o EPS			with EPS			w/o EPS					
Cells/ml	A	B	O(H)	A	B	O(H)	A	B	O(H)	A	B	O(H)	A	B	O(H)
10^9	+++	+++	+++	–	–	–	–	–	–	–	+	–	–	–	–
10^8	+++	+++	+++	–	–	–	–	–	–	–	+	–	–	–	–
10^7	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–

662 +++, strong aggregation; ++, moderate aggregation; +, weak aggregation; −, no aggregation.

663 **Figure Legends**

664

665 **Figure 1.** Detection of histo-blood group antigens (HBGAs) of *Escherichia coli*
666 O86:K61:B7 (ATCC 12701). A plate was coated by GII.6 norovirus-like particles
667 (NoVLPs), and HBGAs in lipopolysaccharide of *E. coli* O86:K61:B7 attached to NoVLP
668 were detected by anti-A, B, and O(H) antibodies. **, $p < 0.01$.

669

670 **Figure 2.** Membrane separation of norovirus-like particles (NoVLPs) with bacterial cells.
671 Reduction of (A) GII.3, (B) GII.4, and (C) GII.6 NoVLP by the filtration with a
672 microfiltration membrane (nominal pore size of 0.45 μm) in the presence of *Enterobacter*
673 sp. SENG-6, *Escherichia coli* O86:K61(B7) (ATCC 12701), and *Staphylococcus*
674 *epidermidis* (ATCC 35984). NoVLP was filtered without bacterial cells in the condition of
675 VLP only. **, $p < 0.01$.

676

677 **Figure 3.** Effect of extracellular polymeric substances on membrane separation of
678 norovirus-like particles (NoVLPs). The mixture/filtrate ratio between the quantity of GII.6
679 NoVLPs in the samples before and after the filtration with a microfiltration membrane
680 (pore size: 0.45μm) in the presence of *Enterobacter* sp. SENG-6 and *Escherichia coli*
681 O86:K61(B7) (ATCC 12701) before and after removal of soluble microbial product,
682 loosely-bound EPS, and tightly-bound EPS. **, $p < 0.01$.

683

684 **Figure 4.** Localization of histo-blood group antigens in mixed liquor suspended solids
685 from a membrane bioreactor pilot plant. Ultrathin section was labeled with immuno-gold

686 after reacting with anti-blood group A, B, and O(H) antibodies (primary antibodies) and
687 observed under transmission electron microscopy. Ultrathin sections without primary
688 antibodies were used as controls. A) The A antigen, B) the B antigen, C) the O(H) antigen,
689 and D) control.

Figure 1

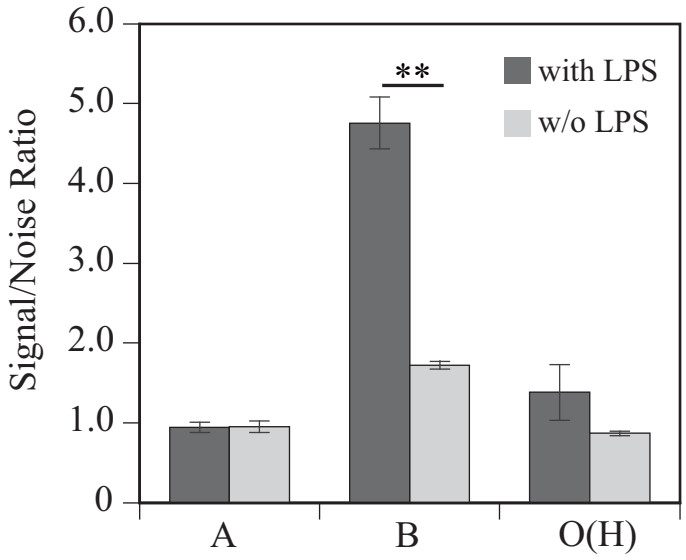

Figure 2

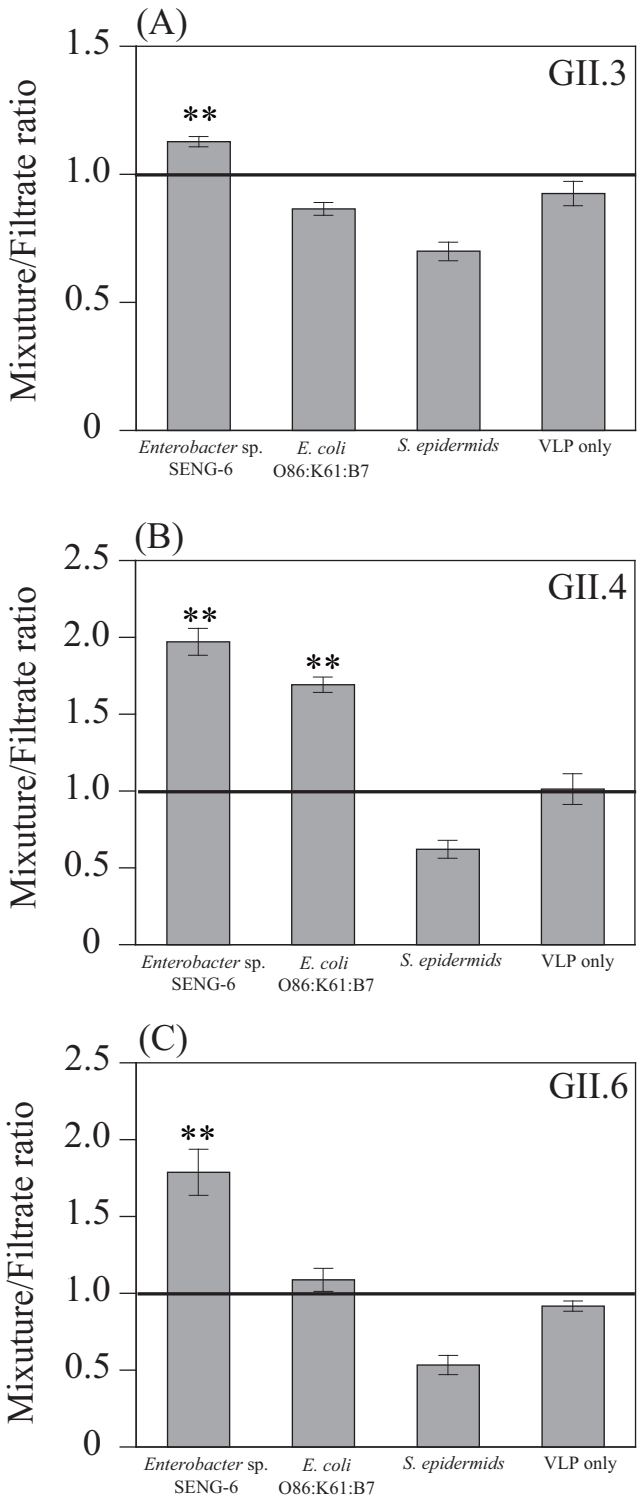
(A)
GII.3
Mixuture/Filtrate ratio
1.5
1.0
0.5
0
**
Enterobacter sp. SENG-6
E. coli O86:K61:B7
S. epidermids
VLP only
(B)
GII.4
Mixuture/Filtrate ratio
2.5
2.0
1.5
1.0
0.5
0
**
**
Enterobacter sp. SENG-6
E. coli O86:K61:B7
S. epidermids
VLP only
(C)
GII.6
Mixuture/Filtrate ratio
2.5
2.0
1.5
1.0
0.5
0
**
Enterobacter sp. SENG-6
E. coli O86:K61:B7
S. epidermids
VLP only

Figure 3

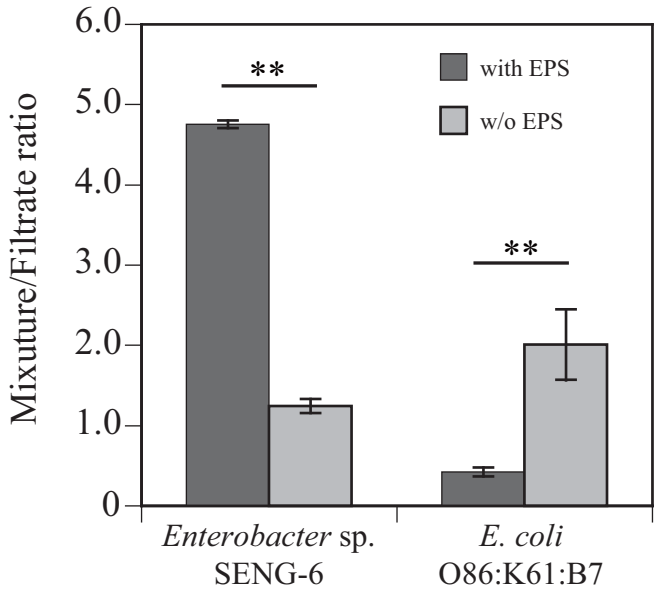
6.0
5.0
4.0
3.0
2.0
1.0
0
Mixuture/Filtrate ratio
**
**
with EPS
w/o EPS
Enterobacter sp.
SENG-6
E. coli
O86:K61:B7

Figure 4