RESEARCH ARTICLE

Lipid IVa incompletely activates MyD88-independent Toll-like receptor 4 signaling in mouse macrophage cell lines

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The manuscript contains interesting novel observation that lipid IVa preferentially stimulates MyD88-associated NFkB signaling in mouse macrophage cell-lines. This information will be useful to those who investigate mechanisms of differential signaling adapter usage by the TLR4/MD2 LPS receptor.

Abstract

We investigated the difference in the effect of synthetic lipid A compounds on MyD88-dependent and -independent Toll-like receptor 4 (TLR4) signaling in mouse macrophage cells. At higher concentrations, *Escherichia coli*-type hexa-acylated lipid A 506, *Salmonella*-type hepta-acylated lipid A 516, the lipid A precursor lipid IVa and monophosphoryl lipid A induced similar levels of production of the MyD88-dependent cytokine IL-1β although their potencies varied, whereas the maximum production of the MyD88-independent cytokine RANTES induced by lipid IVa was less than 50% that of other lipid A compounds. A maximum level of NF-κB activation, which is involved in IL-1β gene transcription, was also induced to a similar level by these four lipid A compounds, while the maximum level of IFN-β promoter activity induced during MyD88-independent signaling was also less than 50% for lipid IVa stimulation compared with other lipid A compounds. Early IκBα phosphorylation activated by MyD88-dependent signaling was similarly induced by 506 and lipid IVa, whereas lipid IVa barely stimulated the phosphorylation of IRF3, a MyD88-independent transcription factor, although efficient phosphorylation was observed with 506 stimulation. These results indicate that lipid IVa has limited activity toward MyD88-independent signaling of TLR4, in macrophage cell lines, despite having efficient activity in the MyD88-dependent pathway.

Introduction

Lipopolysaccharide (LPS) is a structural component of Gram-negative bacteria outer cell wall membranes and triggers innate immune responses through a receptor complex consisting of CD14, Toll-like receptor 4 (TLR4) and MD-2 (Schletter *et al.*, 1995; Ulevitch & Tobias, 1995; Fujihara *et al.*, 2003; Miyake, 2004). LPS often causes fatal septic shock, which is the most common death in intensive care units, with a mortality rate of nearly 20% in the United States (Martin *et al.*, 2003).

Innate immune responses caused by TLR4 stimulation are mediated through two signaling pathways, called the MyD88-dependent and MyD88-independent pathways, which utilize adaptor proteins MyD88/TIRAP and TRIF/TRAM, respectively. The activation of the MyD88-dependent pathway leads to production of pro-inflammatory cytokines such as IL-1β and TNF-α (Kawai *et al.*, 1999),

whereas MyD88-independent pathway activation induces production of IFN-inducible cytokines such as RANTES and IFN-β (Hoebe *et al.*, 2003; Yamamoto *et al.*, 2003). Transcription of many pro-inflammatory cytokine genes is regulated by the transcription factor NF-κB. The early activation of NF-κB mediated through TLR4 depends on the MyD88-dependent pathway but its late activation involves both MyD88-dependent and -independent pathways (Kawai *et al.*, 1999; Hoebe *et al.*, 2003). The activation of both pathways induces phosphorylation of the NF-κB inhibitor IκBα, which triggers proteasome-dependent degradation of IκBα, leading to NF-κB activation (Hayden & Ghosh, 2012). On the other hand, activation of the MyD88-independent pathway induces phosphorylation of the transcription factor IRF3, which induces its nuclear translocation (Kawai & Akira, 2010).

LPS contains a hydrophobic domain known as lipid A, the active moiety of LPS, and its biological activity depends on

the chemical structure. The activity of synthetic lipid A compounds called 506 (hexa-acylated *E. coli*-type lipid A), 516 (hepta-acylated *Salmonella*-type lipid A), and lipid IVa (tetra-acylated lipid A precursor) has well been investigated (see review Raetz & Whitfield, 2002). While all compounds are highly active in mouse macrophage cells, lipid IVa and compound 516 show very little stimulatory activity and act as an antagonist in human macrophages (Tanamoto & Azumi, 2000; Muroi & Tanamoto, 2006). A structure–activity relationship analysis using well-defined synthetic lipid A compounds possessing naturally occurring structures revealed the importance of several structural features such as the presence of a 3-deoxy-D-manno-octulosonic acid moiety and an anomeric phosphate, lipid length, and acylation pattern (Gaekwad *et al.*, 2010). However, only a few studies have reported the differences in the effect of lipid A on the MyD88-dependent and MyD88-independent pathways. In this study, we investigated the activity of four synthetic lipid A-related compounds in MyD88-dependent and -independent signaling.

Material and methods

Reagents

The NF-κB-dependent luciferase reporter plasmid pc4-ELAM-L was constructed by inserting the BglII-ApaI fragment of pcELAM-L (Igarashi *et al.*, 2006) into the BglII-ApaI site of pcDNA4/TO (Life Technologies, Grand Island, NY). A luciferase reporter plasmid (pc4-hIFNβ-luc) containing the human IFN-β promoter was created by inserting the KpnI-ApaI fragment of pchIFNβ-luc, which was constructed by inserting the human IFN-β promoter region (−282 to +24) into the KpnI/HindIII site of pcELAM-L, into the KpnI-ApaI site of pc4-ELAM-L. Recombinant mouse LPS-binding protein (LBP) was prepared using the *Pichia* Expression System (Life Technologies) as described earlier (Muroi *et al.*, 2003). Antibodies against IκBα (C-21; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated (Ser$^{32/36}$) IκBα (5A5; Cell Signaling Technology, Danvers, MA), IRF3 (4962; Cell Signaling Technology), phosphorylated (Ser^{396}) IRF3 (4D4G; Cell Signaling Technology), and β-actin (AC-15; Sigma-Aldrich, St. Louis, MO) were used. Compounds 506, 516, and lipid IVa were from the Peptide Institute (Osaka, Japan), and a synthetic *E. coli*-type monophosphoryl lipid A (MPLA) was obtained from InvivoGen (San Diego, CA).

Cell culture

Mouse macrophage-like cell lines J774A.1 and RAW264.7-γNO(−) (both from ATCC) were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (V/V) heat-inactivated fetal bovine serum (Life Technologies), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). The NF-κB-dependent luciferase reporter cell line (J774-ELAM) and IFN-β promoter–reporter cell line (J774-IFN) stably carrying pc4-ELAM-L and pc4-hIFNβ-luc, respectively, were established as follows. After linearizing

pc4-ELAM-L and pc4-hIFNβ-luc with BglII and SspI, respectively, each plasmid was transfected into J774A.1 cells using X-treamGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany). Stable transfectants were selected for Zeocin resistance at a concentration of 1 mg mL^{-1}. These cell lines were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 1 mg mL^{-1} Zeocin, 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}).

Reporter assay

The luciferase reporter assay was performed as described elsewhere (Igarashi *et al.*, 2006; Ohnishi *et al.*, 2008). Briefly, J774-ELAM or J774-IFN cells (1–3 × 10^5/well) were plated in 12-well plates and on the following day washed twice with phosphate-buffered saline. Cells were then stimulated for 6 h with each lipid A compound in a serum-free medium (FreeStyle 293; Life Technologies) containing 100 ng mL^{-1} mouse LBP. Following stimulation, a lysis buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 5 mM EDTA, 40 mM β-glycerophosphate, 0.5% NP-40, 30 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol) containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) was added for cellular extract preparation. Reporter gene activity in the cellular extracts was then measured according to the manufacturer's instructions (Promega, Madison, WI). The cell viability was determined with the cellular extracts using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega), and the reporter activity was normalized to cell viability to compensate for differences in cell numbers and viabilities between wells.

Cytokine assay

IL-1β and RANTES production was measured as follows. J774A.1 cells (6–8 × 10^5/well) were plated in 12-well plates and washed twice with phosphate-buffered saline 6 h later. Cells were then stimulated for 16 h with each lipid A compound in a serum-free medium (FreeStyle 293; Life Technologies) containing 100 ng mL^{-1} mouse LBP. Following stimulation, the culture supernatant was collected for the cytokine assay, and cellular extracts were prepared by adding the lysis buffer mentioned above containing a protease inhibitor cocktail (Nacalai Tesque). IL-1β and RANTES concentrations were determined using ELISA kits (R&D Systems, Minneapolis, MN). Protein concentrations of the cellular extracts were determined using Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Waltham, MA), and the cytokine levels were normalized to these protein concentrations to compensate for differences in cell numbers and viabilities between wells.

Immunoblotting

J774A.1 or RAW264.7γNO(−) cells (1–3 × 10^6/well) were plated in 6-well plates and washed twice with phosphate-buffered saline 16 h later. Cells were then stimulated with

each lipid A compound in a serum-free medium (FreeStyle 293; Life Technologies) containing 100 ng mL^{-1} mouse LBP. Following stimulation, whole-cell extract was prepared by adding the lysis buffer mentioned above containing 0.4 M NaCl and a protease inhibitor cocktail (Nacalai Tesque), followed by immunoblotting as described previously (Muroi & Tanamoto, 2011).

Results

Cytokine production

We first evaluated the effect of lipid A compounds on the productions of IL-1β and RANTES, which are prototypical MyD88-dependent and MyD88-independent cytokines, respectively (Bjorkbacka *et al.*, 2004; Hirotani *et al.*, 2005). We selected four well-known lipid A-related compounds, *E. coli*-type hexa-acylated lipid A 506, *Salmonella*-type hepta-acylated lipid A 516, lipid A precursor lipid IVa and *E. coli*-type hexa-acylated MPLA. All of these lipid A compounds are known to activate mouse macrophage cells.

The mouse macrophage cell line J774A.1 was stimulated with each of these lipid A compounds for 16 h in the presence of mouse LBP under serum-free conditions, and the amounts of IL-1β and RANTES in the culture supernatant were determined by ELISA. All of the lipid A compounds examined stimulated IL-1β production in a concentration-dependent manner, and the activity (potency) in terms of EC$_{50}$ values varied notably (Fig. 1a). IL-1β production was induced most efficiently with 506, followed by 516, MPLA, and lipid IVa. While compound 516 and MPLA required approximately 10-fold and 100-fold higher concentrations, respectively, to stimulate IL-1β production to a level comparable to that produced by compound 506, similar levels of maximal IL-1β production (maximal activity; efficacy) were achieved by these three lipid A compounds. Lipid IVa required the highest concentration to attain a similar level of IL-1β production compared with other lipid A compounds but at 1000 nM, the production reached the level comparable to the maximum levels achieved by the other three lipid A compounds.

RANTES production by J774A.1 cells was also induced by these lipid A compounds in a concentration-dependent manner with compound 506 again showing the highest potency followed by compound 516 and MPLA, which had almost equivalent activity (Fig. 1b). As with IL-1β production, these lipid A compounds showed a similar maximal activity. Strikingly, lipid IVa induced RANTES production at 10 nM but its maximal activity was found to be less than 50% that of the other lipid A compounds regardless of increased concentration, indicating a distinctive effect of lipid IVa.

Transcriptinal activity

The activation of the MyD88-dependent and MyD88-independent pathways leads to the activation of transcription factors NF-κB and IRF3, respectively (Kawai & Akira, 2010). Thus, we next examined the activity of these transcription
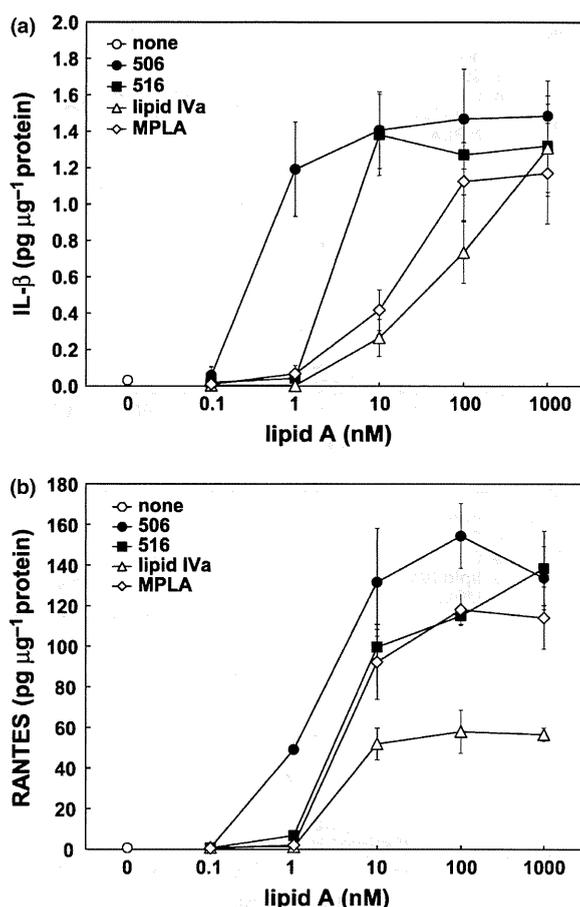

Fig. 1 IL-1β and RANTES production in response to lipid A compounds. The mouse macrophage cell line J774A.1 was stimulated with compound 506 (●), 516 (■), lipid IVa (△), and MPLA (◇) for 16 h in the presence of mouse LBP under serum-free conditions. The amounts of IL-1β (a) and RANTES (b) in the culture supernatant were determined by ELISA and were normalized to protein concentrations of cellular extracts as described in Materials and Methods. Values are the means ± SEM from seven (IL-1β) and three (RANTES) independent experiments.

factors in response to the lipid A compounds by measuring NF-κB-dependent ELAM-1 promoter (Schindler & Baichwal, 1994) and IRF3-dependent IFN-β promoter (Honda & Taniguchi, 2006) activity. J774A.1 cells stably carrying either an ELAM-1 promoter-luciferase reporter gene or IFN-β promoter-luciferase reporter gene were stimulated with each of the lipid A compounds for 6 h in the presence of mouse LBP under serum-free conditions, and the luciferase activity was determined (Fig. 2). For both promoter activities, compound 506 showed the highest potency, followed by compound 516 and MPLA, which had almost equivalent activity. A similar level of maximal activity was induced by these lipid A compounds, although the activity of compound 516 and MPLA toward the IFN-β promoter was slightly lower than that of compound 506 (Fig. 2b). Lipid IVa up to 10 nM stimulated NF-κB-dependent reporter activity to levels
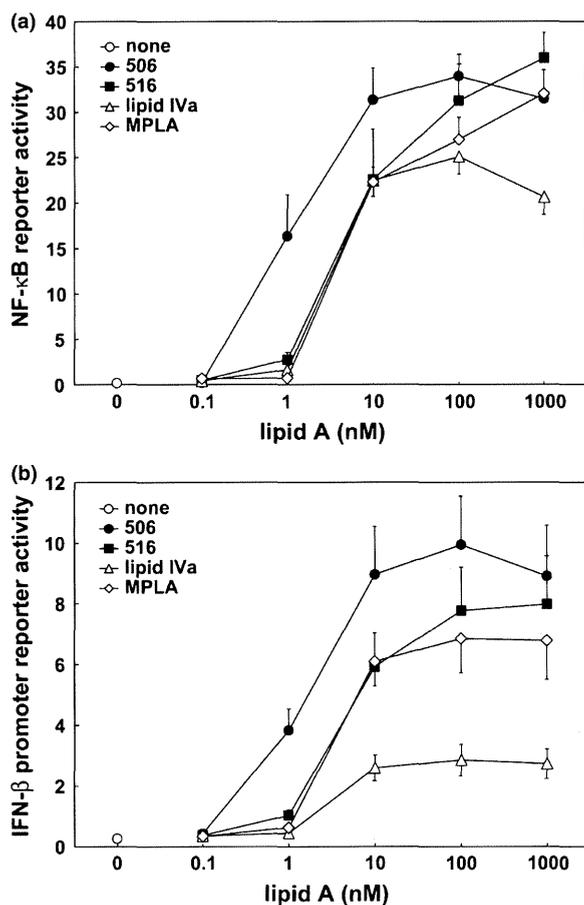
Fig. 2 NF-κB and IFN-β promoter activity in response to lipid A compounds. J774A.1 cells stably carrying either an NF-κB- (a) or IFN-β promoter-dependent luciferase reporter gene (b) were stimulated with compound 506 (●), 516 (■), lipid IVa (△), and MPLA (◇) for 6 h in the presence of mouse LBP under serum-free conditions. The luciferase activity was determined, and the reporter activity was normalized to cell viability. Values are the means ± SEM from five (NF-κB) and eight (IFN-β promoter) independent experiments.

similar to those produced by compound 516 and MPLA, and the maximal activity was approximately 70–78% of that of the other lipid A compounds (Fig. 2a). On the other hand, as observed for RANTES production, lipid IVa increased IFN-β promoter activity at 10 nM and its maximal activity was less than 50% that of the other lipid A compounds regardless of increased concentration.

Activation of transcription factors

Given that activation of NF-κB and the IFN-β promoter involves phosphorylation of IκBα and IRF3, respectively, we next compared the effect of compound 506 and lipid IVa on phosphorylation of these proteins in J774A.1 cells (Fig. 3a and b). J774A.1 cells were stimulated with 1 μM of a given lipid A compound for the indicated time in the presence of mouse LBP under serum-free conditions, and the

phosphorylation of IκBα and IRF3 was determined by immunoblotting using antibodies specific to the phosphorylated forms of these proteins (Fig. 3a). IκBα phosphorylation was observed as early as 5 min following lipid IVa stimulation (Fig. 3a top), and in the following time periods, almost similar levels of the phosphorylation were observed in both stimulations, although the level was decreased at 15 min due to IκBα protein degradation (Fig. 3a second). On the other hand, although IRF3 phosphorylation was clearly observed with 30 min of compound 506 stimulation, phosphorylation in response to lipid IVa was barely detectable (Fig. 3a third) despite comparable expression levels of IRF3 protein (Fig. 3a fourth). The level of β-actin was determined as an indicator of cell viability and was not affected by these stimulations (Fig. 3a bottom).

We also observed IκBα and IRF3 phosphorylation at 90 min following stimulation with 0.1–1000 nM compound 506 and lipid IVa (Fig. 3b). At 1 nM, compound 506 induced higher levels of IκBα phosphorylation compared with lipid IVa; however, a similar phosphorylation level (Fig. 3b top) was observed for both compounds at higher concentrations. The level of IκBα expression was comparable for both treatments (Fig. 3b second). IRF3 phosphorylation was also observed with 10 nM compound 506 stimulation but was barely detectable in response to lipid IVa stimulation (Fig. 3b third) despite the presence of comparable IRF3 expression levels (Fig. 3b fourth). β-actin levels were again not affected by these stimulations (Fig. 3b bottom).

To exclude the possibility that the observed inability of lipid IVa to stimulate IRF3 phosphorylation is unique to J774A.1 cells, the effect of compound 506 and lipid IVa on IκBα and IRF3 phosphorylation was also examined in another mouse macrophage cell line RAW264.7 γNO(−). As was observed in J774A.1 cells, both compounds induced equivalent levels of IκBα phosphorylation, but IRF3 phosphorylation was markedly lower for lipid IVa stimulation (Fig. 3c and d), indicating that the lack of lipid IVa activity was common to both cell lines.

While IRF3 phosphorylation is activated by TLR4 stimulation only through the MyD88-independent pathway, NF-κB activation is mediated through both MyD88-dependent and -independent pathways. To compare the effect of compound 506 and lipid IVa on MyD88-dependent signaling, we examined IκBα phosphorylation induced at 10 min following stimulation because at this early time point, only MyD88-dependent signaling is involved in IκBα phosphorylation. J774A.1 cells were first incubated with the proteasome inhibitor lactacystin to prevent IκBα degradation and then stimulated in the presence of mouse LBP under serum-free conditions with each of the lipid A compounds for 10 min, and IκBα phosphorylation was examined (Fig. 4). Although lactacystin did not inhibit IκBα degradation completely, IκBα levels were comparable for 506 and lipid IVa stimulation (Fig. 4 middle). Both compound 506 and lipid IVa induced a similar level of IκBα phosphorylation in a concentration-dependent manner (Fig. 4 top), indicating that both compounds efficiently activate MyD88-dependent signaling. The level of β-actin was not affected by these stimulations (Fig. 4 bottom). A similar result was obtained when MG-132,
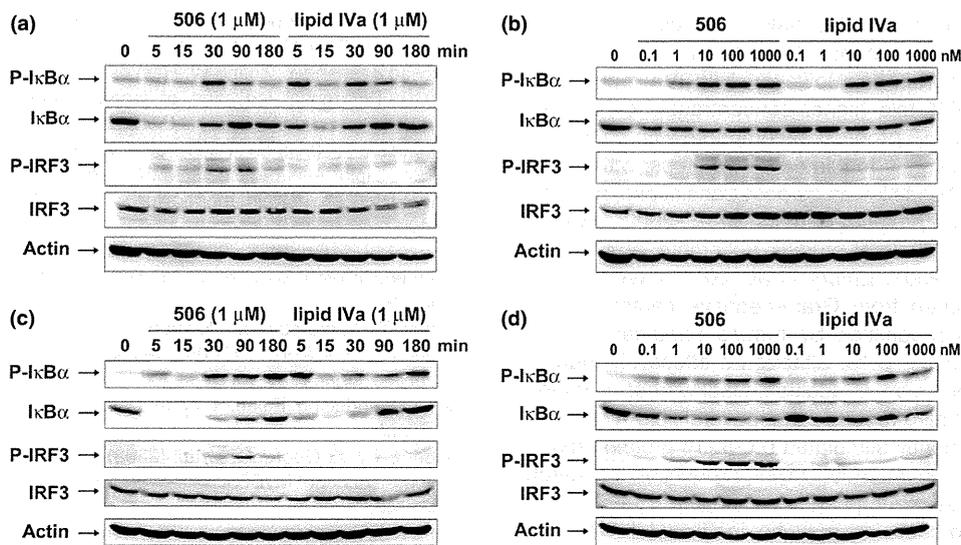
Fig. 3 IκBα and IRF3 phosphorylation in response to lipid A compounds. J774A.1 (a, b) and RAW264.7 γNO(−) (c, d) cells were stimulated with 1 μM compound 506 or lipid IVa for the indicated time (a, c) or with the indicated concentrations of compound 506 or lipid IVa for 90 min (b, d) in the presence of mouse LBP under serum-free conditions. Phosphorylated IκBα (P-IκBα), IκBα, phosphorylated IRF3 (P- IRF3), IRF3 and β-actin were detected by immunoblotting. Data shown are representative of 2–3 independent experiments with similar results.
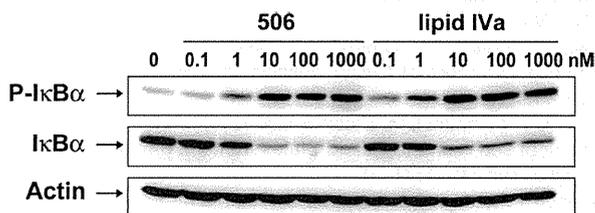

Fig. 4 IκBα phosphorylation at early time points. J774A.1 cells were treated with 20 μM lactacystin for 30 min and then stimulated with compound 506 and lipid IVa for 10 min in the presence of mouse LBP under serum-free conditions. Phosphorylated IκBα (P-IκBα), IκBα, and β-actin were detected by immunoblotting.

another proteasome inhibitor, was used instead of lactacystin (data not shown).

Taken together, these results indicate that lipid IVa has limited ability to activate the MyD88-independent pathway of TLR4 signaling despite having efficient activity in MyD88-dependent signaling.

Discussion

The biological activity of lipid A compounds has widely been studied by examining production of cytokines, including TNF-α, and the structural requirement of the lipid A molecule for this activity has been reported in studies using lipid A preparations isolated from Gram-negative bacteria (Rietschel *et al.*, 1994; Darveau, 1998). However, these preparations often have heterogeneous lipid A structures and can be contaminated with other inflammatory bacterial cell wall components, which makes interpretation of the structure–activity relationship study difficult. Moreover, few

studies reported a difference in the activity of lipid A between the MyD88-dependent and -independent signaling pathways. Gaekwad *et al.* (2010) reported the structure–activity relationship of chemically synthesized hexa-acylated or hepta-acylated lipid A compounds derived from *E. coli*, *Salmonella minnesota*, *Neisseria meningitides*, and *Salmonella typhymurium* by examining various cytokines induced separately through these pathways. However, they found no evidence that a particular lipid A structure is involved in the preferential activation of these signaling pathways. In the present study, we found that lipid IVa, which possesses unique characteristics that allow activity in mouse but not human macrophages, where it acts as an antagonist, preferentially activates the MyD88-dependent pathway.

Lipid IVa fully stimulated IL-1β production and early IκBα phosphorylation, both of which are induced by the MyD88-dependent pathway. However, lipid IVa-induced maximal levels of RANTES production and IFN-β promoter activity, both of which are induced by the MyD88-independent pathway, were less than 50% of that induced by other lipid A compounds. The full agonistic nature of lipid IVa in the MyD88-dependent pathway is consistent with earlier studies observed in mouse macrophages (Golenbock *et al.*, 1991; Perera *et al.*, 1993). However, the partial agonistic nature of the compound found in the MyD88-independent pathway has not been reported. Because activated IRF3 is required for RANTES production and IFN-β promoter activity (Kawai & Akira, 2010), inefficient activation of IRF3 phosphorylation may be responsible for this partial agonistic nature.

The activity of lipid IVa on TNF-α production by mouse macrophages (Tanamoto & Azumi, 2000) or NF-κB activation in HEK293 cells expressing mouse CD14, TLR4, and MD-2 (Muroi & Tanamoto, 2006) is reported to be equal or

slightly lower in terms of EC_{50} value than compound 506. Our result for NF-κB-dependent reporter activity was consistent with these reports. However, lipid IVa required an approximately 1000-fold higher concentration to stimulate similar levels of IL-1β production compared with compound 506. IL-1β production is regulated in two steps, with mRNA expression induction followed by post-translational processing into the mature protein (Martinon *et al.*, 2002; Bryant & Fitzgerald, 2009). Several studies (Okemoto *et al.*, 2006; Mata-Haro *et al.*, 2007; Embry *et al.*, 2011) have reported that MPLA prepared from Gram-negative bacteria poorly stimulated IL-1β production by mouse peritoneal macrophages or RAW264.7 cells, although it effectively induced IL-1β mRNA expression. In addition, Gaekwad *et al.* (Gaekwad *et al.*, 2010) also reported that several lipid A and LPS preparations efficiently stimulated pro-IL-1β protein production but induced no or much lower production of mature IL-1β in the mouse macrophage cell line BAC1.2F5. Therefore, pro-IL-1β processing may be inefficient in lipid IVa stimulation.

TLR4 signaling is initiated first by MD-2 recognition of the lipid A molecule and subsequent formation of the lipid A-MD-2-TLR4 complex, which leads to dimerization of the TLR4 intracellular domain (Miyake, 2004; Kawai & Akira, 2010). This dimerization leads to activation of MyD88-dependent signaling (Miyake, 2004). On the other hand, the activation of MyD88-independent signaling reportedly requires TLR4 to localize to the endosome after internalization through endocytosis (Kawai & Akira, 2010). Although CD14 is reported to play an important role in TLR4 endocytosis (Zanoni *et al.*, 2011), how this endocytosis occurs after lipid A recognition by MD-2 is unclear. Because lipid IVa failed to induce efficient phosphorylation of IRF3, the process prior to its phosphorylation should be involved in the inability of this compound to activate MyD88-independent signaling. The ability of lipid IVa to cause TLR4 endocytosis may thus be restricted compared with compound 506, although further investigation will be needed to confirm this possibility.

Acknowledgements

References

Bjorkbacka H, Fitzgerald KA, Huet F, Li X, Gregory JA, Lee MA, Ordija CM, Dowley NE, Golenbock DT & Freeman MW (2004) The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiol Genomics* 19: 319–330.

Bryant C & Fitzgerald KA (2009) Molecular mechanisms involved in inflammasome activation. *Trends Cell Biol* 19: 455–464.

Darveau RP (1998) Lipid A diversity and the innate host response to bacterial infection. *Curr Opin Microbiol* 1: 36–42.

Embry CA, Franchi L, Nunez G & Mitchell TC (2011) Mechanism of impaired NLRP3 inflammasome priming by monophosphoryl lipid A. *Sci Signal* 4: ra28.

Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H & Ikeda H (2003) Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* 100: 171–194.

Gaekwad J, Zhang Y, Zhang W, Reeves J, Wolfert MA & Boons GJ (2010) Differential induction of innate immune responses by synthetic lipid a derivatives. *J Biol Chem* 285: 29375–29386.

Golenbock DT, Hampton RY, Qureshi N, Takayama K & Raetz CR (1991) Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *J Biol Chem* 266: 19490–19498.

Hayden MS & Ghosh S (2012) NF-κB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev* 26: 203–234.

Hirotani T, Yamamoto M, Kumagai Y, Uematsu S, Kawase I, Takeuchi O & Akira S (2005) Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-β. *Biochem Biophys Res Commun* 328: 383–392.

Hoebe K, Du X, Georgel P *et al.* (2003) Identification of *Lps2* as a key transducer of MyD88-independent TIR signalling. *Nature* 424: 743–748.

Honda K & Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6: 644–658.

Igarashi A, Ohtsu S, Muroi M & Tanamoto K (2006) Effects of possible endocrine disrupting chemicals on bacterial component-induced activation of NF-κB. *Biol Pharm Bull* 29: 2120–2122.

Kawai T & Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11: 373–384.

Kawai T, Adachi O, Ogawa T, Takeda K & Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.

Martin GS, Mannino DM, Eaton S & Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348: 1546–1554.

Martinon F, Burns K & Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory 'and processing of proIL-β. *Mol Cell* 10: 417–426.

Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR & Mitchell TC (2007) The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 316: 1628–1632.

Miyake K (2004) Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol* 12: 186–192.

Muroi M & Tanamoto KI (2006) Structural regions of MD-2 that determine the agonist-antagonist activity of lipid IVa. *J Biol Chem* 281: 5484–5491.

Muroi M & Tanamoto KI (2011) IRAK-1-mediated negative regulation of Toll-like receptor signaling through proteasome-dependent downregulation of TRAF6. *Biochim Biophys Acta* 1823: 255–263.

Muroi M, Ohnishi T, Azumi-Mayuzumi S & Tanamoto K (2003) Lipopolysaccharide-mimetic activities of a Toll-like receptor 2-stimulatory substance(s) in enterobacterial lipopolysaccharide preparations. *Infect Immun* 71: 3221–3226.

Ohnishi T, Yoshida T, Igarashi A, Muroi M & Tanamoto K (2008) Effects of possible endocrine disruptors on MyD88-independent TLR4 signaling. *FEMS Immunol Med Microbiol* 52: 293–295.

Okemoto K, Kawasaki K, Hanada K, Miura M & Nishijima M (2006) A potent adjuvant monophosphoryl lipid A triggers various immune responses, but not secretion of IL-1β or activation of caspase-1. *J Immunol* 176: 1203–1208.

Perera PY, Manthey CL, Stutz PL, Hildebrandt J & Vogel SN (1993) Induction of early gene expression in murine macrophages by synthetic lipid A analogs with differing endotoxic potentials. *Infect Immun* 61: 2015–2023.

Raetz CR & Whitfield C (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71: 635–700.

Rietschel ET, Kirikae T, Schade FU *et al.* (1994) Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J* 8: 217–225.

Schindler U & Baichwal VR (1994) Three NF-κB binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. *Mol Cell Biol* 14: 5820–5831.

Schletter J, Heine H, Ulmer AJ & Rietschel ET (1995) Molecular mechanisms of endotoxin activity. *Arch Microbiol* 164: 383–389.

Tanamoto K & Azumi S (2000) *Salmonella*-type heptaacylated lipid A is inactive and acts as an antagonist of lipopolysaccharide action on human line cells. *J Immunol* 164: 3149–3156.

Ulevitch RJ & Tobias PS (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 13: 437–457.

Yamamoto M, Sato S, Hemmi H *et al.* (2003) Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 301: 640–643.

Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, Granucci F & Kagan JC (2011) CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 147: 868–880.

16S Ribosomal RNA Gene-Based Phylogenetic Analysis of Abundant Bacteria in River, Canal and Potable Water in Bangkok, Thailand

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In Southeast Asian countries, industrialization and urbanization is occurring rapidly, and water pollution in rivers and canals poses serious problems in some areas, especially in cities. Excess inflow of domestic, agricultural, and industrial wastewater to freshwater environments disturbs the aquatic microbial ecosystem, which can further pollute water by inhibiting biodegradation of pollutants. Therefore, monitoring of microbes in freshwater environment is important to identify changes in indigenous microbial populations and to estimate the influence of wastewater inflows on them. Polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) analysis is suitable for monitoring changes in microbial communities caused by human activities, but this method can be difficult in eutrophic freshwater samples that contain PCR inhibitors. In this study, we optimized DNA extraction procedures and PCR conditions for DGGE analysis of bacterial populations in freshwater samples (canal, river, and tap water) collected in Bangkok, Thailand. A simple freeze–thaw procedure was effective for extracting DNA from bacterial cells in the samples, and LA Taq with added bovine serum albumin provided the best PCR amplification. The PCR–DGGE approach revealed that the most common bacteria in freshwater samples belonged to *Gammaproteobacteria*, while a Gram-positive bacterium was present at Bangkok Noi Canal. Temporally and spatially continuous analyses of bacterial populations in Bangkok canals and rivers by PCR–DGGE approach should be useful to recognize disturbances of microbial ecosystems caused by excess inflows of wastewater.

Key words　bacterial monitoring; Southeast Asian country; bacterial population analysis; denaturing gradient gel electrophoresis

In Southeast Asian countries, industrialization and urbanization is occurring rapidly, and water pollution in rivers and canals has become a serious problem in some areas, especially in cities.[1–4] Sewage plants have been constructed in these areas, but the total number is not yet sufficient, and drainpipes are sometimes incomplete. Canals and rivers are therefore polluted by untreated or incompletely treated sewage in these areas. Excess inflow of domestic, agricultural, and industrial wastewater to freshwater environments disturbs aquatic microbial ecosystems, which can further collapse the ecosystem, because bacterial populations are essential in degrading pollutants derived from human activities. Therefore, monitoring of freshwater microbes is important to recognize changes in indigenous microbial populations and to estimate the influence of wastewater inflows on them. Microbial ecology in Southeast Asian countries is therefore being explored; however, current knowledge is limited.

Culture-dependent methods such as plate counting and most probable number (MPN) estimates are often used to evaluate the microbiological quality of freshwater and drinking water.[4] Determination of bacterial numbers and identification of major bacteria based on solely on culturing yields valuable information. However, bacterial colony formation is dependent on growth conditions, such as nutrient media, incubation time, and temperature. Additionally, most bacteria in natural aquatic environments cannot be cultured by conventional techniques.[5,6] Thus, culture-independent methods should be used for microbiological monitoring in addition to culture-dependent methods.[7,8]

Several molecular microbiological techniques have been developed to identify bacterial cells and analyze their diversity and community structure in natural environments without the need for isolation. In particular, denaturing gradient gel electrophoresis (DGGE)[9] has been used to determine the genetic diversity of natural microbial communities and to identify the phylogenetic affiliation of community members.[10–12] Polymerase chain reaction (PCR)-amplified DNA fragments of the eubacterial 16S ribosomal RNA (rRNA) gene that have the same length but different sequences can be separated by DGGE. However, PCR–DGGE analysis is often difficult to implement with freshwater samples from eutrophic environments that contain PCR-inhibitors, such as humic acid.

In this study, we optimized PCR conditions for PCR–DGGE analysis of bacteria in freshwater collected from canals and rivers as well as tap water samples from metropolitan Bangkok, Thailand. The abundant bacteria in these samples were then phylogenetically analyzed using the rRNA-targeted PCR–DGGE approach.

MATERIALS AND METHODS

Sampling Sites　Water samples were collected in Bangkok, Thailand from Bangkok Noi Canal (samples 1–5 and 12 in Table 1), Chao Phraya River (samples 8–11 in Table 1), and Bangkok taps (samples 6 and 7 in Table 1) on February 24, 2002 (Supplementary Fig. 1). The canal is 10–20 m wide, while the river spans more than 100 m. The water quality of the Chao Phraya River has been monitored by the Pollution Control Department of Thailand, and biochemical oxygen demand (BOD) was 1.1–8 mg/L (average 3.5 mg/L) in metro-

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Table 1. Physicochemical Characteristics of Freshwater Samples

Sample	Location	A.T.[a] (°C)	W.T.[b] (°C)	pH	D.O.[c] (mgL⁻¹)
1	Canal; Taling Chan Floating market (13°46′16.5″N, 100°27′22.1″E)	31.3	29.3	7.5	0.9
2	Canal; Bangkok Noi District Office (13°45′31.7″N, 100°28′37.4″E)	31.8	29.3	7.3	1.4
3	Canal; Bangkok Noi (13°48′8.2″N, 100°28′34.4″E)	33.6	29.7	7.4	1.3
4	Canal; Khlong Ban Yai (Nonthaburi) (13°50′47.4″N, 100°25′25.7″E)	37.1	29.8	7.4	2.7
5	Canal; near temple (13°51′3.5″N, 100°25′28.9″E)	36.6	29.7	7.6	3.8
6	Tap water; riverside house (13°51′3.5″N, 100°25′28.9″E)	35.0	29.6	7.5	6.6
7	Tap water; house (reservoir) (13°49′57.5″N, 100°28′22.4″E)	34.8	33.1	7.2	4.5
8	River; Wat Chaloem Phra Kiat (13°50′40.0″N, 100°29′5.4″E)	34.9	30.7	7.4	1.2
9	River; Krung Thon Bridge (13°46′36.4″N, 100°30′3.2″E)	35.6	29.7	7.4	6.7
10	River; Pra Pin Klao pier (13°45′28.4″N, 100°29′25.1″E)	35.6	29.5	7.4	—[d]
11	River; Thonburi station (13°45′15.2″N, 100°29′13.7″E)	35.6	29.2	7.4	—
12	Canal; Thonburi station (13°45′21.1″N, 100°29′9.7″E)	34.6	29.8	7.5	1.3

a) A.T., ambient temperature. b) W.T., water temperature. c) D.O., dissolved oxygen. d) —, not determined.
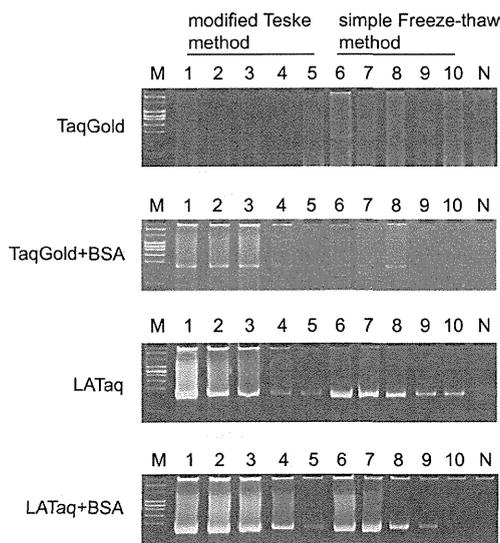

Fig. 1. Amplification of Bacterial 16S Ribosomal RNA Genes in Freshwater Samples Taken in Bangkok, Thailand

Bacterial DNA was extracted by a modified Teske method (lanes 1–5) or a simple freeze–thaw method (lanes 6–10). M: pHY marker. Lanes 1 and 6: Bangkok Noi Canal; lanes 2–4 and 7–9: Chao Phraya River; lanes 5 and 10: tap water. N: Distilled water (negative control).
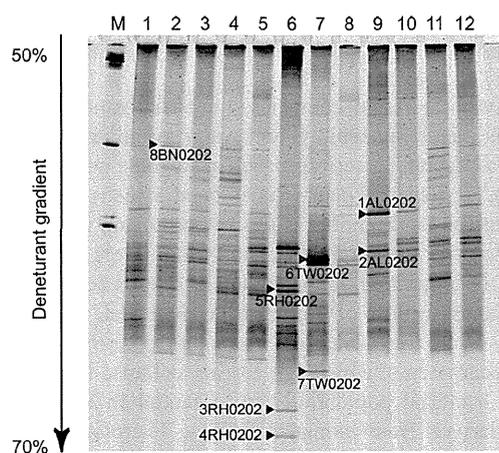

Fig. 2. Denaturing Gradient Gel Electrophoresis Band Patterns of Freshwater Bacteria from Bangkok Noi Canal (Lanes 1–5 and 12), Chao Phraya River (Lanes 8–11), and Tap Water (Lanes 6 and 7) Taken in Bangkok, Thailand, Using the Primer Combination EUBf933-GC/EUBr1387

M: pHY marker. Arrows indicate bands excised for sequencing. Bands 1AL0202 and 2AL0202: common in all freshwater samples; bands 3RH0202, 4RH0202, and 5RH0202: bands specific to purified tap water stored in a plastic tank at a riverside house; bands 6TW0202 and 7TW0202: specific to tap water in a reservoir; band 8BN0202: specific to Bangkok Noi Canal water.

politan Bangkok.[13]

One tap water sample stored in a plastic bucket at a riverside house and one from a water reservoir at a house in the city were also collected as oligotrophic aquatic environmental samples.

The collected samples were stored immediately in ice and used for the following experiments within two hours after sampling.

DNA Extraction for 16S rRNA Gene Analysis Bacterial

DNA was extracted using a simple freeze–thaw[14] or modified Teske method.[15] Bacterial cells in water samples were vacuum-filtered onto polycarbonate white filters (pore size: 0.2 μm). In the simple freeze–thaw method, these filters were placed into sterilized tubes with 400 μL of sterile DNA-free water. The samples were mixed thoroughly, frozen in liquid nitrogen, and then thawed at room temperature. This freeze–thaw cycle was repeated twice, and the extracted DNAs were directly used for PCR.
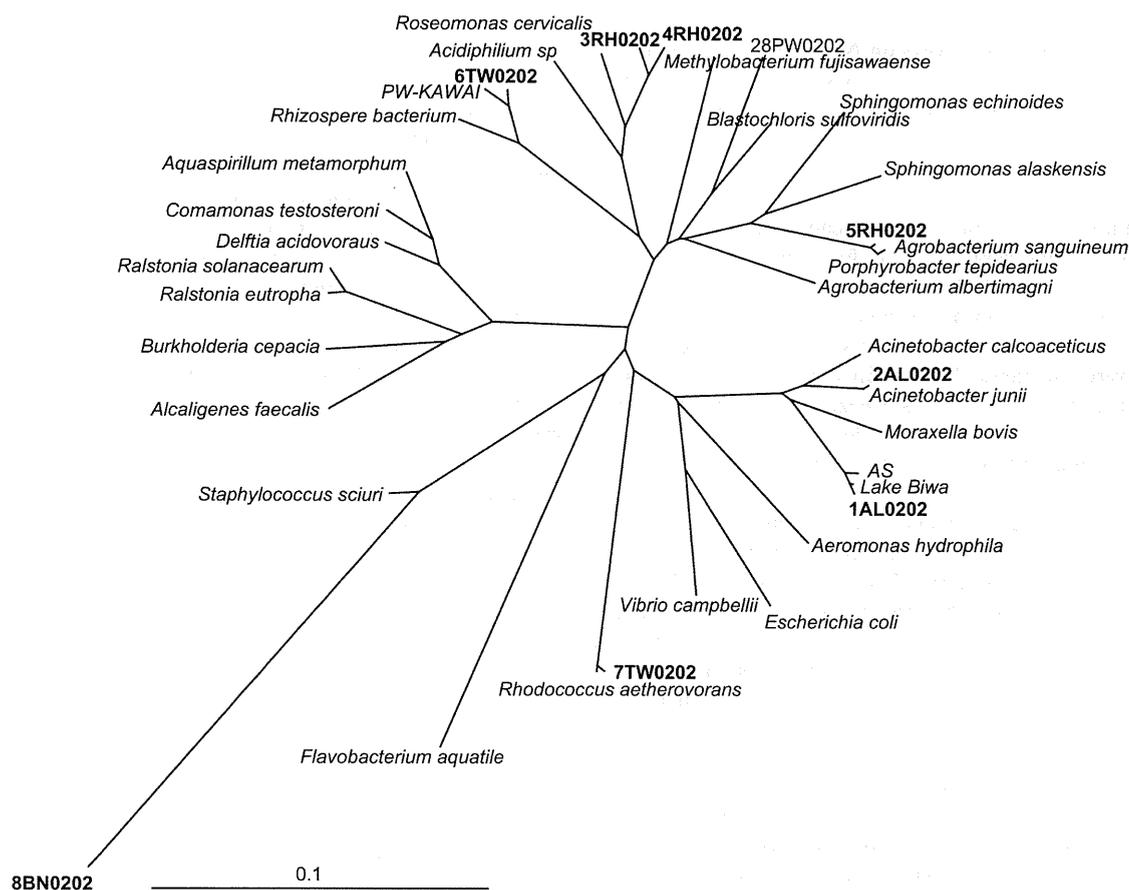
Fig. 3. Phylogenetic Affiliations within the Freshwater Bacteria in Bangkok Noi Canal, Chao Phraya River, and Tap Water Taken in Bangkok, Thailand

Sequences determined in this study are shown in boldface (compare with Fig. 2 for location of bands on the gel). The scale bar corresponds to 0.1 substitutions per nucleotide position.

In the modified Teske method, each filter was added to 2 mL of AE buffer (20 mM sodium acetate (pH 5.5), 1 mM ethylenediaminetetraacetic acid (EDTA)), frozen in liquid nitrogen, and then thawed at room temperature. This freeze–thaw cycle was repeated once. After this lysis treatment, each filter and buffer were added to 6 mL of Tris–EDTA (TE)-buffered phenol–chloroform–isoamyl alcohol (25 : 24 : 1, pH 8.0) and 60 μL of 25% (w/v) sodium dodecyl sulfate. After 5 min of incubation at 60°C, each solution was cooled on ice for 3 min and then centrifuged for 5 min at 3500×g. The supernatant was transferred to a new tube and 250 μL of 2 M sodium acetate (pH 5.2) was added. Nucleic acids were precipitated with 2.5 volumes of 100% ethanol for 3 h at −20°C and then recovered by centrifugation. Each pellet was washed with 75% ethanol, dried, and redissolved in 50 μL of sterile distilled water. The suspensions were used for PCR.

Primers and PCR Amplification Fragments of the 16S rRNA gene, including the V6–V8 region, were amplified with primers GC-clamp-EUB f933 (5′-GC-clamp-GCA CAA GCG GTG GAG CAT GTG G-3′) and EUB r1387 (5′-GCC CGG GAA CGT ATT CAC CG-3′), which are specific for universally conserved bacterial 16S rRNA gene sequences.[15] For DGGE analysis of the PCR products, a GC-clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) was attached to the 5′ end of primer EUB f933. PCR was performed with one of the following enzymes according to the

manufacturer's guidelines: Ampli Taq Gold (Applied Biosystems, Foster City, CA, U.S.A.), LA Taq (TaKaRa Bio, Shiga, Japan), rTaq (Toyobo, Osaka, Japan), KOD Plus (Toyobo), or Platinum Pfx (Invitrogen, Carlsbad, CA, U.S.A.). One or more of the following additives were used to optimize amplification: bovine serum albumin (BSA) solution[16] (0.2 mg/mL; TaKaRa Bio), betaine solution (0.5 M; Sigma-Aldrich, St. Louis, MO, U.S.A.), and dimethyl sulfoxide (DMSO; 1% (v/v); Nacalai Tesque, Kyoto, Japan).

Denaturing Gradient Gel Electrophoresis PCR products were loaded onto a 6.5% (w/v) polyacrylamide gel in 1×TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (pH 8.0)). The polyacrylamide gels (37.5 : 1 acrylamide–bisacrylamide) were made with denaturing gradients ranging from 50–70% (100% denaturant contained 7 M urea and 40% formamide). The gels were run at 55°C for 10 min at 20 V and subsequently for 14 h at 100 V.

Sequencing of DGGE Fragments Bands of 16S rRNA gene fragments in the DGGE gel were excised with a sterile razor blade. The DNA was reamplified with EUB f933 and EUB r1387 primers, and the PCR products were ligated for cloning. Plasmid inserts were reamplified by PCR with primers GC-clamp-EUB f933 and EUB r1387 and analyzed by DGGE. Plasmids inserts that produced a DGGE band at the same position as the excised band were selected and their sequences were analyzed by a sequencer (CEQ-8000, Beckman

Coulter, Indianapolis, IN, U.S.A.).

Nucleotide Sequence Accession Numbers The sequences obtained in this study have been deposited in the DDBJ database under accession Nos. AB769961 to AB769969.

RESULTS AND DISCUSSION

To monitor bacteria in river and canal water in Bangkok with a PCR-based approach, we first optimized the DNA extraction method and PCR conditions for these samples, because eutrophic freshwater samples often inhibit PCR. For DNA extraction, simple freeze–thaw and modified Teske methods were compared. The modified Teske method involves DNA purification steps after a freeze–thaw treatment while the simple freeze–thaw method does not. Different DNA polymerases and additives were tested for effective PCR amplification.

Figure 1 shows electrophoretic gels comparing the DNA extraction procedures and PCR conditions. Rather clear bands were obtained when bacterial DNAs were extracted by the modified Teske method, while the simple freeze–thaw method was effective in almost all samples. Thus, this simple method is suitable for analyzing many samples simultaneously. TaqGold is often used for PCR amplification of eubacterial 16S rRNA genes from freshwater samples[10,11,14,15]; however, this DNA polymerase was not very effective in our study. Among the five DNA polymerases tested, LA Taq yielded the best amplification, while rTaq, KOD Plus, and Platinum Pfx amplified non–specific products (data not shown). The PCR additives BSA, betaine, and DMSO were also examined. Neither betaine nor DMSO was effective (data not shown), while BSA enhanced amplification with both Taq Gold and LA Taq. Thus, we concluded that LA Taq with BSA provided the best amplification for our samples.

Bacterial 16S rRNA genes in the freshwater samples were then amplified with the optimized conditions and analyzed by DGGE (Fig. 2). More than 10 bands appeared in each sample, and eight were selected for further analysis: two that were common in all freshwater samples, one that was specific to all Bangkok Noi Canal water samples, three that were specific to household tap water stored in a plastic bucket, and two specific to household tap water in a reservoir.

Figure 3 shows the phylogenetic positions of the eight clones. The two bacteria common to all freshwater samples belonged to *Gammaproteobacteria*; 1AL0202 had 99% similarity with a partial sequence isolated from Lake Biwa, Japan's largest freshwater lake, and 2AL0202 belonged to *Acinetobacter* sp. Kenzaka used fluorescence *in situ* hybridization to analyze bacterial communities in freshwater samples from Chao Phraya River and reported that 30–40% of bacteria belonged to *Gammaproteobacteria*,[13] similar to our results. The dominant bacterium in Bangkok Noi Canal (8BN0202) belonged to Gram-positive bacteria and was closely related to *Staphylococcus sciuri*. This bacterium is sometimes found in urine and related to urinary tract infections.[17] This sampling site was probably affected by the wastewater inflow. The dominant bacteria in tap water (3RH0202, 4RH0202, 5RH0202, and 6TW0202) belonged to *Alphaproteobacteria*, which are often found in oligotrophic environments.[14] The bacterium found in tap water stored in the reservoir (7TW0202) had 99% similarity with a partial sequence of *Rhodococcus aetherovo-*

rans, which is often found in soil. Interestingly, 6TW0202 was very close to bacteria found in pharmaceutical purified water used in Osaka, Japan, which was prepared from tap water.[14] This bacterium may be widely distributed in tap water.

In this study, we optimized a DNA extraction procedure and PCR conditions for DGGE analysis of bacterial populations in freshwater samples collected in Bangkok, Thailand. Bacterial populations in both eutrophic (*e.g.*, canal and river) and oligotrophic (*e.g.*, tap water) aquatic environments could be analyzed by the optimized PCR–DGGE procedure. DGGE is suitable to detect changes in microbial communities caused by human activities,[15] and temporally and spatially continuous analyses of bacterial populations in Bangkok canals and rivers by PCR–DGGE should be useful to recognize disturbances in microbial ecosystems caused by excess inflow of wastewater.

REFERENCES

1) Hammen VC. Long-time risk of groundwater/drinking water pollution with sulphuric compounds beneath burned peatlands in Indonesia. *Water Sci. Technol.*, **56**, 253–258 (2007).
2) Muyibi SA, Ambali AR, Eissa GS. The impact of economic development on water pollution: Trends and policy actions in Malaysia. *Water Resour. Manage.*, **22**, 485–508 (2008).
3) Pham TA, Kroeze C, Bush SR, Mol APJ. Water pollution by intensive brackish shrimp farming in south-east Vietnam: Causes and options for control. *Agric. Water Manage.*, **97**, 872–882 (2010).
4) Pollution Control Department, Ministry of Natural Resources and Environment, Thailand. "Thailand State of Pollution Report 2011.": ⟨http://www.pcd.go.th/indexEng.cfm⟩. cited March 26, 2013.
5) Yamaguchi N, Nasu M. Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *J. Appl. Microbiol.*, **83**, 43–52 (1997).
6) Baba T, Inoue N, Yamaguchi N, Nasu M. Rapid enumeration of active *Legionella pneumophila* in freshwater environments by the microcolony method combined with direct fluorescent antibody staining. *Microbes Environ.*, **27**, 324–326 (2012).
7) Yamaguchi N, Baba T, Nakagawa S, Saito A, Nasu M. Rapid monitoring of bacteria in dialysis fluids by fluorescent vital staining and microcolony methods. *Nephrol. Dial. Transplant.*, **22**, 612–616 (2007).
8) Yamaguchi N, Torii M, Uebayashi Y, Nasu M. Rapid, semiautomated quantification of bacterial cells in freshwater by using a microfluidic device for on-chip staining and counting. *Appl. Environ. Microbiol.*, **77**, 1536–1539 (2011).
9) Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**, 695–700 (1993).
10) Kawai M, Yamagishi J, Yamaguchi N, Tani K, Nasu M. Bacterial population dynamics and community structure in a pharmaceutical manufacturing water supply system determined by real-time PCR and PCR-denaturing gradient gel electrophoresis. *J. Appl. Microbiol.*, **97**, 1123–1131 (2004).
11) Baba T, Matsumoto R, Yamaguchi N, Nasu M. Bacterial population dynamics in a reverse-osmosis water purification system determined by fluorescent staining and PCR-denaturing gradient gel electrophoresis. *Microbes Environ.*, **24**, 163–167 (2009).
12) Nishimura Y, Kenzaka T, Sueyoshi A, Li P-F, Fujiyama H, Baba T, Yamaguchi N, Nasu M. Similarity of bacterial community structure

between Asian dust and its sources determined by rRNA gene-targeted approaches. *Microbes Environ.*, **25**, 22–27 (2010).

13) Kenzaka T, Yamaguchi N, Prapagdee B, Mikami E, Nasu M. Bacterial community composition and activity in urban rivers in Thailand and Malaysia. *J. Health Sci.*, **47**, 353–361 (2001).

14) Kawai M, Matsutera E, Kanda H, Yamaguchi N, Tani K, Nasu M. 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.*, **68**, 699–704 (2002).

15) Iwamoto T, Tani K, Nakamura K, Suzuki Y, Kitagawa M, Eguchi M, Nasu M. Monitoring impact of *in situ* biostimulation treatment on groundwater bacterial community by DGGE. *FEMS Microbiol. Ecol.*, **32**, 129–141 (2000).

16) Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.*, **62**, 1102–1106 (1996).

17) Stepanovic S, Ježek P, Vukovic D, Dakic I, Petrás P. Isolation of members of the *Staphylococcus sciuri* group from urine and their relationship to urinary tract infections. *J. Clin. Microbiol.*, **41**, 5262–5264 (2003).

MICROBES AND
ENVIRONMENTS

Bacterial Monitoring with Adhesive Sheet in the International Space Station-"Kibo", the Japanese Experiment Module

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Microbiological monitoring is important to assure microbiological safety, especially in long-duration space habitation. We have been continuously monitoring the abundance and diversity of bacteria in the International Space Station (ISS)-"Kibo" module to accumulate knowledge on microbes in the ISS. In this study, we used a new sampling device, a microbe-collecting adhesive sheet developed in our laboratory. This adhesive sheet has high operability, needs no water for sampling, and is easy to transport and store. We first validated the adhesive sheet as a sampling device to be used in a space habitat with regard to the stability of the bacterial number on the sheet during prolonged storage of up to 12 months. Bacterial abundance on the surfaces in Kibo was then determined and was lower than on the surfaces in our laboratory (10^5 cells $[cm^2]^{-1}$), except for the return air grill, and the bacteria detected in Kibo were human skin microflora. From these aspects of microbial abundance and their phylogenetic affiliation, we concluded that Kibo has been microbiologically well maintained; however, microbial abundance may increase with the prolonged stay of astronauts. To ensure crew safety and understand bacterial dynamics in space habitation environments, continuous bacterial monitoring in Kibo is required.

Key words: International Space Station (ISS), bacterial monitoring, adhesive sheet, Japanese Experiment Module "Kibo"

The International Space Station (ISS) has been staffed continuously since the first resident crew entered the facility on 2 November 2000, thereby providing a permanent human presence in space. During space flight, a variety of physiological and psychological stressors associated with the space environment, and spacecraft conditions, potentially contribute to detrimental alterations in the human immune system (1). In addition, microorganisms that might present a health hazard to the crew and potentially damage flight hardware have been recognized in the ISS (3, 13, 14). Space agencies have attempted to avoid microbiological problems by developing strategies to limit contamination aboard the ISS (11) (Japan Aerospace Exploration Agency [JAXA] website, ISS/Kibo utilization scenario toward 2020, http://iss.jaxa.jp/kiboexp/news/2020_kibo.html [in Japanese]; Towards Human Exploration of Space: a European Strategy [THESEUS] website, THESEUS Roadmap, http://theseus.hd20.hosting.punkt.de/fileadmin/Docs/Eg_reports_roadmap/RoadMap_web.pdf).

For microbial monitoring of interior surfaces of the ISS, sampling has been performed with swabs or agar-based media (10, 13), and laboratory analysis of bacterial genera is carried out back on Earth. These conventional sampling methods have some problems that should be resolved: use of water for swabbing methods; potential risk of injury if the handle of the swab is broken during sampling; contamination of surfaces with culture medium components; and difficulty of sampling from curved surfaces. For sampling microbial cells on solid surfaces, we have developed a microbe-collecting adhesive sheet (20). This sheet has high operability, needs

no water for sampling, and is easy to transport and store. In the present study, we evaluated via a laboratory test on ground the applicability of the adhesive sheet for microbial monitoring in a space habitat by determining the effect of preservation of the adhesive sheet on the collected microbes, because sample storage is necessary before transporting back to the laboratory on Earth.

We then used the adhesive sheet for microbial monitoring in the ISS. The environment in the ISS is different from that on Earth, and the microbial ecosystem may also differ. Therefore, investigation is required of the relationship between humans and microbes, as well as how microbes influence the materials and systems in this closed environment. We have been continuously monitoring microbes in the Japanese Experiment Module "Kibo" on the ISS, in cooperation with the Japan Aerospace Exploration Agency (JAXA) (research title: "Microbe": http://iss.jaxa.jp/en/kiboexp/news/101101_microbe-2_start.html). The objective of "Microbe" is to monitor changes in microbial abundance and species diversity in Kibo, which entered operation in June 2008, and to clarify microbial dynamics in space habitation systems. In 2010, microbes were collected by applying the adhesive sheets to the surfaces of the interior and the equipment in Kibo. After the samples were transported to our laboratory, bacterial abundance and taxonomic distribution were determined.

Materials and Methods

Bacterial strains

The following bacterial species have been detected in the ISS, and reference strains of these species were used in this study: *Acinetobacter lwoffii* ATCC15390, *Bacillus subtilis* 168,

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  Tel: +81–6879–8172; Fax: +81–6879–8174.

Pseudomonas putida ATCC12633 and *Staphylococcus epidermidis* IFO3762. Each bacterial strain was incubated in LB broth at 37°C until reaching the stationary phase.

Sample preparation for evaluating recovery rate and preservation experiment

A painted stainless plate was provided by JAXA (Tokyo, Japan) for the following ground experiments. The plate was disinfected by spraying 70% ethanol on all surfaces and wiping with a paper towel immediately before performing each experiment.

To evaluate the recovery rate, 1.0×10^7 mixed cultured cells (2.5×10^6 cultured cells each) suspended in sterilized water were spread on a 6.25 cm² area on the plate. After the cells were air-dried, they were recovered with an adhesive sheet (Fig. 1) or a swab (Large Alpha Swab; TexWipe, Kernersville, NC, USA). To achieve a high recovery rate of bacterial cells with the adhesive sheet, the adhesive face was pressed down three times (repeatedly placing and removing the same sheet) in exactly the same area (20). Swabbing was optimized and performed as described previously (18). Recovered cells were fluorescently stained with 1×SYBR Green II (Invitrogen, Carlsbad, CA, USA) and counted under an epifluorescent microscope (Eclipse E400; Nikon, Tokyo, Japan) at a magnification of ×1,000. Recovery rate was calculated by dividing the number of recovered bacterial cell by the spread cell number (1.0×10^7).

For the preservation experiment, 1.8×10^5 mixed cultured cells (4.5×10^4 cultured cells each) suspended in sterilized water were spread on a 6.25 cm² area on the plate and recovered with the adhesive sheet. Sampling with the adhesive sheet was performed as described above. The adhesive sheets on which bacterial cells were collected were packed in small Ziplock bags and stored at −80°C, 4°C or room temperature (approx. 20°C) for one to 12 months. Samples without any preservation were used as the time zero control. Bacterial cells on the adhesive sheet were fluorescently stained with 1×SYBR Green II after storage. Recovered cells in 20 microscopic fields were counted under the epifluorescent microscope at ×1000 magnification. Recovery rate was calculated by dividing the number of fluorescently stained cells after storage by that on the initial day.

Sampling in Kibo

In Kibo, sampling using adhesive sheets was performed by an astronaut on 29 October 2010 at 1830 JST (880 d after launch) as part of the "Microbe" research program. The surfaces of the CBEF (Cell Biology Experiment Facilities; relatively low frequency of astronaut contact), PC palm rest (routine contact by astronauts), return air grill (intake of air) and handrail (high frequency of astronaut contact) were selected as sampling sites. Samples were stored in the freezer (−80°C) installed in Kibo for seven months and transferred to our laboratory via NASA Kennedy Space Center and JAXA Tsukuba Space Center at −80°C. We received the samples on 6 June 2011 (JST).

Total direct counting of microbes collected with adhesive sheet

Center of the adhesive area (5 mm×5 mm) was excised aseptically with a sterilized razor. This piece of sheet was used for total direct counting of microbes. The other area was used for DNA extraction as described below. Collected cells on the adhesive sheet were fluorescently stained with 1×SYBR Green II. Fluorescently stained cells in 50 microscopic fields were counted under the epifluorescent microscope at ×1,000 magnification.

DNA extraction from adhesive sheet sample

The adhesive area used for DNA extraction (6 cm²) was excised aseptically with scissors. DNA was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA). The excised sheet for DNA extraction was inserted into a Lysing Matrix E tube provided as part of the kit. After insertion, 2 μL luciferase gene fragment was added as an internal control for quantitative PCR (12), and then DNA extraction was performed following the manufacturer's instructions. For dilution of DNA, 100 μL DES was used, provided as part of the kit.

Quantitative PCR

For determination of bacterial abundance, the 16S rRNA gene was quantified by quantitative PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) (19). Quantitative PCR amplification was performed with the reagents supplied with the LightCycler DNA Master SYBR Green I (Roche Diagnostics). The quantitative PCR mixture, containing 4 mM MgCl₂, 0.5 μM each primer (EUB f933, EUB r1387; Table 1) and 4.5 ng μL⁻¹ 8-methoxypsoralen was made up to 8 μL with DNA-free water. The 10×LightCycler DNA Master SYBR Green I and the DNA suspension were added last in 1 μL volume each after irradiation of the PCR mixture with UV light (7). After an initial denaturing
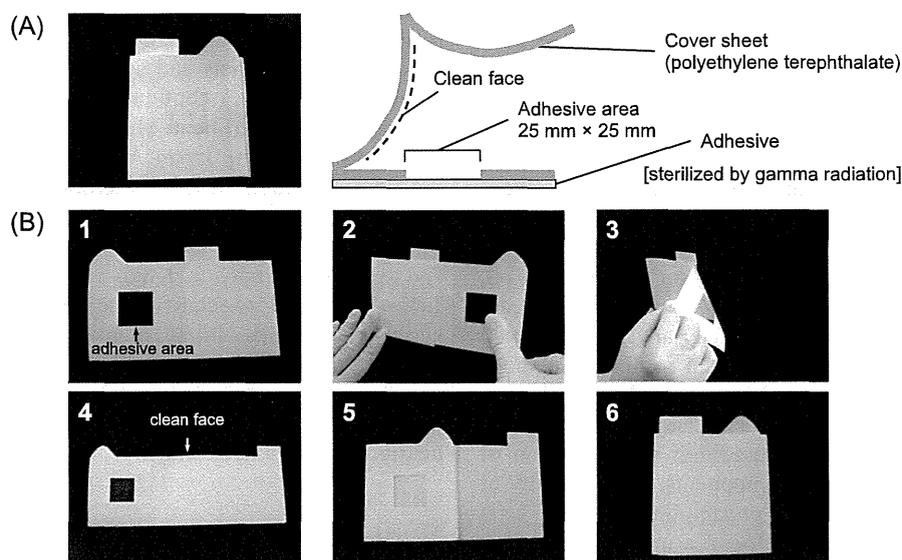

Fig. 1. Adhesive sheet for microbial monitoring in space habitat. Sheets were sterilized by gamma radiation and packed individually in small bags. (A) Photograph and schematic illustration of adhesive sheet. (B) Procedure for microbial sampling: 1, open triangular tab; 2, attach adhesive area to sampling site and press three times; 3, peel adhesive sheet off sampling site; 4, open rectangular tab; 5, close triangular tab; 6, close rectangular tab.

Table 1. Primers and probes used in this study

Primer	Target gene and position	Sequence (5' to 3')	References
8f	16S rRNA, 8–27[c]	AGAGTTTGATCCTGGCTCAG	(9)
1492r	16S rRNA, 1510–1492[c]	GGTTACCTTGTTACGACTT	(9)
EUB f933	16S rRNA, 933–954[c]	GCACAAGCGGTGGAGCATGTGG	(6)
EUB f933-GC-clamp	16S rRNA, 933–954[c]	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGG-EUB f933	(6)
EUB r1387	16S rRNA, 1387–1368[c]	GCCCGGGAACGTATTCACCG	(6)
pgL1908f	*luc* gene, 1908–1927[d]	AGGAAGCTTTCCATGGAAGA	(12)
Luc175r	*luc* gene, 2063–2082[d]	CAGCGTAAGTGATGTCCACC	(12)
n-LucHP1[a]	*luc* gene, 2008–2029[d]	TGAAGAGATACGCCCTGGTTCC	(12)
n-LucHP2[b]	*luc* gene, 2030–2058[d]	GGAACAATTGCTTTTACAGATGCACATA	(12)

[a] 3' FITC labeled; [b] 5' LightCycler Red640 labeled; [c] *Escherichia coli* numbering system; [d] Numbering bases of pGeneGRIP-Luc.

step at 95°C for 10 min, 45 cycles were performed as follows: denaturing at 95°C for 15 sec, annealing at 60°C for 10 sec, extension at 72°C for 30 sec, and signal detection at 86°C for 5 sec.

To determine the rate of recovery of DNA during extraction, known amounts of PCR products of the luciferase gene (*luc*) were inoculated into the samples as an internal standard and quantified after DNA extraction according to Nishimura et al. (12). The DNA recovery rate was calculated by comparing the copy number of the inoculated *luc* gene before and after DNA extraction. The copy number of the 16S rRNA gene quantified by quantitative PCR was calibrated based on the recovery rate.

Nested PCR-DGGE analysis

16S rRNA gene fragments were amplified using the primers EUB f933-GC-clamp and EUB r1387 following amplification of nearly the full-length 16S rRNA gene with 8f and 1492r primer set (Table 1). First and second PCR amplifications were performed as described by Buchholz-Cleven et al. (2) and Iwamoto et al. (6), respectively. Denaturant gradient gel electrophoresis (DGGE) was run as described by Iwamoto et al. (6). Briefly, 200 ng PCR product was loaded onto a 6.5% (w/v) polyacrylamide gel cast in 1×TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA; pH 8.0). Polyacrylamide gels (acrylamide : bisacrylamide, 37.5:1) were made with denaturing gradients ranging from 45 to 65%. After electro-phoresis, DGGE gel was soaked in 1×SYBR Gold (Invitrogen) for 30 min.

The bands in the DGGE gel were excised with a sterilized razor blade under blue excitation light, and then placed in 100 μL nucleic acid-free water. After overnight incubation at 4°C, the supernatant was used as a template for re-amplification with EUB f933 and EUB r1387 primer set (Table 1). Because a single DGGE band would not represent a single bacterial strain (17), we constructed small clone libraries using the pGEM-T Easy Vector System II (Promega, Madison, WI, USA) and inserted fragments of randomly selected six clones were sequenced (Hokkaido System Sciences, Hokkaido, Japan). Nucleic acid sequences were analyzed by the ribosomal database project (4) in order to determine the taxonomic distribution. A phylogenetic tree was constructed by the neighbor-joining method.

Nucleotide sequence accession numbers

The sequences obtained from the DNA clone library were deposited in the DNA Data Bank of Japan (DDBJ) database under accession numbers AB720834 to AB720837 (CBEF surface), AB720838 to AB720850 (handrail), AB720851 to AB720853 (return air grill) and AB720854 to AB720871 (PC palm rest).

Statistical analysis

Student's t test was carried out using an online statistical analysis program MEPHAS (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/).

Results and Discussion

Sampling is one of the most important processes in environmental microbiology. In this study, we first evaluated the applicability of our adhesive sheet for bacterial monitoring in a space habitat. Sampling in the ISS is not performed by specialists in microbiology but by those who are not familiar with this field, such as astronauts; thus, any sampling technique should have high operability. The microbe-collecting adhesive sheet developed for the present study is shown in Fig. 1A. This sheet was 7 cm×8.5 cm (adhesive area: 2.5 cm×2.5 cm). To avoid any microbial contamination of the clean face, the cover sheet is folded in half. Sheets were packed individually in a small bag and sterilized by gamma radiation (10 kGy; Koga Isotope, Shiga, Japan). The sampling procedure is shown in Fig. 1B. Before sampling, we confirmed by fluorescent staining that there were no microbial cells on the adhesive area. In order to protect the adhesive area of the sheet from any contamination, the clean face (inside of the cover sheet) is covered during sampling, as shown in Fig. 1B (step 1–3). After sampling, the adhesive area faces the clean face (Fig. 1B, step 4, 5), and is stored (Fig. 1B, step 6).

Microbial cells collected on adhesive sheets were directly stained with 1×SYBR Green II (Fig. 2). Four mixed species of cultured bacteria and microbes collected from the vertical surface of the rack in our laboratory (ground control of CBEF surface in Kibo) were used as samples. As shown in Fig. 2, microbial cells stained with SYBR Green II were clearly observed with an epifluorescent microscope.

Next, we evaluated the recovery rate of bacterial cells spread on the painted stainless plate with the adhesive sheet. A swab was also used for sampling and the results were compared. The recovery rates of bacterial cells with the adhesive sheet and the swab were 78±12% (*n*=5) and 69±11% (*n*=10), respectively. The ability of the adhesive sheet to collect microbes from a solid surface was equivalent to that of the swab (*P*>0.05; Student's t test). We also evaluated the recovery rate of bacterial cells spread on the laptop palm rest (plastic, rough surface) with the adhesive sheet. The rate was calculated as 71±6.1% (*n*=3). Using the adhesive sheet, the recovery rate from the plastic surface was not significantly different from that from the painted stainless plate (*P*>0.05; Student's t test). The adhesive sheet was therefore an alter-native device for sampling in a space habitat.
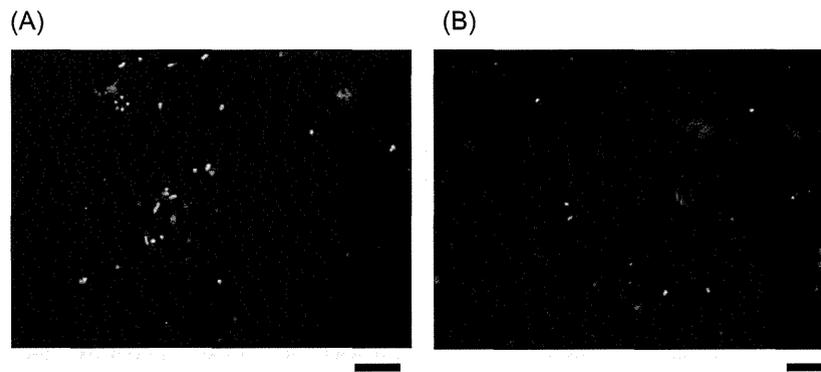
(A) (B)

Fig. 2. Fluorescent microscopic image of microbes collected with an adhesive sheet. Microbial cells were stained with 1×SYBR Green II (scale bars, 10 μm). (A) Mixture of *A. lwoffii* ATCC15390, *B. subtilis* 168, *P. putida* ATCC12633 and *S. epidermidis* IFO3762; (B) sample from vertical surface of the rack in our laboratory.
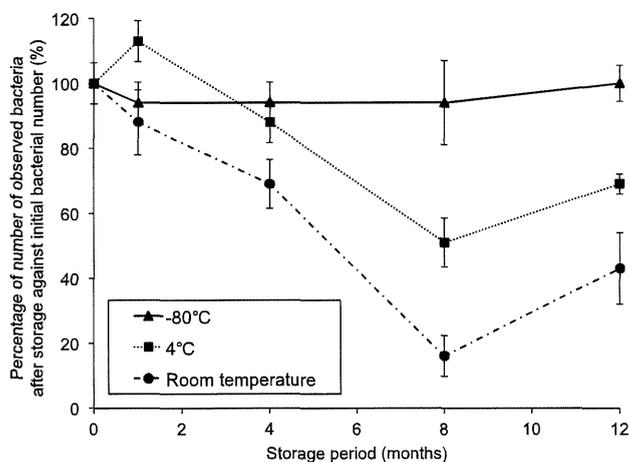

Fig. 3. Changes in numbers of recovered bacterial cells on adhesive sheets during prolonged storage. Initial bacterial number was converted to 100%. Error bars show standard deviations ($n=3$).

Table 2. Bacterial abundance on the interior surfaces in Kibo determined with fluorescent staining and quantitative PCR

Sampling point	Bacterial abundance (cells [cm²]⁻¹)	
	Fluorescent staining	Quantitative PCR[a,b]
CBEF surface	$<2.0\times10^4$	$<7\times10^4$ ($<7\times10^4$)
PC palm rest	2.6×10^4	3×10^3-5×10^4 (5×10^4)
Return air grill	Not countable	5×10^6-8×10^7 (8×10^7)
Handrail	2.8×10^4	2×10^3-3×10^4 (3×10^4)

[a] Bacterial cells carry 1 to 15 copies of the 16S rRNA gene in their genome (8).

[b] Numbers in parentheses show 16S gene copy numbers (unit: copies [cm²]⁻¹).

For bacterial monitoring in the ISS, it is difficult to analyze the samples immediately because transportation to the ground laboratory strictly depends on the availability of the return vehicle. In some cases, long-term storage in the ISS will be required. Changes in bacterial number during prolonged storage at various temperatures were therefore determined (Fig. 3). The number of SYBR Green II-stained bacterial cells that could be counted by microscopy on adhesive sheets stored at room temperature and 4°C markedly decreased during storage. In contrast, the number of bacteria collected on the adhesive sheet and stored at −80°C was >90% of the initial number and did not significantly change during this period (until 12 months) ($P<0.05$; Student's t test). We also evaluated the effect of long-term storage at −80°C for eight months on the number of 16S rRNA genes, which was stable for one to eight months, as was the bacterial number determined by fluorescent microscopy. Therefore, after storing samples at −80°C, the counts obtained reflect the abundance of bacteria collected on the day of sampling (time zero). The sampling sheet enables us to analyze the bacteria in the ISS precisely, even after long-term storage when kept at −80°C.

We then used the adhesive sheet for bacterial monitoring

in Kibo. Samples were collected as part of the "Microbe" research program. We determined bacterial numbers with different approaches of fluorescence microscopy and quantitative PCR targeting the 16S rRNA gene to confirm the reliability of the results. Table 2 shows the bacterial abundance in Kibo determined with these two methods. Before this experiment, we confirmed that the number of 16S rRNA genes on an unused adhesive sheet (negative control) was below the quantification limit. Using fluorescent staining, bacterial abundance at each site was equivalent to or less than the quantification limit. Using quantitative PCR, a similar result to fluorescent staining was obtained, except on the return air grill. The return air grill sample collected a lot of dust on the adhesive face; therefore, we were not able to discriminate bacterial cells from other particles clearly under an epifluorescent microscope. Quantitative PCR was more effective when the adhesive face was covered with dust.

Finally, we estimated the taxonomic distribution of bacteria collected from surfaces at four sites in Kibo. The yield of PCR products with a 8f/1492r primer set or an EUB f933-GC-clamp/EUB r1387 primer set was too low to construct 16S rRNA gene clone libraries or perform PCR-DGGE followed by sequencing, respectively; therefore, we used nested PCR-DGGE followed by sequencing to obtain phylogenetic information of bacteria present in Kibo. This approach showed the presence of bacteria, although it did not reflect the dominance of any particular species (15). Fig. 4 shows the phylogenetic tree of the 16S rRNA gene fragments retrieved from a DGGE gel. Their phylogenetic
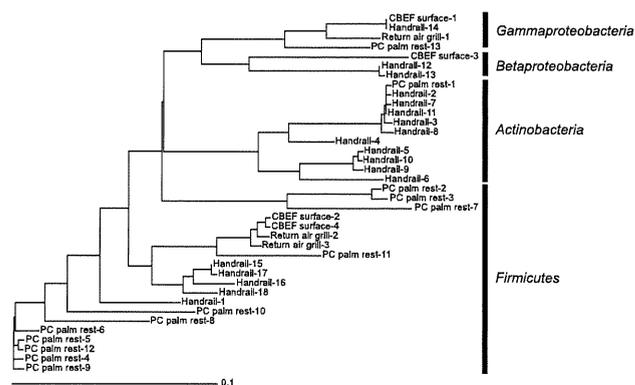
Fig. 4. Neighbor-joining tree of the 16S rRNA gene sequences retrieved from bacteria collected in Kibo, Japanese Experiment Module on the ISS.

affiliations determined by a BLAST search are also shown in Table S1. Bacteria in the phyla *Actinobacteria* and *Firmicutes* were frequently detected on the surface of the PC palm rest and handrail, which astronauts touch frequently. Most of these bacteria have been detected on the hands as human skin microbiome (5); thus, bacterial cells might transfer to the surfaces in Kibo via astronaut contact. Furthermore, this result was consistent with previous research in other modules of the ISS (13). Other bacteria detected in Kibo, such as *Beta-* and *Gammaproteobacteria*, are also part of human skin microflora (5).

The abundance of bacteria in Kibo, except on the return air grill, was equivalent to or lower than that on the surfaces in our laboratory (10^5 cells [cm^2]$^{-1}$) (20), and bacteria detected in Kibo was related to the human skin microflora. Furthermore, with regard to fungal biota analyses, Satoh *et al.* (16) reported that the degree of cleanliness in Kibo during the first 460 d was equivalent to that in a clean room environment on the ground. The surfaces of equipment installed in Kibo are wiped with disinfectant once a week; however, on the return air grill it is easy for dust to accumulate, so the number of microbes can be higher on its surface. From these aspects of microbial abundance and their phylogenetic affiliation, Kibo has been microbiologically well maintained during the first 880 d; however, microbial abundance in Kibo may increase with the prolonged stay of astronauts. To ensure crew safety and understand bacterial dynamics in space habitation environments, continuous bacterial monitoring in Kibo is required.

Acknowledgements

References

1. Borchers, A.T., C.L. Keen, and M.E. Gershwin. 2002. Microgravity and immune responsiveness: Implications for space travel. Nutrition 18:889–898.

2. Buchholz-Cleven, B.E.E., B. Rattunde, and K.L. Straub. 1997. Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizing bacteria using DGGE and whole-cell hybridization. Syst. Appl. Microbiol. 20:301–309.

3. Castro, V.A., A.N. Thrasher, M. Healy, C.M. Ott, and D.L. Pierson. 2004. Microbial characterization during the early habitation of the International Space Station. Microb. Ecol. 47:119–126.

4. Cole, J.R., Q. Wang, E. Cardenas, *et al.* 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37:D141–D145.

5. Fierera, N., M. Hamady, C.L. Lauber, and R. Knight. 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proc. Natl. Acad. Sci. U.S.A. 105:17994–17999.

6. Iwamoto, T., K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi, and M. Nasu. 2000. Monitoring impact of in situ bio-stimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol. Ecol. 32:129–141.

7. Kawai, M., E. Matsutera, H. Kanda, N. Yamaguchi, K. Tani, and M. Nasu. 2002. 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 68:699–704.

8. Klappenbach, J.A., P.R. Saxman, J.R. Cole, and T.M. Schmidt. 2001. rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29:181–184.

9. Lane, D.J. 1991. 16S/23S rRNA sequencing, p 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New Jersey.

10. Maule, J., N. Wainwright, A. Steele, L. Monaco, H. Morris, D. Gunter, M. Damon, and M. Wells. 2009. Rapid culture-independent microbial analysis aboard the International Space Station (ISS). Astrobiology 9:759–775.

11. NASA Aeronautics and Space Engineering Board. 2012. NASA Space Technology Roadmaps and Priorities: Restoring NASA's Technological Edge and Paving the Way for a New Era in Space. The National Academic Press, Washington D.C.

12. Nishimura, Y., T. Kenzaka, A. Sueyoshi, P. Li, H. Fujiyama, T. Baba, N. Yamaguchi, and M. Nasu. 2010. Similarity of bacterial community structure between Asian dust and its sources determined by rRNA gene-targeted approaches. Microbes Environ. 25:22–27.

13. Novikova, N., P. De Boever, S. Poddubko, E. Deshevaya, N. Polikarpov, N. Rakova, I. Coninx, and M. Mergeay. 2006. Survey of environmental biocontamination on board the International Space Station. Res. Microbiol. 157:5–12.

14. Ott, C.M., R.J. Bruce, and D.L. Pierson. 2004. Microbial characterization of free floating condensate aboard the Mir space station. Microb. Ecol. 47:133–136.

15. Posas, M.B., and K. Toyota. 2010. Mechanism of tomato bacterial wilt suppression in soil amended with lysine. Microbes Environ. 25:83–94.

16. Satoh, K., Y. Nishiyama, T. Yamazaki, T. Sugita, Y. Tsukii, K. Takatori, Y. Benno, and K. Makimura. 2011. Microbe-I: fungal biota analyses of the Japanese experimental module KIBO of the International Space Station before launch and after being in orbit for about 460 days. Microbiol. Immunol. 55:823–829.

17. Sekiguchi, H., N. Tomioka, T. Nakahara, and H. Uchiyama. 2001. A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnol. Lett. 23:1205–1208.

18. Yamaguchi, N., H. Hieda, and M. Nasu. 2010. Simple and reliable swabbing procedure for monitoring microbes in the International Space Station. Eco-Engineering 22:27–30.

19. Yamaguchi, N., T. Ichijo, A. Sakotani, T. Baba, and M. Nasu. 2012. Global dispersion of bacterial cells on Asian dust. Sci. Rep. 2:525.

20. Yamaguchi, N., A. Ishidoshiro, Y. Yoshida, T. Saika, S. Senda, and M. Nasu. 2003. Development of an adhesive sheet for direct counting of bacteria on solid surfaces. J. Microbiol. Methods 53:405–410.

Microchip-Based Terminal Restriction Fragment Length Polymorphism for On-Site Analysis of Bacterial Communities in Freshwater

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Assessing microbiological quality assurance by monitoring bacteria in various sources of freshwater used for human consumption, recreation, and food preparation is important for a healthy life. Bacterial number and their community structure in freshwater should be determined as quickly as possible, and "real-time" and "on-site" microbiological methods are required. In this study, we examined the protocol for microchip-based terminal restriction fragment length polymorphism (T-RFLP) analysis, which uses microchip electrophoresis for rapid microbial community analysis. The availability of microchip-based T-RFLP was compared with conventional T-RFLP analysis, which uses a capillary electrophoresis system, with freshwater samples (spring water, river water, groundwater, and hydroponics solution). The detection limit of targeted bacteria by on-chip T-RFLP analysis was 1% (10^3 cells/mL). The fragment sizes determined by the two analysis methods were highly correlated ($r^2=0.98$). On-chip T-RFLP analysis was completed within 15 min. T-RFLP profiles of nine hydroponics solution samples were analyzed by multidimensional scaling. Considerable changes and stability in bacterial community structure during hydroponic culture were detected by both analyses. These results show that on-chip T-RFLP analysis can monitor changes in bacterial community structure, as well as conventional T-RFLP analysis. The present results indicate that on-chip T-RFLP analysis is an effective tool for rapid and "on-site" bacterial community profiling in freshwater environments, as well as freshwater used for medical and industrial purposes.

Key words bacterial community analysis; freshwater; microchip electrophoresis; terminal restriction fragment length polymorphism; bacterial monitoring

Assessing microbiological quality assurance by monitoring bacteria in various sources of freshwater used for human consumption, recreation, and food preparation is important for a healthy life. In addition, regeneration of used water and seawater desalination are important to save freshwater resources. Microorganisms that survive the water purification process can become a source of microbial contamination.[1] Bacterial number and their community structure in freshwater should be determined as quickly as possible, and "real-time" and "on-site" microbiological methods are therefore required.

Determination of total bacterial number is a basis for routine monitoring and microbiological quality assurance. Microchip-based systems have been developed for rapid quantification of bacteria in freshwater.[2,3] These systems enable counting of bacteria in freshwater within 1 h using a microfluidic device following fluorescent staining, without concentration or other time-consuming preparation steps.

For microbiological quality assessment of freshwater, assessment is required to determine whether the number of harmful bacteria is increasing when total bacterial numbers are rapidly changing. Terminal restriction fragment length polymorphism (T-RFLP) targeting the bacterial 16S ribosomal RNA (rRNA) gene as a universal genetic marker is widely used for characterization of bacterial community composition.[4] T-RFLP is semi-quantitative and has a high sensitivity, resolution, and reproducibility. T-RFLP has been applied for community analysis of bacteria in natural environments (*e.g.*, lakes,[5] ocean,[6] and soil[7]) as well as human oral[8] or intestinal[9] microbiota, because this method is suitable for evaluation of temporal and spatial changes of targeted microbial communities. The terminal restriction fragments

(T-RFs) of digested polymerase chain reaction (PCR) products are usually analyzed by capillary electrophoresis, and the relative abundance and size of each T-RF are measured. DNA sequence information of each T-RF can be obtained by comparing its size and type of the used restriction enzymes with several specified databases.[10,11] However, conventional capillary electrophoresis systems are large and expensive for on-site T-RFLP analysis.

The present study examined the use of microchips, which have been developed during decades of progress in microfabrication technologies. Microchip-based analyses are faster, performed on a smaller scale, and consume less sample and reagents than conventional approaches, and are thus applied to microbiological studies.[12,13] Application of these new and useful techniques in environmental microbiology is highly expected.[14] In this study, we examined the protocol for microchip-based T-RFLP analysis and compared its availability with conventional T-RFLP analysis with freshwater samples.

MATERIALS AND METHODS

Freshwater Samples Spring water was collected at Suma spring, Sawanoi spring, Jyurinji Temple (Hyogo Prefecture, Japan), and Tarumi Shrine (Osaka Prefecture, Japan). River water samples were collected at the Minoh River (Osaka Prefecture, Japan). Bottled potable groundwater (natural mineral water) was purchased from a retail store.

Hydroponics solution of the Closed Ecology Experiment Facilities (CEEF; Aomori Prefecture, Japan) was also analyzed. The CEEF were developed to study stable material circulation by the physicochemical system and long-term closed habitation experiments based on material balance between plants, animals, and humans.[15]

The authors declare no conflict of interest.

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The collected samples were kept on ice and immediately analyzed after sampling.

Bacterial Strains Two bacterial strains, *Bacillus cereus* ATCC 14579 and *Pseudomonas aeruginosa* ATCC 10145, were used to estimate the detection limit of on-chip T-RFLP analysis. *B. cereus* or *P. aeruginosa* was inoculated to potable groundwater with the ratio of 0%, 0.1%, 0.25%, 0.5%, 1%, 5%, 10%, and 20% of indigenous bacterial number.

Escherichia coli W3110 was used to prepare the upper marker for on-chip T-RFLP analysis.

These bacterial cells were grown aerobically at 37°C in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl).

DNA Extraction Approximately 100–1000 mL of freshwater or hydroponics solution was filtrated on filters (0.2 μm pore polycarbonate filter, Toyo-Roshi, Tokyo, Japan) to collect bacteria in the samples. These filters were then cut in small pieces and put in beat bead vials with a 1/2 volume of 0.1 mm sterilized glass beads (Biospec Products, Bartlesville, U.S.A.). Genomic DNA of each bacterium and total DNAs from environmental samples were extracted by using a bead-beating method.[16]

PCR Reaction mixtures for PCR contained PCR buffer, each deoxynucleoside triphosphate (dNTP) at a concentration of 200 mM, 2.5 mM $MgCl_2$, each primer at a concentration of 0.1 mM, and 2.5 U of Taq DNA polymerase (Applied Biosystems, New Jersey, U.S.A.) in a final volume of 100 μL. The 16S rRNA fragments were amplified with the primers EUB 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 926R (5′-CCG TCA ATT CCT TTR AGT TT-3′), which are specific for the universally conserved bacterial 16S rRNA gene sequence.[4] The 5′ end of the EUB 8F primer was labeled with fluorochrome Cy5. DNA amplification was performed with the PTC-200 thermal cycler (MJ Research, Massachusetts, U.S.A.) using the following program: 9 min hot start at 95°C, followed by 20 cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 65°C, touch down: 0.5°C per cycle), and extension (3 min at 72°C), and then 15 cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (3 min at 72°C), and a final extension at 72°C for 10 min. PCR products were purified by the MinElute PCR Purification Kit (Qiagen, Maryland, U.S.A.).

Klenow Fragment and Restriction Enzyme Treatment PCR products were treated with Klenow fragment as follows[17] to avoid the production of pseudo-T-RFs. Approximately 1 μg of PCR products were incubated with 5 U of Klenow fragment exonuclease minus in the reaction buffer (TaKaRa, Shiga, Japan) and 50 μM of each of the four dNTPs in a total volume of 100 μL for 1 h at 20°C in the dark.

After Klenow fragment treatment, DNA was recovered by adding 2.5-fold volume of 100% ethanol and 1/10 volume of 3 M sodium acetate. This solution was kept for 1 h at −80°C, followed by centrifugation at 13000×*g* for 30 min (ethanol precipitation). The pellet was washed with 70% ethanol, and then dried and resuspended in 20 μL of distilled water.

These Klenow-treated PCR products were incubated for 2.5 h at 37°C with the restriction enzymes *Hha*I, *Mbo*I or *Msp*I (TaKaRa), followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid (EDTA)).

T-RFLP Analysis by Capillary Electrophoresis The precise lengths of the T-RFs were determined by electrophoresis with the CEQ 8000 automated sequencer (Beckman Coulter, Indianapolis, U.S.A.). For analysis, 1 μL of digested DNA from each sample (DNA concentration was 1 ng/μL, measured by the Quant-iT dsDNA HS Assay Kit [Invitrogen, Carlsbad, U.S.A.]) was mixed with 24.875 μL of loading buffer (Beckman Coulter) and 0.125 μL of DNA fragment length standard (Beckman Coulter). These mixtures were loaded onto the CEQ 8000. After electrophoresis, the lengths of fluorescently-labeled T-RFs were determined by comparison with internal standards.

on-Chip T-RFLP Analysis For on-chip T-RFLP analysis, Cosmo-I (size: 30 cm×45 cm×30 cm; Hitachi High-Technologies, Tokyo, Japan) was used as the microchip electrophoresis system, using i-chip 12 (size: 9.2 cm×6.6 cm×0.15 cm; Hitachi Chemical, Tokyo, Japan) and i-chip gel 3 (Hitachi Chemical). The analysis conditions were as follows: separation voltage, 650 V; temperature, 30°C; and separation time: 650 s. A volume of 1–8 μL of digested DNA (the DNA concentration was 100 ng, measured by the Quant-iT dsDNA HS Assay Kit) was mixed with the lower and upper markers, applied to the well on the chip, and analyzed. The lower marker was prepared by annealing two complementary single strand oligonucleotides of 100 bases labeled by Cy5 (Cy5-MARKER 100s [5′-GTA CGG TCA TCA TCT GAC ACG TAC GGT CAT CAT CTG ACA CGT ACG GTC ATC ATC TGA CAC GTA CGG TCA TCA TCT GAC ACG TAC GGT CAT CAT CTG ACA C-3′] and Cy5-MARKER 100a [5′-GTG TCA GAT GAT GAC CGT ACG TGT CAG ATG ATG ACC GTA CGT GTC AGA TGA TGA CCG TAC GTG TCA GAT GAT GAC CGT ACG TGT CAG ATG ATG ACC GTA C-3′]). The upper marker was developed by amplifying DNA extracted from *E. coli*, using Cy5-labeled EUB8F and Eco607R (5′-TCA CAT CTG ACT TAA CAA ACC G-3′) primers with the above-mentioned PCR conditions, followed by purification by the MinElute PCR Purification Kit (Qiagen).

Multi-Dimensional Scaling Analysis To visualize changes in bacterial community structure, multidimensional scaling (MDS) was performed on the Euclidean distance.[18] The similarity of each T-RF was estimated by fragment size and the ratio of the peak area to the total peak area. The software SPSS Categories 10.0J (SPSS, Tokyo, Japan) was used for analysis.

RESULTS AND DISCUSSION

Detection Limit of on-Chip T-RFLP Analysis and Similarity of Fragment Sizes Determined by Capillary and on-Chip T-RFLP Analyses The conditions of microchip-electrophoresis were first optimized for on-chip T-RFLP analysis. Temperature (15°C, 30°C, and 45°C) and voltage (650 V, 800 V, and 950 V) were examined. A high temperature and high voltage reduced the time for the analysis. However, some peaks overlapped and the resolution was decreased. The best separation was obtained at 30°C with 650 V, and this condition was used for the following experiments.

Groundwater samples inoculated with different numbers of *B. cereus* or *P. aeruginosa* were analyzed by on-chip T-RFLP, and the detection limit of this technique was determined (Fig. 1). The number of indigenous bacteria in this groundwater was 10^5 cells/mL and the detection limit of targeted bacteria by on-chip T-RFLP analysis was 1% (10^3 cells/mL). Bacteria

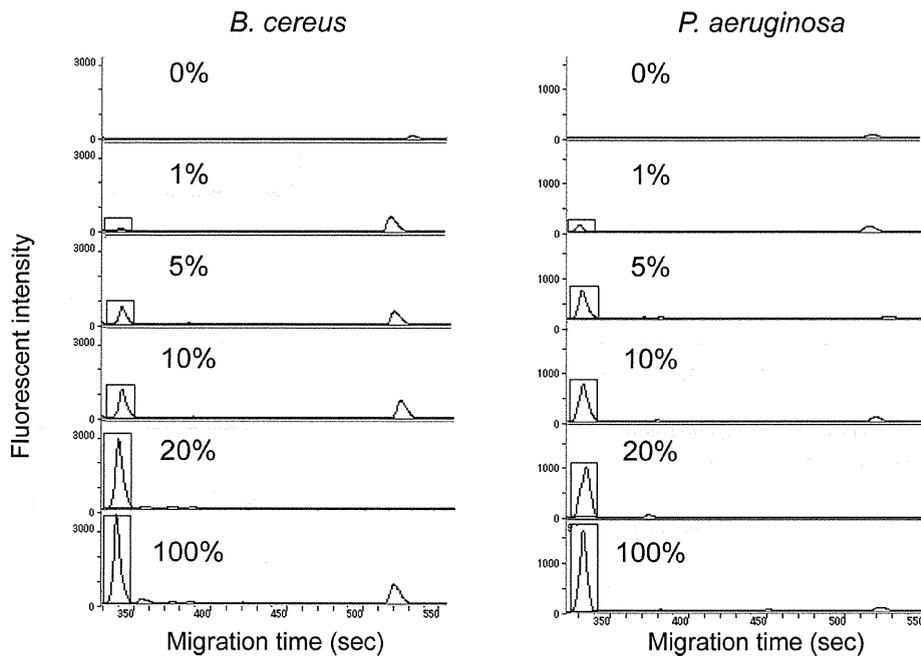### B. cereus

P. aeruginosa

Fig. 1.   on-Chip T-RFLP Profiles of Bacteria Inoculated in Groundwater with Different Ratios

Black squares represent T-RF derived from *B. cereus* or *P. aeruginosa*. PCR products were digested with *Msp*I. The experiments were repeated four times.

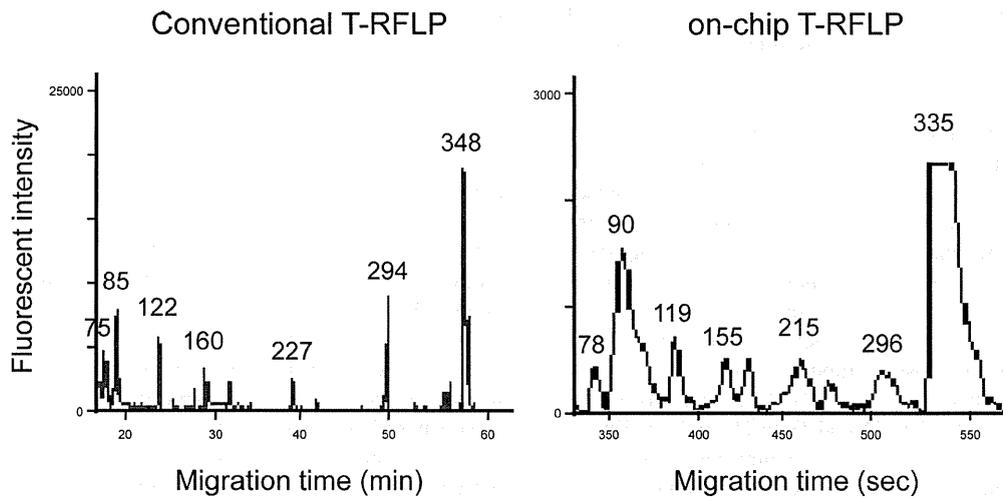### Conventional T-RFLP

on-chip T-RFLP

Fig. 2.   Similarity of T-RFLP Profiles of the Bacterial Community in Groundwater Measured by Conventional T-RFLP with Capillary Electrophoresis and on-Chip T-RFLP Analyses

PCR products were digested with *Hha*I. Numbers above the profiles indicate the size of the fragment determined by each system.

of 0.1%, 0.25%, and 0.5% of the total cells were not clearly detected by on-chip T-RFLP analysis because it was difficult to distinguish bacterial peaks from baseline. The detection limit of both Gram-positive and Gram-negative bacteria was 1% in these experiments (repeated four times).

Profiles for bacterial DNA in freshwater (spring water, river water, and hydroponics solution) were analyzed by conventional T-RFLP with capillary electrophoresis and on-chip T-RFLP, and we examined the similarity of fragment sizes obtained by these different systems. T-RFLP profiles of a freshwater sample obtained by the two analysis methods were similar (Fig. 2). The fragment sizes determined by these two methods were highly correlated (Fig. 3; $r^2=0.98$).

on-Chip T-RFLP enabled rapid analysis. Conventional T-

RFLP required longer than 1 h for the analysis with our condition, while on-chip T-RFLP analysis was completed within 15 min.

Bacterial Community Profiling in Hydroponics Solution
Changes in the bacterial community in hydroponics solution were monitored to estimate the usefulness of on-chip T-RFLP for bacterial community profiling of freshwater samples. This was carried out because bacterial community composition in hydroponics solution is simple and changeable compared with that in natural freshwater environments,[19] and thus is suitable for bacterial monitoring. Changes in T-RFLP profiles of bacterial DNA in hydroponics solution in a 4-week-old culture are shown in Fig. 4. We assumed that certain bacterial species consistently existed and changed their ratio in hydroponics