Biodegradation of microcystins by bacterial communities co-existing with the flagellate *Monas guttula* and concurrent succession of community structures

Jieming Li, Kazuya Shimizu, Yulin Zhou, Motoo Utsumi, Meena Kishore Sakharkar, Zhenya Zhang, Hongwen Sun and Norio Sugiura

ABSTRACT

Grazing on *Microcystis* by the flagellate *Monas guttula* causes simultaneous degradation of microcystins (MCs) produced by *Microcystis* in culture. Although the MC-degrading bacterial strains that co-exist with *M. guttula* have been isolated, it is still unknown if the MC-degrading bacteria can degrade MCs within the indigenous bacterial community co-existing with *M. guttula*. To investigate this, we separated two indigenous bacterial communities (free-living and cell-bound) from *M. guttula* culture to test the ability of each community to degrade MCs. Results showed that MCs were rapidly degraded to undetectable level, and earlier MC exhaustion due to biodegradation was evident after re-spiking with MCs in both communities. These findings show that the MC-degrading bacteria are distributed over both communities, and can degrade MCs within the indigenous bacterial community co-existing with *M. guttula*. Denaturing gradient gel electrophoresis (DGGE) revealed differences in species diversity and structure between the two communities. Cluster analysis for DGGE patterns indicated that cell-bound community structures responded more sensitively than free-living community during degradation, and the two community structures evolved closer genetically with each other along the degradation period.

Key words | bacteria, community, degradation, denaturing gradient gel electrophoresis, microcystin, *Monas guttula*

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INTRODUCTION

The proliferation of cyanobacterial blooms poses a serious threat to ecosystem functioning, adversely affects the wildlife exposed to it and severely compromises public health (Falconer 1999; Azevedo *et al*. 2002; Furukawa *et al*. 2008). Hence, effective control of blooms is of particular concern to the water industry. Among the cyanobacterial species, *Microcystis* is the commonest genus during water blooms (Carmichael 1992) and many strains of it produce virulent hepatotoxic microcystins (MCs), which have been frequently reported to exceed guideline levels in water sources used for potable supply.

Over recent decades, several species of protists and multicellular organisms have been identified as active grazers on *Microcystis*, for example, the amoeba *Penardochlamys* spp. (Nishibe *et al*. 2004), the flagellates *Collodictyon triciliatum* (Nishibe *et al*. 2002) and *Monas guttula* (Sugiura *et al*. 1992), the oligochaete *Aeolosoma hemprichi* and rotifer *Philodina erythrophthalma* (Sugiura *et al*. 1990, 1991; Park *et al*. 1998),

doi: 10.2166/aqua.2011.069

as well as the mixotrophic golden algae *Poterioochromonas* and *Ochromonas* (Cole & Wynne 1974; Zhang *et al*. 2009). These grazers have the potential to remove blooms, and a few are capable of simultaneously degrading MCs while feeding on *Microcystis*. One investigation demonstrated that *Poterioochromonas* can degrade MCs while preying on *Microcystis* cells (Zhang *et al*. 2008). Also, a rapid decrease in MC concentration is observed accompanied by simultaneous *M. guttula* predation on *Microcystis*, suggesting that MCs can be degraded in the presence of *M. guttula* (Saitou *et al*. 2003a). In contrast, some grazers feed on *Microcystis* without degrading MCs. Inamori *et al*. (1998) studied predation of *A. hemprichi* and *P. erythrophthalma* on *Microcystis* cultures and found that there was no significant degradation of microcystin-RR (MC-RR) on addition of these organisms; however, when MC-degrading bacteria were also added to the cultures, the intracellular MC-RR concentration decreased. This implies that MC-RR eluted from *Microcystis* cells was degraded by the added bacteria.

In aquatic environments, the population density of *Microcystis* has been reported to decrease dramatically within a short period in autumn, and the MC concentration in the water body to decrease simultaneously with fading out of the bloom (Sugiura *et al*. 2002). This might be related to predation by *Microcystis* grazers (Sugiura *et al*. 1990, 1992; Iwami *et al*. 1999, 2000; Okano *et al*. 2009; Chen *et al*. 2010). Until recently, few investigations have reported why some specific species of grazers such as the flagellate *M. guttula* have the potential for simultaneous degradation of MCs while feeding on *Microcystis*.

Bacterial communities co-exist naturally with *M. guttula*, as they cannot be removed during the isolation process of *M. guttula* (Fujimoto *et al*. 2007); the communities include a free-living community, and a cell-associated community, made up of intracellular organisms and those adhered to surface mucilage of *M. guttula* cells (*M. guttula* cell-bound, abbreviated as cell-bound). Fujimoto *et al*. (2007) isolated two MC-degrading bacterial strains from the indigenous bacterial community co-existing with *M. guttula*, and confirmed the degradation potential of these strains. This suggests that some bacteria co-existing with *M. guttula* contribute to MC degradation when *Microcystis* is preyed on by *M. guttula*. However, until now the ability of the co-existent bacteria to degrade MCs has not been clarified within the context of indigenous bacterial communities, and no knowledge is available about the structure of the communities. Lack of such information limits in-depth understanding of the ecological function of this flagellate. Therefore, it is imperative to attempt to separate the two pools, free-living and cell-bound, of indigenous co-existing bacterial communities from *M. guttula* culture, aiming to analyse the MC degradation potential of these communities, and the dynamics of the community structures during degradation.

This study focused on investigating and characterising MC degradation by these communities in culture, to clarify if MC degrading bacteria are distributed in either one or both of the communities. Furthermore, to rapidly assess the community structure, denaturing gradient gel electrophoresis (DGGE) was performed to compare the difference in species diversity and structure between the two communities, and to present a global view of the temporal succession of the individual community structure during the biodegradation period.

MATERIALS AND METHODS

Organisms and materials

Microcystis viridis (culture collection No. NIES-102) was obtained from the National Institute for Environmental Studies, Japan. This axenic strain can produce MC-RR, microcystin-YR (MC-YR) and microcystin-LR (MC-LR), and was employed as prey of the flagellate *M. guttula*. *M. guttula* was maintained in culture where it grew from an isolated individual cell via *Microcystis*-predation and was periodically sub-cultured.

MC-RR (≥95% purity), MC-YR and MC-LR (≥90% purity) standard materials were purchased from Wako Pure Chemical Industries, Japan. Solvents used for MC extraction, and standards and samples preparation were of high performance liquid chromatography (HPLC) grade.

All glassware was autoclaved at 121 °C for 20 min prior to use.

Preparation of *M. guttula* culture

M. viridis was incubated in 740 mL of M-11 medium (100 mg L^{-1} $NaNO_3$, 75 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 40 mg L^{-1}

$CaCl_2{\cdot}2H_2O$, 20 mg L^{-1} Na_2CO_3, 1 mg L^{-1} $FeSO_4{\cdot}7H_2O$, and 1 mg L^{-1} $Na_2{\cdot}EDTA{\cdot}2H_2O$, autoclaved at 121 °C for 20 min, pH 8.0) at 30 °C in 12 h:12 h light-dark cycles (12:12 LD cycle) to obtain a suspension culture. The *M. viridis* suspension was divided into two equal portions; 30 mL of *M. guttula* culture was inoculated into one of the suspension fractions, and 30 mL of autoclaved M11 medium was added into the other fraction as a control. To evaluate MC removal in the presence of *M. guttula*, aliquots (4.5 mL) were periodically removed aseptically. Both intracellular and extracellular MCs were extracted from the aliquot and detected. The aliquot was pre-treated as described by Nicholson *et al.* (1994) with minor modification. Briefly, acetic acid was added to the aliquot at a final concentration of 5% to elute intracellular MCs into the aqueous phase. Total MCs in the aliquot were solid-phase extracted by ODS C_{18} cartridge (Wako, Japan) primed beforehand with methanol and Milli-Q water successively, then eluted from the cartridge by 10 mL of methanol. The eluate was evaporated to dryness at 40 °C and the residue was dissolved in 1.0 mL of methanol.

Simultaneously, *M. viridis* and *M. guttula* cell densities, and pH in culture were periodically determined by directly counting under microscopy (BX50, Olympus, Japan) using an eosinophil counter (Kayagaki Irika Kogyo Co., Ltd.), and a pH meter (Mettler Toledo, MP 220), respectively.

At the stationary phase of *M. guttula* culture when no MCs could be detected in the aliquot, co-existing bacterial communities were separated from the culture.

MC analysis

MCs were analysed by HPLC (Shimadzu 10A series, Shimadzu, Japan) using the following conditions: 50 μL of filtered (pore size: 0.2 μm, PTFE Hydrophilic, Millipore) sample was injected into a 3.0 × 250 mm SunFire™ C_{18} column with 5 μm diameter particle size (Waters, Ireland) with an oven temperature of 40 °C. The mobile phase was 50% methanol in 0.05 M phosphate buffer (pH 2.5), with a flow rate of 0.58 mL min^{-1}. The concentration of MCs was measured by calibrating the peak areas (at wavelength of 238 nm) with corresponding external standards. The HPLC system had a detection limit of 0.1 μg L^{-1}.

Experimental procedure

Separation of co-existing bacteria and MC-biodegradation test

Two co-existing bacterial communities, free-living and cell-bound, were separated from *M. guttula* culture. Under a low vacuum, 160 mL of stationary-phase *M. guttula* culture was filtered through membranes of pore size 3.0 and 0.45 μm (Nitrocellulose, Millipore) in sequence to remove the cells of *M. guttula* and residual *M. viridis* from the culture, respectively. Three individual membranes of the same pore size were employed successively during each filtration process to enable effective filtration. Free-living bacterial cells in flow-through filtrate were retrieved onto membranes of pore size 0.22 μm (Nitrocellulose, Millipore) by vacuum. Then the 0.22 μm-pore membranes were transferred into a sterilised tube containing autoclaved M11 medium to allow the trapped bacterial cells to resuspend by shaking the tube. Neither *M. guttula* nor *M. viridis* cells can be observed in the bacterial suspension by microscopy, thus indicating that the free-living bacterial community has been separated.

M. guttula and *M. viridis* cells collected on the 3.0 μm-pore membranes were then allowed to resuspend in autoclaved M11 medium. Simultaneous cell counting demonstrated that *M. guttula* cell numbers did not dramatically decrease, suggesting *M. guttula* cells can tolerate the vacuum when trapped onto the membranes. For separation of the cell-bound community from the flagellate, the resuspended *M. guttula* cells in medium were thoroughly disintegrated by vortex agitation. Complete disintegration was subsequently checked by microscopy. Both the intracellular and mucilage-adhered community associated with *M. guttula* were presumably released. The cell lysate of *M. guttula* was then filtered through 0.45 μm-pore membrane to remove the *M. viridis* and *M. guttula* cell residues. The downstream procedure for obtaining the cell-bound bacterial community suspension, with volume equal to the free-living community suspension, was identical to the procedure described above.

MC-RR, -YR, and -LR used in biodegradation test were extracted from a stationary-phase axenic culture of *M. viridis*. The MC biodegradation test was carried out in a series

of 50-mL glass test tubes sealed with rubber stoppers. Each community suspension was homogenised well and 1 mL of the suspension was aseptically transferred into 9 mL of autoclaved M11 medium to give a final volume of 10 mL in a test tube. MC extraction was spiked at an initial concentration of 100 μg L^{-1} for MC-RR and -LR, and 25 μg L^{-1} for MC-YR in culture. A sterile control was set up for the loss of MCs due to any abiotic factors. All the tubes were incubated at 30 °C in a 12:12 LD cycle and samples were taken periodically. At each sub-sampling, 1.0 and 0.5 mL aliquots were removed from the test tube for MCs analysis and cell counting, respectively. The 1.0 mL aliquot was filtered through a membrane (pore size: 0.22 μm, PTFE Hydrophilic, Millipore). To remove any residual fraction of MCs adsorbed on bacterial cells, 0.5 mL of methanol was passed slowly through the membrane to rinse cells trapped on the membrane. The two filtrates were thoroughly mixed and the MC concentration was immediately measured using HPLC. The remaining volume of culture in the test tube was used for total DNA extraction. Such sub-sampling was conducted in duplicate.

MCs were re-spiked in the remaining test tubes to restore the initial concentration when exhaustion of initially-spiked MCs was observed in duplicate. All glassware was autoclaved at 121 °C for 20 min prior to use.

Bacterial counts

Bacterial density in the culture was determined by direct counting. Bacterial cells in the diluted aliquot were vacuum-filtered onto Poretics® black polycarbonate membrane (pore size: 0.2 μm, Funakoshi Company) and covered with 1.0 mg mL^{-1} of ethidium bromide solution for 15 min. Next, the membrane was air-dried and mounted on a slide with non-fluorescence immersion oil (Olympus). Cell counts were performed by epifluorescent microscopy (BX51, Olympus). Ten replicate fields of view containing between 30 and 300 cells were counted and cells mL^{-1} were determined.

DNA extraction

The bacterial cells in culture were vacuum-collected on 0.22 μm-pore membranes (Nitrocellulose, Millipore). Total DNA was extracted with a bead beating kit according to manufacturer's instructions (ISOIL Bead Beating; Nippon Gene, Japan). The resulting DNA was suspended in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the DNA concentration was determined spectrophotometrically at 260 nm by GeneQuant (Amersham Biosciences).

PCR amplification and DGGE

For DGGE analysis, a 193-bp 16S rRNA gene fragment was amplified with the primers 341F-GC clamp and 518R (Muyzer *et al.* 1993). Duplicate total DNA extracts from each sub-sampling were pooled to use in a polymerase chain reaction (PCR). The PCR was performed on a GeneAmp® 9700 PCR system (Applied Biosystems). Each 20 μL reaction mixture contained 1× Ex *Taq* buffer, 2.0 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.5 U *Taq* DNA polymerase (Takara Bio, Japan), 5.0 pmol of each primer and 1.0 μL of DNA templates. Thermal cycling commenced with an initial denaturation at 94 °C for 5 min, followed by a touchdown procedure consisting of denaturation at 94 °C, annealing and primer extension at 72 °C; each step was conducted for 30 s and the initial annealing temperature of 65 °C was subsequently decreased by 0.5 °C per cycle until the touchdown temperature of 55.5 °C was reached. Ten additional cycles were then conducted at a constant annealing temperature of 55 °C. The reaction mixtures were incubated at 72 °C for 7 min.

DGGE was performed with a Bio-Rad Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA) (Pedro *et al.* 2001). Equal amounts of PCR products (approximately 200 ng) were loaded onto 8% (*w/v*) polyacrylamide gel cast in 0.5× TAE buffer [20 mM Tris base, 10 mM acetic acid, 0.5 mM EDTA (pH 8.0)]. The polyacrylamide gel (acrylamide:bisacrylamide, 37.5:1) was made with denaturing gradient ranging from 30% to 70% (100% denaturant contained 7.0 M urea and 40% formamide). DNA fragments were separated by electrophoresis at 60 °C with constant voltage of 60 V for 10.5 h. Following electrophoresis, the gel was stained for 30 min in Gel-Red (Biotium) solution in 0.5× TAE and immediately documented by UV trans-illumination. The 16S rRNA gene fragment of *Sphingomonas* spp. strain MD-1 (Saitou *et al.* 2003b) was used to confirm the co-migration of the identical DNA

sequence. Reproducibility of DGGE was tested by a parallel independent PCR-DGGE analysis, obtaining identical patterns (data not shown).

Statistical analysis

A one-way repeated-measure analysis of variance (ANOVA) (Origin 6.0) was used to determine whether there were significant differences between the treatments of different factor levels; a p-value < 0.05 was considered significant.

Analysis of DGGE patterns

DGGE banding patterns were analysed with Quantity One version 3.0 (Bio-Rad laboratories, Hercules, California, USA) to generate a densitometric profile for each lane. Bands that migrated to the same position in different lanes of the gels were identified by normalising the gel to two standard patterns (marker).

The initial species diversity of the two communities and the change in diversity during the course of MC biodegradation were estimated with the Shannon-Wiener diversity index (H') using Equation (1):

$$H' = -\sum(P_i)(\ln P_i) \quad (1)$$

where P_i is the relative intensity of band i in a DGGE lane and can be calculated as $P_i = n_i/N$, n_i is the height of a peak and N is the sum of all peak heights of bands in the densitometric profile (Shannon & Weaver 1963). A higher H' value corresponds to greater diversity in the community. Phylotype richness (R) was evaluated from the number of bands detected in a DGGE lane (Fromin *et al.* 2002). Species evenness, or species equability (E), was calculated using Equation (2) (Pielou 1969):

$$E = H'/\ln R \quad (2)$$

Cluster analysis was used to assess the temporal succession in both bacterial communities during the degradation period. For this purpose, the DGGE banding patterns for all lanes were converted into a binary matrix. The presence or absence of individual bands sharing identical migration position in different lanes was taken into account and marked with G or A, respectively. The resulting binary (GA) data was then used to generate two distance matrices for the respective communities by computing pairwise distances with the software package Molecular Evolutionary Genetics Analysis version 4.0 (MEGA, Arizona State University, Tempe, AZ, USA). Finally, dendrograms representing succession of community structure were constructed by unweighted-pair group method using arithmetic averages (UPGMA) in the MEGA. The robustness of the grouping bootstrap analysis was assessed with 1,000 replicates.

mlrA gene cluster detection

The *mlrA* gene encodes the MC-degrading enzyme responsible for cleaving the ADDA-Arg peptide bond of cyclic MC structures (Bourne *et al.* 1996, 2001). *mlrA* has been characterised in many isolates and successfully used for detecting the presence of MC-degrading bacteria (Bourne *et al.* 2001; Saito *et al.* 2003; Shimizu *et al.* 2009). To verify if the enzymatic pathway of MC degradation by bacteria co-existing with *M. guttula* was similar to those conserving *mlrA* homologues, *mlrA*-targeted PCR assays was conducted for each sub-sample using the primers MF-MR and MF2-MR2 (5′-GCATACGAA GACAGCGATGA-3′) (Saito *et al.* 2003). MC degrading isolates *Sphingomonas* spp. strains MD-1 and Y2 (Park *et al.* 2001) and *Sphingopyxis* spp. strain C-1 (Okano *et al.* 2009) were the positive controls. Each 20 μL reaction mixture contained 1× Ex *Taq* buffer, 2.0 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.5 U *Taq* DNA polymerase (Takara Bio Inc., Japan), 5.0 pmol of each primer and 2.0 μL of DNA templates. Thermal cycling comprised an initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s and primer extension at 72 °C for 1 min (MF-MR) or 30 s (MF2-MR2).

RESULTS AND DISCUSSION

Estimation of MCs and *M. viridis* cell density in the presence of *M. guttula*

The *M. viridis* cell density decreased in *M. guttula* culture, compared with the control in which it increased

consistently. Moreover, a remarkable increment in *M. guttula* cell density commenced from day 2 due to predation (Figure 1). *M. guttula* were observed grazing on *Microcystis* cells under microscopy. This predation triggered a 0.79 order of magnitude increment in *M. guttula* cell density. On day 5, *M. guttula* cell density achieved the highest value of 12.08×10^4 ($\pm 4.69 \times 10^4$) cells mL^{-1}. The pH continuously fell to nearly neutral in *M. guttula* culture from day 2, whereas pH in the control remained consistently alkaline (Figure 1). The fall of pH probably correlates to the decrease in photosynthesis due to decreasing cyanobacteria population (Okano *et al.* 2009), and implies that *M. guttula* may alleviate the high rise in pH during cyanobacterial blooms, relating to the water quality improvement.

Moreover, the total concentration of each analogue of MC (MC-RR, -YR, and -LR) simultaneously decreased with the decrease of *Microcystis* cell density in *M. guttula* culture. The concentration of MC-RR and -YR decreased to undetectable level by day 2, and MC-LR decreased to undetectable level by day 4. However, total concentration of each analogue of MCs increased continuously in the control (Figure 2).

On day 5, separation of the two co-existing bacterial communities from *M. guttula* culture was performed to test the potential for MC degradation.

MC-biodegradation characteristics of bacteria communities

As shown in Figure 3, no obvious losses of MCs were observed in parallel sterile controls, indicating that any

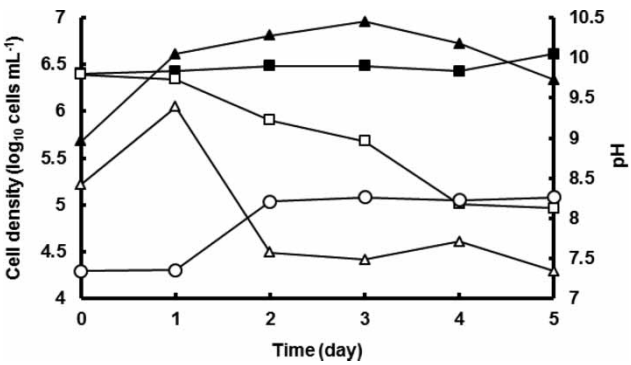

Figure 1 | Dynamics of cell density and pH in control and *M. guttula* culture (—■—: *Microcystis* cell density in control; —□—: *Microcystis* cell density in *M. guttula* culture; —○—: *M. guttula* cell density in *M. guttula* culture; —▲—: pH in control; —△—: pH in *M. guttula* culture).

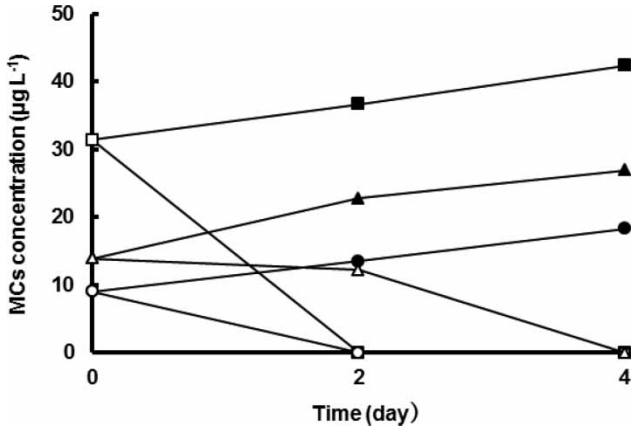

Figure 2 | Dynamics of MC concentration in control and *M. guttula* culture (—■—: MC-RR in control; —□—: MC-RR in *M. guttula* culture; —●—: MC-YR in control; —○—: MC-YR in *M. guttula* culture; —▲—: MC-LR in control; —△—: MC-LR in *M. guttula* culture).

removal of MCs in culture samples resulted from biological degradation. A lag period of 1 day was evident prior to each community initiating degradation of the three MC analogues. Fifty percent of initial concentrations for MC-RR, -YR and -LR were degraded by around day 1.9, day 2.0 and day 2.1 in the presence of the free-living community, respectively. In contrast, in the presence of the cell-bound community, 50% of initial concentration for MC-RR, -YR and -LR were degraded by around day 3.4, day 2.3 and day 3.2, respectively. Complete removal of MCs was observed by day 3 for the free-living community (Figure 3(a)), and day 5 for the cell-bound community (Figure 3(b)).

MCs were then re-spiked into remaining samples of respective community to restore initial concentration. In the free-living community sample, MC-RR and -YR were both completely removed on day 1 after re-spiking, while MC-LR degraded below 10% of initial concentration at the same time, and no MC-LR was detected on the following day. This successive addition led to an earlier exhaustion in comparison with the complete removal achieved at day 3 after initial spiking (Figure 3(a)). Similar degradation profile was also exhibited in cell-bound community sample, in which complete MC removal was achieved on day 3 after re-spiking, earlier than that after initial spiking (Figure 3(b)). After re-spiking, 50% of initial concentrations for MC-RR, -YR, and -LR were degraded by around day 0.6, day 0.5 and day 0.6, respectively, in the presence of the free-living community, while 50% of initial concentration for MC-RR, -YR and -LR

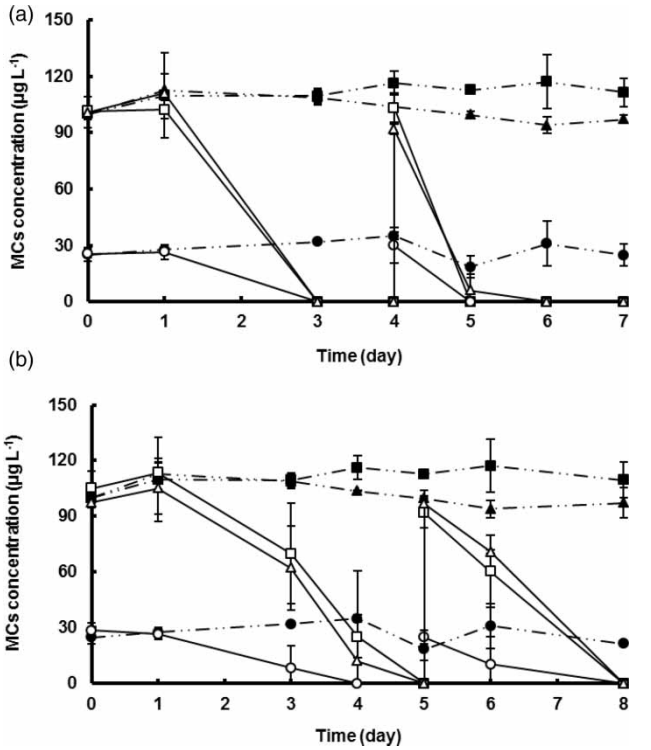

Figure 3 | Dynamics of MC concentration in culture samples of bacterial communities throughout the biodegradation test period. (a) Free-living; (b) Cell-bound. Bars represent the standard errors of the means (-■-: MC-RR in control; —□—: MC-RR in sample; -●-: MC-YR in control; —○—: MC-YR in sample; -▲-: MC-LR in control; —△—: MC-LR in sample).

were degraded by around day 1.3, day 0.8 and day 1.5, respectively, in the presence of the cell-bound community.

M. guttula has been maintained for decades in *Microcystis* culture since its isolation from Lake Kasumigaura, Japan. During this coculture, bacterial communities co-existing with *M. guttula* either survived or further acclimatised to MCs. Our results clearly demonstrate that both bacterial communities from *M. guttula* can effectively degrade three MC analogues (MC-RR, -YR and -LR). Thus, it can be concluded that MC-degrading bacteria are distributed over both communities.

After initial spiking with MC, minor acclimatisation was required for communities to establish the specific enzyme activities, despite pre-exposure to MCs produced by *M. viridis* during the preparation of *M. guttula* culture. In contrast, re-spiking of MCs triggered an elimination of lag period before biodegradation commencement and led to earlier MC exhaustion (Figure 3). Our results concur with the findings of Varelia *et al.* (2006), who reported on MC degradation by *Sphingomonas* spp. CBA4 isolation in culture. These findings suggest that once expression of MC-degrading enzymes has been previously induced, efficient production of corresponding enzymes is able to proceed as long as re-exposure to MCs occurred (Ho *et al.* 2007).

Based on this study, we can speculate that when *M. guttula* filter feeds on *M. viridis*, MCs eluted intact are degraded *in vivo* by intracellular MC-degrading bacteria, while the MCs released from *M. guttula* cells and originally dissolved in culture can be utilised *in vitro* by some mucilage-adhered and free-living bacteria.

Although the MC biodegradation by co-existing bacteria has been confirmed, it is still premature to conclude that MC degradation in the presence of *M. guttula* can exclusively be attributed to these bacteria. Further tests are required to clarify the contribution of *M. guttula* to MC-degradation in culture with removal of the effect of bacterial community. Additionally, it would be interesting to investigate why some MC-degrading bacteria specially prefer to co-exist with certain protist species such as the flagellate *M. guttula*, rather than many other protist species.

Bacterial cell growth corresponding to MC degradation

The free-living community showed a higher initial cell density of 3.72×10^6 cells mL^{-1}, compared with the initial density of 0.34×10^6 cells mL^{-1} in the cell-bound community. In addition, cell density in the free-living community was consistently higher than in the cell-bound community throughout the degradation period (Figure 4), with significantly ($p < 0.05$) higher density than cell-bound community on day 0, 1, 3, and 4, corresponding to more rapid MC biodegradation in the free-living community. Along the entire degradation course, 1.31 and 2.20 orders of magnitude increments in cell density were observed for the free-living and cell-bound communities, respectively.

The increment patterns of bacterial cell density in both communities are in agreement with Hoefel *et al.* (2009), who reported a continuous increment of bacterial counts within sand filter biofilm during water treatment process for MCs by using 16S rRNA-targeted qPCR. This implies that MCs may provide associated bacteria in communities with a significant carbon and/or nitrogen source for their

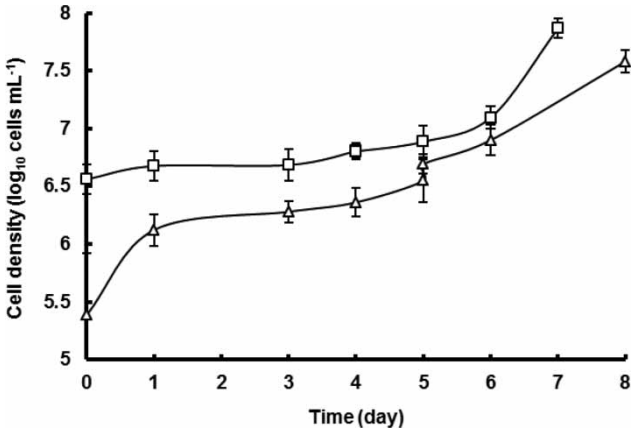

Figure 4 | Dynamics of bacterial cell density in communities throughout the biodegradation test period. Bars represent the standard errors of the means (—□—: Free-living; —△—: Cell-bound).

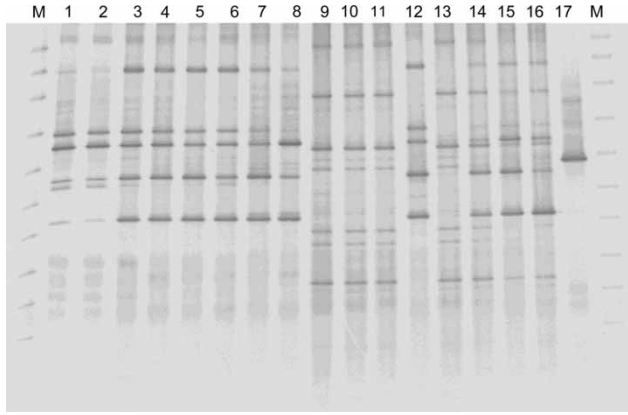

Figure 5 | DGGE banding patterns of bacterial 16S rRNA gene fragments for the culture samples of bacterial communities at various days of biodegradation test period. Lane 1–8: samples for the free-living community on day 0, day 1, day 3, day 4, day 4 (RS)[a], day 5, day 6 and day 7, respectively. Lane 9–16: samples for the cell-bound community on day 0, day 1, day 3, day 4, day 5, day 5 (RS), day 6 and day 8, respectively. Lane 17: sample for 16S rRNA gene fragment of *Sphingomonas* sp. strain MD-1. M: DNA reference marker. [a]RS: re-spiking; the sample had been subjected to MC re-spiking on the same day.

proliferation, which in turn facilitates MC removal. Difference in cell density during the entire study period may trigger distinct degradation rates in both communities. Also, increase in cell density probably correlates with elevated activity of associated bacteria, which play a role in MC biodegradation. Compared with the MC degradation profiles, the cell density of both communities increased without a lag period, suggesting that the bacterial population is required to accumulate prior to initiating MC degradation.

Analyses of DGGE patterns

Diversity comparison of bacteria communities

DGGE patterns with intense bands indicated that many species were present in temporal succession of the two communities along the degradation process (Figure 5). The cell-bound community displayed greater values of species richness (13) and evenness (0.97) in initial culture on day 0, than the values of species richness (10) and evenness (0.95) in the free-living community on day 0 (Table 1). A time-dependent decrease and subsequent rebound in richness value were exhibited in both communities, with the lowest richness values (8 for free-living community on day 3, 5 for cell-bound community on day 4), during the period before re-spiking of MCs. Interestingly, the highest value of richness (11 for free-living community day 4, 14 for cell-bound community on day 5) was displayed for both communities on the re-spiking with MCs (Table 1). The free-living community showed lower evenness than the cell-bound community throughout study period, except for a slightly higher evenness of 0.96 on day 6 (Table 1).

H' is a useful first-line approach to estimate the diversity of a community. A greater diversity was present in the initial cell-bound community (2.49) than the free-living community (2.18) (Table 1). Similar to richness values, a common fluctuating trend is apparent in diversity for both communities, with the lowest H' values (1.93 for free-living community on day 3, 1.59 for cell-bound community on day 4) before re-spiking with MCs, and the highest H' values (2.29 for free-living community on day 6, 2.54 for cell-bound community on day 5) after re-spiking (Table 1).

Temporal succession of bacterial community structures

Two dendrograms were constructed to interpret the succession of community structure by cluster analysis (Figure 6(a) and (b)). Each of these dendrograms contained two clusters. In the dendrogram for the free-living community, at least 55% similarity was shared amongst all the patterns. The patterns of the first five days (day 0 to day 5) were located in one cluster, with at least around 90% similarity with one another, while those of day 6 and day 7 were grouped

Table 1 | Dynamics of Shannon-Wiener diversity index (H'), evenness (E) and richness (R) in communities throughout the biodegradation test period

Item	Community	Time elapsed Day 0	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Shannon-Wiener index (H')	Free-living	2.18	2.08	1.93	2.22 2.22 (RS)[a]	2.15	2.29	2.10	
	Cell-bound	2.49	2.32	2.15	1.59	2.33 2.54 (RS)	2.18		2.21
Evenness (E)	Free-living	0.95	0.95	0.93	0.93 0.93 (RS)	0.93	0.96	0.91	
	Cell-bound	0.97	0.97	0.98	0.99	0.97 0.96 (RS)	0.95		0.96
Richness (R)	Free-living	10	9	8	11 11 (RS)	10	11	10	
	Cell-bound	13	11	9	5	11 14 (RS)	10		10

[a]RS: re-spiking, representing the sample had been subjected to MCs re-spiking on the same day.

in another cluster with nearly 80% similarity (Figure 6(a)). In contrast, more variation was evident in community structure succession of the cell-bound community along the entire study period (Figure 6(b)), the patterns forming one cluster were largely distinct from the remaining patterns grouped into the other cluster. Even in the same cluster, some patterns shared less than 60% similarity. This suggests that the cell-bound community structure is more dynamic than the free-living community during MC degradation.

To compare the succession in community structures between the two communities, one dendrogram was further constructed using the initial patterns on day 0 and patterns at the end of study period (Figure 6(c)). Interestingly, the resulting dendrogram hinted that the community structures of the two communities greatly differed from each other on day 0, while the community structures shared approximately 60% similarity at the end of study period. This result suggests that the structures of the two communities evolved closer genetically, with a decrease in genetic distance, during the degradation process.

To the best of our knowledge, this is the first attempt to elucidate the community structures of co-existing bacterial communities with *M. guttula* and dynamic shifts of structures responding to MC-degradation. As the two communities differed in the initial diversity and structure, as well as in the cell counts, it is envisaged that the two communities may be differentiated due to the microbiological environment of *M. guttula* cells. Especially for the cell-bound community, DGGE patterns showed that some bands appearing during the later phase of degradation period were absent on day 0, suggesting the species represented by these bands were in low abundance within the initial community and were enriched upon MC spiking.

DGGE analysis revealed a complex profile for each community throughout the degradation process, with no band becoming conspicuously predominant, implying that the MC biodegradation perhaps involved many bacteria within each community. In general, present profiles may have resulted from either cross-feeding with MC and its metabolites between different members of bacteria in community along the degradation pathway, or several bacteria capable of totally degrading MCs.

mlrA gene cluster detection

No *mlrA* amplification was detected at each sub-sampling, whereas *mlrA* homologues products were obtained in all positive controls using each primer set (data not shown). This suggests that novel enzymatic pathways for MC-degradation, not related to *mlrA* gene, may be harboured

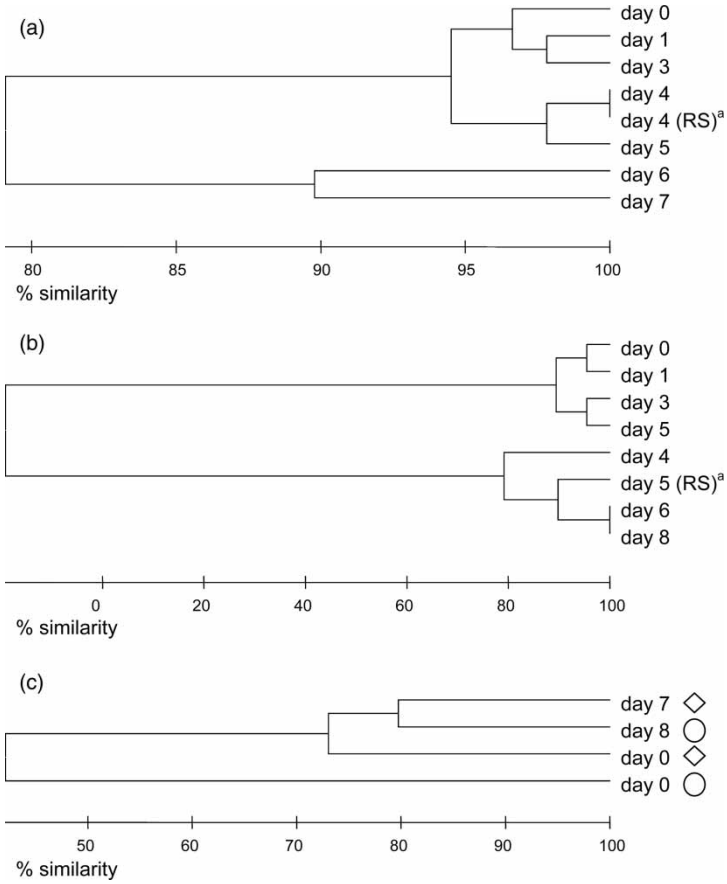

Figure 6 | Cluster analyses of the DGGE banding patterns for the culture samples of bacterial communities. The succession of free-living and cell-bound community structure throughout the biodegradation test period is represented by dendrograms (a) and (b), respectively. Dendrogram (c) represents the comparison of succession from day 0 (initial) to the end of biodegradation test period between both communities (◇: Free-living; ◯: Cell-bound). Bars represent percentage similarity. [a]RS: re-spiking; the sample had been subjected to MC re-spiking on the same day.

in the co-existing MC degrading bacteria. It is also possible that *mlrA* homologues maybe present but the sites are not well-targeted by primers due to some sequence differences (Manage *et al.* 2009; Chen *et al.* 2010). The isolation and identification of MC-degrading bacteria from *M. guttula* culture are underway to substantiate this.

The current study provides new insights on the scenario of MC degradation in the presence of *M. guttula*, and emphasises the ecological function of bacterial communities co-existing with *M. guttula*. The findings presented here will facilitate understanding the cooperative pathways for MC removal involved in co-existing systems comprising different species in nature. Further, optimisation of growth conditions for *M. guttula* deserves future investigation, because it can enable the *M. guttula* bacterial co-existing system to act more effectively in bloom removal. Ultimately, optimisation of this cooperative process may realise a cost-effective application of *in situ* remediation technology.

CONCLUSIONS

This is the first study to demonstrate MC degradation by two co-existing bacterial communities and the distribution of MC-degrading bacteria associated with *M. guttula*. Results show that the two communities differ in the degradation rates probably due to distinct MC-degradating bacterial populations and community structures, but both indicate earlier MC exhaustion after re-spiking. Moreover, community structure analysis by DGGE has revealed: (1) distinct

diversities and composition structures between the two communities, (2) more variation of structure succession in the cell-bound than the free-living community, and (3) that the structures of the two communities evolve closer genetically to each other along MC-degradation period. DGGE profiles compromise the identification of predominant species probably responsible for MC biodegradation. On the other hand, the profiles suggest a range of unidentified bacteria in community may be involved in the pathway of MC degradation.

ACKNOWLEDGEMENTS

The authors thank Dr Hideaki Maseda in the University of Tokushima, Japan for helpful discussions. This study was fund by a Grant-in-Aid for Scientific Research Category 'B' (21310049) from the Japan Society for the Promotion of Science, and the Japan Securities Scholarship Foundation.

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First received 10 October 2010; accepted in revised form 14 June 2011