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

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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 *Limurus* 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 lwoffii* 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.

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 1–10, 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. *Limurus* 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

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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 the 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., *Methylobacterium* sp., *Pseudomonas* sp., and *Flavobacterium* sp. in distributed water samples. Furuhata et al. (Furuhata, 2004) also reported that *Methylobacterium* 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 bacteria/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 1
Bacterial strains tested in this investigation.

Tested bacteria	Strain	Class	Proliferation site	Culture condition	Reference
Escherichia coli	NBRC 3301	γ-*Proteobacteria*	–	LB broth, 37 °C, 30 h	
Microcystis aeruginosa	NIES-44	*Cyanophyceae*	Water resources	CB medium, 20 °C, 30 days	(Rapala et al., 2002)
Synechococcus sp.	NIES-946	*Cyanophyceae*	Water resources	C medium, 20 °C, 20 days	(Hoson et al., 2002)
Pseudomonas fluorescens	ATCC 49642	γ-*Proteobacteria*	Distribution systems	R2A broth, 20 °C, 5 days	(Yano et al., 2009)
Pseudomonas aeruginosa	NBRC 12689	γ-*Proteobacteria*	Distribution systems	NBRC 802, 20 °C, 12 days	(Yano et al., 2009) (Szita et al., 2007)
Aquabacterium commune	ATCC BAA-209	β-*Proteobacteria*	Distribution systems	ATTC 2261, 20 °C, 7 days	(Kalmbach et al., 1999)
Acidovorax delafieldii	ATCC 17606	β-*Proteobacteria*	Distribution systems	Nutrient broth, 20 °C, 4 days	(Lee et al., 2010)
Acinetobacter lwoffii	JCM 6840	γ-*Proteobacteria*	Distribution systems	Nutrient broth, 20 °C, 7 days	(Scott and Pepper, 2010)
Methylobacterium fujisawaense	NBRC 16843	α-*Proteobacteria*	Distribution systems	NBRC 352, 20 °C, 7 days	(Furuhata et al., 2006)