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Development of an *mlrA* Gene-Directed TaqMan PCR Assay for Quantitative Assessment of Microcystin-Degrading Bacteria within Water Treatment Plant Sand Filter Biofilms[▽]

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We report for the first time a quantitative *mlrA* gene-directed TaqMan PCR assay for the rapid detection of microcystin-degrading bacteria. This was applied, in combination with 16S ribosomal DNA-directed quantitative PCR and denaturing gradient gel electrophoresis, to study virgin sand filter column biofilm development and to correlate *mlrA* gene abundance with microcystin removal efficiency.

It has been predicted that the changing climatic conditions around the world are likely to increase both the occurrence and the intensity of blue-green algal (cyanobacterial) blooms (16). Of particular concern to the water industry are the blooms of *Microcystis*, *Anabaena*, *Nostoc*, and *Planktothrix* species, which are capable of producing microcystin toxins within surface water storages used for potable water supply (4, 5, 12). In dissolved (extracellular) form, microcystins are not efficiently removed by conventional water treatment processes (6), and more advanced treatment options, such as activated carbon application or ozonation, are usually employed. However, these are expensive alternatives, and removal efficiencies are often compromised by the presence of natural organic matter (15). Biological filtration of microcystins is now recognized as an alternative treatment barrier (1, 7, 8, 14) and is favored by water utilities, as the process is generally low technology, chemical free, and requires little maintenance, where retrofitting of the process into existing water treatment plant (WTP) infrastructure is often feasible.

To date, 10 different microcystin-degrading bacteria have been isolated from rivers, lakes, and biofilters (7), and the *mlr* gene cluster has been demonstrated to encode proteins involved in the initial steps of microcystin biodegradation by such organisms (2, 3). The MlrA protein is responsible for the initial hydrolytic cleavage of the cyclic microcystin structure, and conventional *mlrA* gene-directed PCR has been employed for qualitative detection of microcystin-degrading bacteria from lakes (18) and within the biofilm of biofilters (1, 7, 8). However, these conventional PCR assays do not allow for accurate quantitation of *mlrA* gene abundance and have not been designed with degenerate primer sequences to allow for variations that exist between different *mlrA* homologues. In this study, we report for the first time a quantitative *mlrA* gene-directed TaqMan PCR assay, including degenerate oligonucleotides targeting conserved DNA regions, for the rapid detection of microcystin-degrading bacteria.

Using all available *mlrA* nucleotide sequences to date (GenBank accession numbers DQ112243, AF411068, AB114203, AB161685, and AB114202), primers qmlrAf (5′-AGCCCKGG CCCRCTGC-3′) and qmlrAr (5′-ATGCCARGCCCACCAC AT-3′) and the TaqMan probe qmlrA-tm, which was labeled with 6-carboxyfluorescein (FAM) at the 5′ end and labeled with black hole quencher 1 (BHQ1) at the 3′ end (5′-FAM-TGCCSCAGCTSCTCAAGAAGTTTG-BHQ1-3′), were designed to target highly conserved regions of the *mlrA* gene for quantitative TaqMan PCR. Reactions resulted in the amplification of a 120-bp product and were carried out in quadruplicate on a Rotor Gene 6000 (Corbett Research, New South Wales, Australia) thermal cycling system. Each 25-μl reaction mixture contained 200 μM of each deoxynucleoside triphosphate, 2.0 mM of $MgCl_2$, 1× PCR buffer, 0.5 μM of primers qmlrAf and qmlrAr, 0.25 μM of TaqMan probe qmlrA-tm, 400 μg/ml bovine serum albumin, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 2.5 μl of either a DNA standard or sample template. Thermal cycling conditions consisted of an initial denaturation at 95°C for 2 min followed by 45 cycles of denaturation at 95°C for 5 s and annealing/extension at 62°C for 25 s. Data were collected in the FAM channel (gain set to 6) at the end of each annealing/extension step.

Quantitation was performed using DNA standard curves constructed from a serial dilution, in Milli-Q water, of an 807-bp *mlrA* gene fragment (18) from the two microcystin-degrading bacteria *Sphingomonas* sp. strain ACM-3962 (2) and *Sphingopyxis* sp. strain LH21 (7). Results were linear over the range of 1×10^1 to 1×10^9 *mlrA* gene copies/μl with linear coefficient values (R^2) for ACM-3962 and LH21 of 0.999 and 0.998 and reaction efficiencies of 0.97 and 0.99, respectively. Specificity of the *mlrA* TaqMan assay was verified by the absence of signals for genomic DNA from the non-microcystin-

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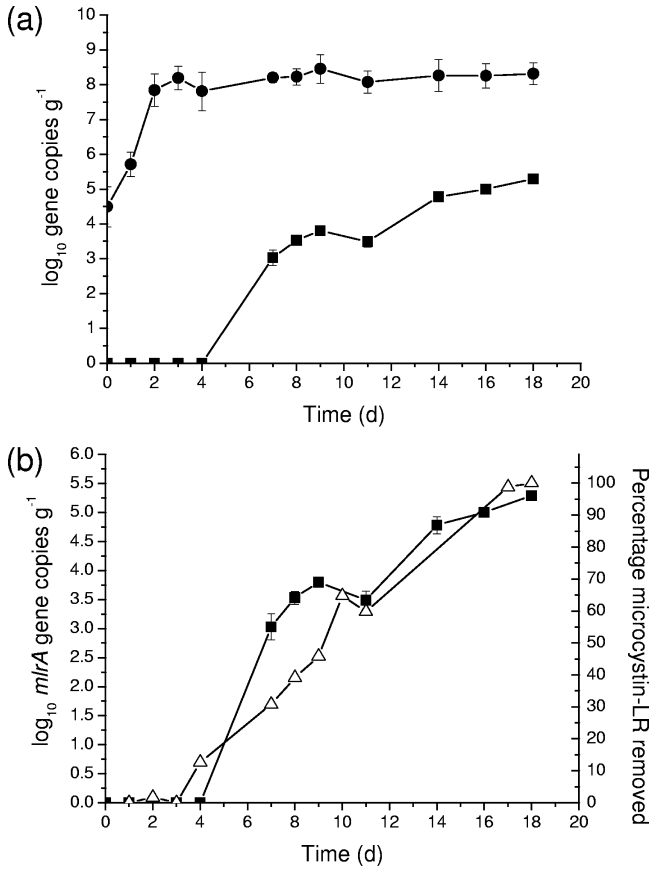

FIG. 1. (a) Abundance of 16S rDNA (closed circles) and *mlrA* gene copies (closed squares) within the sand filter column biofilm. (b) Abundance of *mlrA* gene copies (closed squares) within the sand filter column biofilm and the percentage of microcystin-LR removed (open triangles) through the column. Error bars represent standard deviations of the results for triplicate analyses.

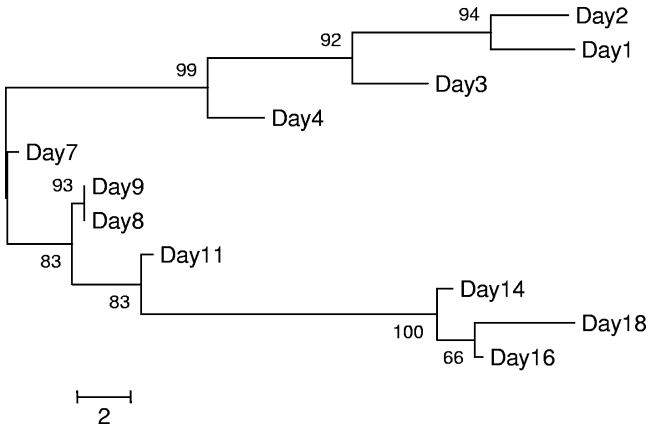

FIG. 2. Cluster analysis of DGGE band profiles for the sand filter column study. Scale bar represents two band differences.

degrading bacteria *Aeromonas hydrophila* (ATCC 7966), *Bacillus subtilis* (ATCC 10145), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145), and *Staphylococcus epidermidis* (ATCC 12228).

Determination of the DNA extraction efficiency, linear range, and the limit of detection of the TaqMan PCR assay under environmentally relevant conditions was achieved by the spiking of known amounts of *Sphingopyxis* sp. strain LH21 into Myponga reservoir water and Morgan WTP filter sand (containing a biofilm) in $\log_{10}$-fold increments ranging from 0 to 1×10^8 cells/ml or g, respectively. DNA from each water (0.5 ml) and sand preparation (0.5g) was then extracted in triplicate using the UltraClean soil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA). For reservoir water, the TaqMan assay was linear over 6 $\log_{10}$ orders of magnitude, with an R^2 of 0.998 and a limit of detection equivalent to 10^2 *mlrA* copies/ml. For sand filter medium, the TaqMan assay was linear over 5 $\log_{10}$ orders of magnitude, with an R^2 equivalent to 0.998 and a limit of detection equivalent to 10^3 *mlrA* copies/g. These results are comparable to other quantitative PCR (qPCR) data cited in the literature, where the detection limit was 2×10^2 cells/ml for *Methylocystis* sp. from water (13) and approximately 10^3 cells/g for *Escherichia coli* O157:H7 (11) and *Rhodococcus* sp. (17) cells from soil.

The *mlrA* TaqMan assay was then applied, in conjunction with 16S rDNA qPCR (10) and 16S rDNA PCR-denaturing gradient gel electrophoresis (DGGE) cluster analysis (9), to investigate the attachment and subsequent biofilm formation upon virgin sand particles within a laboratory scale sand filter and also to investigate the previously unknown relationship between *mlrA* gene copy abundance and microcystin removal through biofiltration processes. Virgin sand (effective particle size, 1.25 mm; uniformity coefficient, 1.4; particle density, 1.62 g/cm^3) (Riversands Pty Ltd, Carbrook, Queensland, Australia) was washed and sterilized by autoclaving. Sand was then packed into a glass column (length, 30 cm; internal diameter, 2.5 cm) at a bed height of 15 cm, and the column was continually fed with Myponga reservoir water (empty bed contact time, 15 min; UV_{254}/cm, 0.412; dissolved organic carbon, 11.8 mg/liter; specific UV absorbance, 3.5 liters/mg-m; and pH, 6.7; South Australia) and spiked daily with microcystin-LR at a target concentration of 5 μg/liter. Full details of the sand column apparatus are described by Ho et al. (8). Influent and effluent water samples were taken at regular intervals for microcystin-LR determination by high-performance liquid chromatography (8). Sand samples (0.5 g) were sampled in triplicate from the top surface of the sand bed, and the biofilm DNA samples were extracted using the UltraClean soil DNA isolation kit (Mo Bio).

As shown in Fig. 1a, bacterial biofilm development (determined by 16S rDNA qPCR) appeared to begin within the first 24 h of start-up, and following day 3, the 16S bacterial abundance remained steady at approximately 1×10^8 copies/g. Despite the limited change in total bacterial abundance following day 3, 16S rDNA PCR-DGGE cluster analysis revealed a dynamic shift in the overall bacterial community composition throughout the 18-day study (Fig. 2). There was also a 3-day period before removals of microcystin were observed through the column (Fig. 1b). At day 4, there was a 12.6% removal of microcystin, although the abundance of *mlrA*-containing bacteria on day 4 remained below the limit of detection of the TaqMan assay (Fig. 1b). By day 7, the removal of microcystin had increased to 30.8% and was accompanied by the detection

of 1.17×10^3 *mlrA* gene copies/g of sand. For the remainder of the study, there was a close association between the trend of microcystin removal and the abundance of *mlrA* gene copies/g of sand (Fig. 1b). These data suggested that the efficiency of microcystin removals by biofiltration processes was directly related to the abundance of microcystin-degrading bacteria within the sand filter biofilm. Within biofilters that have had no preexposure to microcystins, lag periods have been reported to be as short as 2 to 4 days (1, 8), but they have been reported to be up to 211 days for a filter containing virgin sand (19). In most cases, 100% removal of microcystin through biofilters usually occurs rapidly within several days following the lag period (8, 19), although complete removal of microcystin in this study was achieved 15 days following the 3-day lag period (Fig. 1b).

A clone library was then constructed to investigate the diversity of *mlrA* homologues detected by the *mlrA* TaqMan PCR assay within the sand filter column on day 18. Following sequence analysis of 50 cloned DNA fragments, three unique *mlrA* gene sequences, MC-A, MC-B, and MC-C, were obtained. Each sequence had greater than 98% similarity to previously described *mlrA* gene sequences, where MC-A, MC-B, and MC-C represented 22, 72, and 6% of the clone library, respectively.

In summary, this study has demonstrated the development of an *mlrA* gene-directed TaqMan PCR assay for the assessment of microcystin-degrading bacteria within biologically active sand filter biofilm. The data presented here revealed that during the early phase of operation of a sand filter column, the removal of microcystin was directly related to the abundance of microcystin-degrading bacteria within the biofilm, although removals began only once an adequate number of microcystin-degrading bacteria were established. It is envisaged that the TaqMan PCR assay will be valuable for WTP operators who wish to investigate the abundance of microcystin-degrading bacteria within their biofilters and, in turn, assess the capacity of the biofilters for removing microcystin toxins—especially as such episodes are particularly transient in nature.

Nucleotide sequence accession numbers. The three unique *mlrA* gene sequences, MC-A, MC-B, and MC-C, obtained in this study have been deposited in GenBank under accession numbers FJ438525, FJ438526, and FJ438527, respectively.

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