Inflammatory responses and potencies of various lipopolysaccharides from bacteria and cyanobacteria in aquatic environments and water supply systems

Yumiko Ohkouchia, *, Satoshi Tajimab, 1, Masahiro Nomurab, 2, Sadahiko Itohb

* Department of Global Ecology, Graduate School of Global Environmental Studies, Kyoto University, Kyoto Daigaku-Katsura CI-2-233, Nishikyo-ku, Kyoto, 615-8540, Japan
1 Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, Kyoto Daigaku-Katsura CI-2-233, Nishikyo-ku, Kyoto, 615-8540, Japan

ABSTRACT

Inflammatory substances derived from indigenous bacteria in aquatic environments or water systems are of great concern. Lipopolysaccharides (LPSs), one of the major inflammatory substances in water, are usually identified using Limulus amoebocyte lysate (LAL) assay on the basis of their endotoxic activity, but endotoxin levels do not accurately represent their inflammatory potency in humans. In this investigation, the cellular endotoxin contents of pure-cultured bacteria/cyanobacteria, which are frequently detected in water sources and distribution systems, and of indigenous bacteria in a river and in biologically activated carbon (BAC) effluent, were investigated. The indigenous bacteria showed the highest endotoxin contents exceeding 10^3 EU/cell. The LPSs were then purified from those samples, and their inflammatory potencies were examined using a human monocytic cell line. The LPSs from Acinetobacter lwofii culture, the river water, and the BAC effluent sample revealed a unique cytokine secretion pattern; they induced both IL-8 and TNF-α more strongly than the other tested bacterial LPSs. These results suggest that natural bacterial/cyanobacterial flora in aquatic environments and water distribution systems have the potential to induce relatively strong inflammatory responses in humans; therefore, further accumulation of data on water quality from the perspective of not just endotoxins but inflammatory potency is needed.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

There are various naturally occurring substances that are toxic to humans in aquatic environments. Cyanobacterial toxins, such as microcystins, are one of the major toxic compounds in aquatic environments (Carmichael and Bent, 1981). Lipopolysaccharides (LPSs), which are the outer membranes of gram-negative bacterial or cyanobacterial cells, are also widely known to cause strong innate immune reactions in humans via Toll-like receptors on the cell surface. LPSs are also called endotoxins because of their biological activity. Information on endotoxin levels in various water samples has been accumulated. Just as an example, drinking water in Japan, China, and Australia ranges 1–104, 4–10, and 72–186 EU/mL, respectively, while the endotoxin levels in source water varied largely, but normally are in the range of 10–1000 EU/mL (Ohkouchi et al., 2007, 2009; Can et al., 2013; O’Toole et al., 2008). Rapala et al. (2002) also reported that water bloom sample with dense cyanobacteria reached at 3.8 × 10^4 EU/mL in Finland. Limulus amoebocyte lysate (LAL) assay is widely used to determine endotoxin levels in aquatic environments. This assay, based on coagulating reactions of LAL with endotoxins, is very sensitive and can detect small amounts of endotoxins. In this assay, the endotoxic activity of each bacterial LPS was expressed in terms of its endotoxic equivalence with the LPS of Escherichia coli-type strains. However, several studies have implied that there are big differences between the endotoxic activities of various bacteria determined by LAL assay.
and their toxic effects on human health (Hansen et al., 1999; Dehus et al., 2006). Our previous study indicated that the cellular assay system using a monocytic cell line, THP-1, could be useful for detecting changes in inflammatory potencies caused by bacterial communities in aquatic environments (Ohkouchi et al., 2012). However, the information on their inflammatory potencies as caused by indigenous bacteria in various water samples is still insufficient, as is that on their endotoxic activities.

In water supply systems, there are three sites where endotoxins can increase: at water sources as previously described (Rapala et al., 2002), in a treatment process using biologically activated carbon (BAC) (Ohkouchi et al., 2007; Can et al., 2013), and in distribution systems (Ohkouchi et al., 2009). In general, cyanobacteria are recognized as major contributors to the increase of endotoxins in water sources, while gram-negative bacteria could be a dominant factor in treatment processes and distribution systems. Ohkouchi et al. (2007) compared the endotoxic activities derived from Microcystis aeruginosa, Synechococcus sp. and E. coli cultured in the laboratory. The results showed that the Synechococcus sp. could be a major contributor to endotoxin increase in the Lake Biwa - Yodo River Basin due to their abundance in the natural aquatic environment, especially in summer. The survey results also suggested that effluents from sewage treatment plants could be sources of endotoxin contamination other than cyanobacterial bloom in that basin. The second site for the increase of endotoxins could be BAC processes, which provide habitats for bacteria. Bacteria can multiply on granular activated carbon (GAC) by using nutrients in water. The proteobacteria, i.e. gram-negative bacteria, were found to be dominant inhabitants in the bacterial community created on the surface of BAC (Poitelon et al., 2010). In contrast, it is known that the bacterial populations in water distribution systems vary from system to system depending on their conditions. In many cases, the bacteria in biofilm accumulated inside the pipes or in tap water samples have been detected using culture techniques, but some researchers have applied DNA-based molecular techniques. Various species of gram-negative bacteria that belong to alpha-, beta-, and gamma-proteobacteria were frequently detected. For example, Hirata et al. (1993) detected Alcaligenes sp., Bacillus sp., Methyllobacterium sp., Pseudomonas sp., and Flavobacterium sp. in distributed water samples. Furuhata et al. (Furuhata, 2004) also reported that Methyllobacterium sp. and Pseudomonas sp. were dominant species in water samples taken at a hospital via a storage tank. Scott and Pepper (2010) identified frequently detected bacterial species in tap water samples in the United States as Sphingomonas sp., Acidovorax sp., Aquabacterium sp., and Acinetobacter sp. based on the homology of 16S rRNA sequences. The bacterial regrowth of these various species, except for Sphingomonas sp., whose membrane structure differs from typical LPSs (Kawasaki et al., 1994; Kawahara et al., 1999), could cause increases in endotoxin and inflammatory potency during water distribution. Therefore, identifying and prioritizing the major contributors to increases in endotoxin and inflammatory potency in water supply systems would help in the development of a novel strategy of water quality management.

This investigation focused on several bacterial or cyanobacterial strains which ubiquitously inhabit water sources or distribution systems to determine what kind of bacteria could contribute to an increase in inflammatory potencies as well as endotoxins in water supply systems. Firstly, the cellular LPS contents of each pure cultured bacteria/cyanobacteria or indigenous bacteria in river water or BAC effluent were compared. Secondly, their endotoxic activities and the inflammatory potencies of LPSs after purification were also compared using a monocytic cell line based on the levels and patterns of cytokine secretion to narrow down which strains can impact human health. Also, a possibility that contaminant substances contribute to inflammatory potencies by the purified LPSs pretreated with/without polymixin B was examined. Lastly, the importance of water quality in terms of inflammatory potency was discussed based on the data obtained.

2. Materials and methods

2.1. Bacterial/cyanobacterial culture conditions

The bacteria/cyanobacteria used for the LPS preparation and their cultivation conditions are listed in Table 1. These bacterial/cyanobacteria were chosen based on published data regarding their occurrence rates in water sources or distribution systems. Microcystis aeruginosa and Synechococcus sp., which are frequently detected in water sources (Rapala et al., 2002; Hoson et al., 2002), were cultured at 20 °C for 20–30 days with illumination of 1500–2500 lux at 12-h intervals using CB and C media, respectively. Gram-negative bacteria, except Escherichia coli NBRC 3301, which are frequently detected in tap water samples or in biofilm accumulated in distribution systems, were cultured at 20 °C for 4–12 days using a medium recommended by each culture collection. E. coli NBRC 3301 was cultured at 37 °C for 30 h using LB medium.

2.2. Water sampling

A 21.8 L volume of river water was taken from the Yodo River downstream of Osaka (N34.72.48.47, E135.51.30.37) in December 2010. The sampling site was adjacent to the intake area of a drinking water purification plant. A 20 L volume of effluent water from the BAC adsorption process was taken at the water purification plant on the Yodo River. The purification plant has coagulation-sedimentation, mid-ozonation, rapid sand filtration, post-ozonation, BAC, and chlorination processes. The containers used for water samplings were soaked with PyroCLEAN (Alerchek, Inc., Portland, ME, USA) for more than one hour to degrade and solubilize endotoxins attached on the inner surface and rinsed thoroughly with endotoxin-free Milli-Q water. The endotoxin-free Milli-Q water was produced using a Milli-Q Academic equipped

<table>
<thead>
<tr>
<th>Tested bacteria</th>
<th>Strain</th>
<th>Class</th>
<th>Proliferation site</th>
<th>Culture condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>NBRC 3301</td>
<td>γ-Proteobacteria</td>
<td>Water resources</td>
<td>LB broth, 37 °C, 30 h</td>
<td>(Rapala et al., 2002)</td>
</tr>
<tr>
<td>Microcystis aeruginosa</td>
<td>NIES-44</td>
<td>Cyanophyceae</td>
<td>Water resources</td>
<td>CB medium, 20 °C, 30 days</td>
<td>(Hoson et al., 2002)</td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>NIES-946</td>
<td>Cyanophyceae</td>
<td>Water resources</td>
<td>C medium, 20 °C, 20 days</td>
<td>(Scott and Pepper, 2010)</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>ATCC 49642</td>
<td>γ-Proteobacteria</td>
<td>Distribution systems</td>
<td>R2A broth, 20 °C, 5 days</td>
<td>(Yano et al., 2009)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>NBRC 12689</td>
<td>γ-Proteobacteria</td>
<td>Distribution systems</td>
<td>NBRC 802, 20 °C, 12 days</td>
<td>(Yano et al., 2009)</td>
</tr>
<tr>
<td>Aquabacterium commune</td>
<td>ATCC BAA-209</td>
<td>β-Proteobacteria</td>
<td>Distribution systems</td>
<td>ATCC 2361, 20 °C, 7 days</td>
<td>(Szita et al., 2007)</td>
</tr>
<tr>
<td>Acidovorax delafieldii</td>
<td>ATCC 17606</td>
<td>β-Proteobacteria</td>
<td>Distribution systems</td>
<td>Nutrient broth, 20 °C, 4 days</td>
<td>(Kalmbach et al., 1999)</td>
</tr>
<tr>
<td>Acinetobacter lwoffi</td>
<td>JCM 6840</td>
<td>γ-Proteobacteria</td>
<td>Distribution systems</td>
<td>Nutrient broth, 20 °C, 7 days</td>
<td>(Lee et al., 2010)</td>
</tr>
<tr>
<td>Methyllobacterium fujisawaense</td>
<td>NBRC 16843</td>
<td>α-Proteobacteria</td>
<td>Distribution systems</td>
<td>NBRC 352, 20 °C, 7 days</td>
<td>(Scott and Pepper, 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Furuhata et al., 2006)</td>
</tr>
</tbody>
</table>
with a BioPak cartridge as a final filter. The water sample was transported to our laboratory under refrigerated conditions at 4 °C.

2.3. Enumeration of bacterial cells

The bacterial cell counts cultured in the laboratory were determined by pour-plating using agar plates under the conditions recommended by each culture collection. The Microcystis cell count was determined by microscopic examination at x400 magnification after pressurization. The Synechococcus cell count was determined by epifluorescence microscopy observation of intrinsic red fluorescence under G-excitation. The culturable heterotrophic bacteria were enumerated on R2A agar plates at 20 °C for 16 h at 37 °C to degrade RNA in the samples, an equal volume of 90% phenol-water solution was added again and stirred vigorously. The THP-1 cells were stimulated with the pretreated LPSs at 500 EU/mL in the same way. The commercial LPS of E. coli O55:B5 (Sigma-Aldrich Japan, Tokyo, Japan) and the extracted LPS samples were dissolved in endotoxin-free Milli-Q water and filtered through a sterilized 0.2-μm syringe filter (DISMIC-25CS, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). After preincubation, the culture medium in each well was removed and 10 μL of fresh media was added. The THP-1 cells were stimulated with LPS samples for six hours by adding 10 μL LPS suspension (Ohkouchi et al., 2012).

2.4. LPS purification

The bacterial cells and Synechococcus sp. cultured in our laboratory were harvested by centrifugation at 8000 rpm for 10–20 min and then washed three times with sterilized 50 mM phosphate buffer (pH 7.2). Only Microcystis aeruginosa was harvested by centrifugation at 6000 g for 10 min and then washed three times with sterilized 15 mg/L NaHCO₃ solution. The Yodo River sample and the BAC effluent water sample were enumerated using R2A agar plates at 20 °C with 7 days incubation. The total bacterial cell counts were determined by epifluorescence microscopic enumeration under UV excitation after 4',6-diamidino-2-phenylindole (DAPI) staining.

2.5. Endotoxin determination

The endotoxins in pure bacterial/cyanobacterial culture, the water samples, and the purified LPSs were determined by kinetic-chromogenic LAL assay using Pyrochrome with Glucashield buffer (Seikagaku Biobusiness Corporation, Tokyo, Japan). The unintentional reaction with β-glucan remaining in the samples was blocked using Glucashield buffer. LPS purified from Escherichia coli strain O113:H10 (Seikagaku Biobusiness Corporation, Tokyo, Japan) was used for calibration, and the results were given in endotoxin units (EU). Each sample was diluted with endotoxin-free Milli-Q water. Pipette chips and 96-well microplates guaranteed to be endotoxin-free were used for the assay.

For determination of endotoxin in pure culture, the culture media samples were taken at late logarithmic growth phases. The endotoxins of media themselves for cultivation in Table 1 were also determined before inoculation. The known weight of the purified LPS samples were dissolved in sterilized endotoxin-free Milli Q water, and then the endotoxins were also determined in the same way.

2.6. Cell culture

A monocytic cell line, THP-1 (JCRB0112.1), purchased from the Japanese Collection of Research Bioresources Cell Bank, was cultured in RPMI1640 medium with 5% heat-inactivated FBS. All culture flasks were incubated at 37 °C in humidified 5% CO₂.

2.7. Endotoxin stimulation

The non-adherent THP-1 cells were suspended in each medium at 1.0 × 10⁵ cells/mL. Aliquots of 100 μL cell suspensions were seeded in flat-bottomed 96-well culture plates and were incubated for three hours prior to LPS stimulation. The commercial LPS of E. coli O55:B5 (Sigma-Aldrich Japan, Tokyo, Japan) and the extracted LPS samples were dissolved in endotoxin-free Milli-Q water and filtered through a sterilized 0.2-μm syringe filter (DISMIC-25CS, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). After preincubation, the culture medium in each well was removed and 100 μL of fresh media was added. The THP-1 cells were stimulated with LPS samples for six hours by adding 10 μL LPS suspension (Ohkouchi et al., 2012).

2.8. Contaminant assay by blocking endotoxin activity

Polymixin B, cationic polypeptide, can bind to mono or diphosphogroup of the lipid A (Coyne and Fenwick, 1993), and so it is often used to neutralize biological activity of LPSs. The inhibition of cytokine secretions by addition of polymixin B can exclude the possibility that contaminants remaining in the purified samples contribute to cytokine production. The E. coli O55:B5 LPS (Sigma-Aldrich Japan), the LPSs extracted from Acinetobacter lwofii JCM 6840, the river water, and the BAC effluent samples (5000 EU/mL) were pretreated with 50 μg/mL polymixin B sulphate (Sigma Aldrich Japan) for 1 h at room temperature. Then, THP-1 was stimulated with the pretreated LPSs at 500 EU/mL in the same way as described above.

2.9. Determination of cytokine levels and cell counts

After stimulation with LPS samples, the culture supernatants were collected and stored at −80 °C until cytokine determination. An aliquot of 100 μL fresh medium was added to each well again, and the cells were incubated for 30 min. Then, viable cell counts were determined using Cell counting Kit-8 (Dojin Laboratories, Tokyo, Japan). The cytokines, TNF-α and IL-8, were measured by indirect sandwich ELISA assays (Diaclone, Gen-Probe Inc., San Diego, CA, USA) following the manufacturer’s instructions.

2.10. Statistical analysis

Data were compared by t-test using GraphPad Prism version 5.0 (GraphPad Prism Inc., San Diego, CA, USA), and significant differences were determined with a level of p < 0.05. Principal component analysis was also applied to data set of 3 variables (cellular
endotoxin contents, two types of cytokine secretions at LPS doses of 1000 EU/mL) obtained from 11 different LPS samples using the Microsoft Excel add-in software, Mac Taken ver. 1.0a (ESUMI Co., Ltd., Tokyo, Japan).

3. Results and discussion

3.1. Comparison of endotoxic activities derived from different bacterial strains and water samples

The endotoxic activities of the pure bacteria/cyanobacteria strains cultured in our laboratory were determined before LPS extraction. The results in terms of endotoxins per cell in each culture medium are compared in Fig. 1. The endotoxin in each cultivation medium was negligible less than 0.23 EU/mL. The endotoxic activities of indigenous bacteria in river water/BAC effluent samples are also shown in Fig. 1. The endotoxic activities per cell were calculated by dividing the endotoxins determined by LAL assay by the total cell counts in the samples determined by DAPI staining, because LPSs on cell surfaces exhibit endotoxic activity regardless of the viability of bacterial cells. Aquabacterium commune and Pseudomonas aeruginosa showed the highest endotoxic activities, greater than 10^(-3) EU/cell. The endotoxic activities per cell of Acinetobacter lwofii, Synechococcus sp., Pseudomonas fluorescens, Microcystis aeruginosa, Acidovorax delafieldii, and Escherichia coli followed in descending order. In contrast, significantly lower endotoxic activities were detected in Methylobacterium fujisawaense, less than 10^(-5) EU/cell.

The endotoxin levels of the river water and the BAC effluent were found to be 440 and 13 EU/mL, respectively. The endotoxic activity per cell of indigenous bacteria in the BAC effluent water was slightly higher than that in the river water (river water 1.1 x 10^(-3); BAC effluent 1.5 x 10^(-3) EU/cell). Both water samples were taken in the winter season; therefore, gram-negative bacteria rather than cyanobacteria were considered to be major contributors to the endotoxic activities. This result showed that the cellular endotoxin content was somewhat increased in the BAC effluent, whereas 97% of endotoxins in the river water regarded as source water were removed through rapid sand filtration and ozonation-BAC processes. The increase can be attributed to the shift in the bacterial community during treatment processes, especially the BAC adsorption process, which is known to be a place for bacterial regrowth. Kasuga et al. (2011) reported that the bacterial community structures in the BAC can shift with the maturing process. However, the water purification plant where we conducted sampling replaced a subset, not all, of the BAC columns with new GAC every 5 years in order to attain constant organic removal. Therefore, the effect of the operation period of BAC does not need to be taken into account in this case. The result also indicates that bacteria ubiquitously present in the water source or even after the water has undergone drinking-water treatment have relatively high endotoxic activities.

Based on the comparison of the cellular endotoxin contents of each bacteria/cyanobacteria, there is a possibility that Aquabacterium commune, Pseudomonas spp., and Acinetobacter lwofii, which exhibited endotoxin contents around 10^(-3) EU/cell, may be major contributors to endotoxin increases that occur during water distribution. Two Pseudomonas spp. strains were frequently detected in biofilm samples or tap water samples in many countries (Yano et al., 2009; Szita et al., 2007). Additionally, Kalimbach et al. (1999) reported that Aquabacterium commune is a dominant and ubiquitous community member in biofilms accumulated in several distribution systems in Hamburg, Berlin, Mainz, and Stockholm, which had different water sources and treatment processes. Acinetobacter lwofii, which is known as an opportunistic pathogen, was also widely detected in water supply systems (Scott and Pepper, 2010). If these ubiquitously present bacteria, which have potent endotoxic activity, multiply in water distribution systems, significant elevations of endotoxin levels in water could occur.

3.2. LPS purification from pure bacterial cultures, river water, and BAC effluent

Table 2 shows the changes in endotoxic activities and dry weights during the LPS purification steps from the pure bacterial cultures and the indigenous bacterial cells in the river water or the BAC effluent samples. The results showed that the endotoxic activities per dry weight were not always increased by purification in the case of pure bacterial cultures. Theoretically, the total endotoxic activities per dry weight should have increased as the purity increased due to the removal of many of the coexisting cellular components. In fact, the dry weights after purification were largely reduced by 0.8–10%. However, the total endotoxins were increased in some cases in pure culture samples after phenol extraction, which destroys cell membranes and removes proteinaceous compounds contained in bacterial cells by denaturing. In contrast, the endotoxic activities per unit weight of indigenous bacteria in water samples were increased step-by-step during purification. The different behaviors of the endotoxic activities in the environmental samples from the cultured samples during purification might arise due to changes in the association state of the LPSs and in the damage to the cell membrane, or due to a non-specific LAL reaction with some components contained in the bacterial cultures, but the exact reason is still unclear. An additional data accumulation would be needed.

The endotoxic activities per dry weight in the purified LPSs are compared in Fig. 2. Synechococcus sp. exhibited the highest endotoxic activity of 50 EU/µg, and the activities of Aquabacterium commune and Escherichia coli followed at levels greater than 10 EU/µg. Generally, the endotoxic activities of purified LPSs from Escherichia coli were reported in the range of 5–10 EU/µg (Anderson et al., 2003; Bernardova et al., 2008). The endotoxic activity of the purified LPS from E. coli NBRC 3301 cultured in our laboratory was consistent with that value, and this consistency suggests that the purification procedure was adequate.

The endotoxic activity of the LPS purified from the river water sample was comparable with the LPSs purified from two Pseudomonas species. In contrast, the LPS purified from the BAC effluent
sample showed much lower endotoxic activity, less than 0.1 EU/ng. Also Microcystis aeruginosa LPS revealed much lower endotoxic activity than E. coli LPS as reported by Bláhová et al. (Bláhová et al., 2013).

3.3. Comparison of inflammatory responses caused by the purified LPSs

The IL-8 and TNF-α secretions from THP-1 after six hours stimulation with each purified LPS are presented in Fig. 3 and Fig. 4, respectively. The cell counts of THP-1 after six hours stimulation showed no significant change in the examined range of LPSs purified from different lab-cultured bacteria/cyanobacteria. Each bacterial/cyanobacterial LPS induced different potencies and patterns of inflammatory responses in THP-1. Most LPSs induced higher IL-8 secretions than TNF-α, whereas only Acinetobacter Iwoffi LPS induced TNF-α secretions that were twice as high as IL-8. *Methyllobacterium fujisawaense* LPS, which showed very little endotoxic activity, induced IL-8 at 36 pg/10⁶ cells, even at 0.2 EU/mL. Because of its extremely low endotoxic activities, the LPS from *Methyllobacterium fujisawaense* was excluded from the following analyses. The IL-8 secretion levels caused by the other LPSs were categorized into three groups: the first group including Acinetobacter Iwoffi and Microcystis aeruginosa showed high levels of IL-8 secretion over 1000 pg/10⁶ THP-1 cells at a dose of 1000 EU/mL. The second group including Escherichia coli and Synechococcus sp. showed moderate IL-8 secretions (approximately 100–400 pg/10⁶ cells) at a 1000 EU/mL dose. The last group including two Pseudomonas sp., *Aquabacterium commune*, and *Acidovorax delafeldii* exhibited few IL-8 secretions over the entire endotoxin range. Regarding the two Pseudomonas spp. and *Aquabacterium commune*, very slight increases in IL-8 secretion were observed at the extremely high endotoxin dose of 10000 EU/mL, but no significant dose–response relationship was observed.

As described previously, the TNF-α secretions were much lower than the IL-8 secretions in most bacterial samples other than Acinetobacter Iwoffi LPS. These results are consistent with previous reports concluded that gram-negative bacteria more strongly induced IL-10-, IL-8-, and IL-6-type cytokines than gram-positive bacteria, while gram-positive bacteria induced more of other types of cytokines such as IFN-γ, TNF-α, and IL-1β (Skovbjerg et al., 2010). However, only Acinetobacter Iwoffi showed greater TNF-α secretion in the examined endotoxin range. Erridge et al. (2007) reported that Acinetobacter baumannii and Acinetobacter ‘genomespecies 9’ LPSs showed high potential to induce IL-8 and TNF-α in differentiated THP-1 cells. They indicated that stimulation with Acinetobacter LPSs induced significantly higher TNF-α secretion even at 1 ng/mL LPS. They did not mention the levels of endotoxic activity of the Acinetobacter LPSs in that research, but they explained that Acinetobacter baumannii LPSs exhibited endotoxic activity and cytokine secretions equal to those of *Escherichia coli* LPS. In our investigation, Figs. 3 and 4 clearly show that the purified *A. Iwoffi* LPSs induced 8- and 20-times higher secretions of *E. coli* LPS at a dose of 1000 EU/mL, although *A. Iwoffi* LPS had one-tenth the endotoxic activity per dry weight of *E. coli* LPS as shown in Fig. 2. Acinetobacter spp. including *A. Iwoffi* are known not just as opportunistic pathogens but also ubiquitous inhabitant in biofilm in water distribution systems. (Scott and Pepper, 2010; Vaz-Moreira et al., 2013; Kelly et al., 2014). This result suggests that inflammatory potency can increase with the regeneration of Acinetobacter sp. in distribution systems even though the endotoxic activity remains low.

3.4. Comparison of inflammatory potencies of river water and BAC effluent

The IL-8 and TNF-α secretions from THP-1 stimulated with the LPSs purified from the river water and the BAC effluent samples are also presented in Figs. 3 and 4, respectively. Recently data on endotoxin levels in various aquatic environments has been collected all over the world. For example, several studies reported that endotoxin levels in drinking water derived from treated surface water were in the range of 1–10 EU/mL (Ohkouchi et al., 2009; Can et al., 2013). Usually the endotoxin levels in surface water were a few hundred EU/mL, but they rose above 1000 EU/mL if the surface water was impacted by effluents from sewage treatment plants.

<table>
<thead>
<tr>
<th>Bacteria/water samples</th>
<th>Harvested</th>
<th>After phenol extraction</th>
<th>Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endotoxin (EU)</td>
<td>Dry weight (mg)</td>
<td>Endotoxin (EU)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>4.1 × 10⁴</td>
<td>280</td>
<td>1.48 × 10⁻¹</td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td>3.9 × 10⁵</td>
<td>347</td>
<td>1.12 × 10⁻¹</td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>1.4 × 10⁶</td>
<td>194</td>
<td>7.12 × 10⁻¹</td>
</tr>
<tr>
<td>River water</td>
<td>9.6 × 10⁶</td>
<td>203</td>
<td>4.72 × 10⁻¹</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>1.9 × 10⁷</td>
<td>516</td>
<td>3.67 × 10⁻⁸</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>9.3 × 10⁹</td>
<td>339</td>
<td>2.74 × 10⁻⁸</td>
</tr>
<tr>
<td><em>A. commune</em></td>
<td>6.2 × 10⁵</td>
<td>106</td>
<td>5.84 × 10⁻⁸</td>
</tr>
<tr>
<td><em>A. delafeldii</em></td>
<td>2.0 × 10⁹</td>
<td>266</td>
<td>7.51 × 10⁻²</td>
</tr>
<tr>
<td><em>A. lwofii</em></td>
<td>1.4 × 10⁸</td>
<td>111</td>
<td>1.26 × 10⁻⁴</td>
</tr>
<tr>
<td><em>M. fujisawaense</em></td>
<td>7.3 × 10⁷</td>
<td>140</td>
<td>5.20 × 10⁻⁴</td>
</tr>
<tr>
<td>BAC effluent</td>
<td>4.3 × 10⁵</td>
<td>6.0</td>
<td>7.13 × 10⁻²</td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of endotoxin contents based on dry weight in purified LPS samples from pure-cultured bacteria/cyanobacteria and water samples.
(Ohkouchi et al., 2007) or cyanobacterial bloom (Rapala et al., 2002). Based on our result, relatively strong cytokine secretions caused by stimulation with river water and BAC effluent were observed at normal endotoxin levels in an aquatic environment, around 100 EU/mL (10^4–10^5 cells/mL as total cells). Of special note is that the BAC effluent LPS induced a moderate inflammatory response (approx. 250 pg/10^6 THP-1 cells) even at 10 EU/mL, which is the same endotoxin level as in drinking water. At LPS doses above 500 EU/mL, cytokine secretions were increased in a dose-dependent manner by stimulation with the river water LPS, whereas cytokine levels did not increase significantly by stimulation with the BAC effluent LPS.

Principal component analysis (PAC) was then applied to group the purified LPS samples, except Methylobacterium fujisawaense, based on their cytokine profiles at a dose of 1000 EU/mL and their cellular endotoxin contents. The endotoxin dose of 1000 EU/mL was chosen by considering actual endotoxin levels in aquatic environments. The results of the analysis are presented in Fig. 5. The PCA score plot showed that the river water LPS, the BAC effluent LPS, and Acinetobacter lwofii LPS formed a group distinct from the other bacterial/cyanobacterial LPSs. This group represented bacteria that induced strong cytokine secretions in human monocytes although their endotoxic activities per cell were moderate (approx. 10^3 EU/cell). In contrast, Aquabacterium commune LPS exhibited very high endotoxin activity but very low induction of inflammatory responses and was distant from the other LPSs. These results prove that Acinetobacter lwofii and indigenous bacteria in aquatic environments have relatively high cellular endotoxin contents and reveal a common pattern by which significant cytokine secretions can be induced in human cells.

Thus, LPSs purified from both the river water and the BAC effluent induced much stronger inflammatory responses at doses of 10–5000 EU/mL than did the LPSs of most lab-cultured bacteria/cyanobacteria. At the same time, both water samples induced very high TNF-α secretions as well as IL-8 secretions like Acinetobacter LPS rather than the LPSs purified from other lab-cultured bacteria/cyanobacteria. The reasons for such stronger responses by stimulation with the indigenous bacterial LPSs have not yet been identified, but there are several possibilities including the following:

1) In aquatic environments, bacteria like Acinetobacter lwofii, which have LPSs that cause strong inflammatory responses in human monocytes, are present in abundance; 2) low water temperature or low nutritional/osmotic conditions in aquatic environments can increase the potency of indigenous bacterial LPSs; and 3) some substances that enhance inflammatory responses exist in those purified samples. The third possibility will be discussed in a later section. Kawahara et al. (2002) and Dentovskaya et al. (2008) reported that the changes in the structure and inflammatory potency of Yersinia pestis LPS depend on the growth temperature. According to their results, the purified LPS from Y. pestis grown at 25 or 27 °C could induce stronger TNF-α secretion in mouse and human macrophages than that grown at 37 °C. They
also proved that hexaacylated lipid-A, which is synthesized in bacteria only grown at low temperatures, contributes to the potentiation of inflammatory responses. In addition, Ernst et al. (2006) indicated that the LPS structure of Pseudomonas aeruginosa change depending on temperature for cultivation. Especially, hexa-acylated LPS was dominant at 15 °C while after growth at elevated temperature penta-acylated LPS was dominant. They suggest that the dominance of hexa-acylated LPS at lower temperature could be attributed to lower deacylase activity, which is deficient in one-third of clinical isolates. In our investigation, both the water samples from the river and from the BAC process were taken in winter when the water temperature was below 10 °C, whereas all bacteria/cyanobacteria except for Escherichia coli were cultured at 20 °C in the laboratory in order to make conditions closer to the natural aquatic environment. Based on the above information, as a consequence of a decrease in deacylase activity caused by water temperature or other environmental conditions in the aquatic environment, LPS structures of indigenous bacteria may shift toward the more potent.

3.5. Effect of contaminants in purified LPSs on TNF-α secretions

As discussed above, there is a possibility that contaminated substances that remained in the purified LPSs of the river water and the BAC effluent contribute to the elevation of TNF-α secretions. In order to exclude this possibility, the cytokine secretions from THP-1 stimulated with each LPS with/without polymixin B as an inhibitor of endotoxins were evaluated. The results are presented in Fig. 6.

Except for the LPS samples from the BAC effluent, the addition of polymixin B significantly inhibited both cytokine secretions. This result proved that the LPS components in the purified sample from river water greatly contributed to the cytokine elevations, including TNF-α, as with the Acinetobacter LPS. In contrast, the cytokine levels stimulated with the BAC effluent LPS were not affected by the addition of polymixin B. The contribution of contaminant substances such as peptidoglycan or lipoteichoic acid to the strong cytokine secretions was suspected. However, the contribution of contaminant substances in the BAC effluent LPS was unlikely because several researches reported that more than 100-fold amounts of contaminated substances such as peptidoglycan or lipoteichoic acid were required to induce the same level of cytokine secretions with LPSs. Another reason that polymixin B failed to inhibit the secretions of both types of cytokines, IL-8 and TNF-α, in that sample may be the lower affinity of polymixin B with the LPSs in the BAC effluent. Kasuga et al. (2011) reported that different bacterial communities from those in the water sources were developed on the BAC surface installed after the ozonation process. Such a difference could have resulted in the diversity in the LPS chemical structures in the BAC effluent sample. At the same time, several researchers also suggested that the surrounding conditions such as the water temperature or osmosis can also contribute to the diversity of the chemical structures of LPSs in the environmental samples (Merino et al., 1998; Suomalainen et al., 2010). On the other hand, Coyne and Fenwick (1993) reported that the ability of polymixin B to inhibit TNF-α synthesis in macrophages had considerable variability. For example, they showed that polymixin B of 1 IU/mL completely inhibited TNF-α synthesis in macrophages in the presence of LPS from Escherichia coli B4:O11. The relative

Fig. 5. Principal component analysis of various LPS samples purified in this investigation. The first and second principal components explained 63.8% and 33.4% of the total variability, respectively.

Fig. 6. Effects of polymixin B addition as an inhibitor of endotoxin on cytokine secretion in the THP-1 cell line. IL-8 and TNF-α were determined after 6 h stimulation with LPS samples and with LPS samples pretreated with polymixin B, respectively. The cytokine data represent means with standard deviations of four independent assays. Asterisks indicate a significant difference in comparison to the samples without the polymixin B pretreatment.
TNF-α synthesis with polymyxin B to the one without polymyxin B was 25.3% for *Salmonella minnesota*, whereas much higher TNF-α syntheses were observed in the presence of LPSs from *S. Typhimurium* (96.5%), *Pseudomonas aeruginosa* (73.4%). They also proposed that the efficiency of inhibition was affected by the saturated carbon chains of LPSs and the phosphate groups of the lipid A core moiety. Based on the above information, it is more likely that the LPSs with various chemical structures in the BAC effluent led to a decrease in the inhibition ability of polymyxin B as a consequence of a lower capability to form complexes with LPSs.

### 4. Conclusions

Our results indicated that the LPSs of some bacteria that are ubiquitous in aquatic environments or distribution systems can induce stronger inflammatory responses in human cells than *E. coli* LPS, which is commonly used as a reference standard for endotoxin determination. Some of these LPSs, such as *Acinetobacter baumannii*, indigenous bacteria in river water and BAC effluent, also exhibited a unique response pattern to induce TNF-α secretions comparable to or higher than the IL-8 secretions, whereas most other LPSs induced more IL-8 than TNF-α. These results suggest that natural bacterial/cyanobacterial flora in aquatic environments and water distribution systems have the potential to induce relatively strong inflammatory responses in humans and that water quality monitoring based on endotoxin levels is not sufficient for evaluating health impacts. The discussion of the inflammatory potency of water samples is in its early stages (Wichmann et al., 2004; Bláhová et al., 2013; Marghani et al., 2014), whereas the health impacts of cyanobacteria/bacteria have been discussed for a long time from the perspectives of their pathogenicity, exotoxins, and drug resistance. A current climate change we are facing can affect bacterial/cyanobacterial flora in aquatic environments and thus their inflammatory potency in response to the flora shifts. An effort to accumulate data on the inflammatory potency of various water samples in aquatic environments, especially those used for recreation or tap water, should be continued.

### Acknowledgments

This research was supported by a GSGES Research Grant for Young Researchers 2011, Kyoto University.

### Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.02.003.

### References


Skovbjerg, S., Martiner, A., Hynso, L., Hesse, C., Olsen, I., Dewhirst, F., Tham, W., Wold, A.E., 2010. Gram-positive and gram-negative bacteria induce different...


