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Isolation and Identification of Novel Microcystin-Degrading Bacteria[▽]

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Of 31 freshwater bacterial isolates screened using the Biolog MT2 assay to determine their metabolism of the microcystin LR, 10 were positive. Phylogenetic analysis (16S rRNA) identified them as *Arthrobacter* spp., *Brevibacterium* sp., and *Rhodococcus* sp. This is the first report of microcystin degraders that do not belong to the *Proteobacteria*.

A number of studies have reported biological degradation of microcystin in samples from lakes and sediments (3, 4, 12, 15), but only a few bacterial strains with the ability to degrade microcystins have been isolated and characterized (6). Previously identified bacteria belonged to the *Proteobacteria*, and with the exception of one isolate (*Sphingomonas* sp. strain CB4), they were all shown to degrade microcystin LR (MC-LR) via the same degradation pathway: formation of linear MC-LR following cleavage at the 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda)–Arg bond and hydrolysis at Ala-Leu to yield a tetrapeptide with Adda as the final product detected (1, 10, 11). Genes encoding enzymes involved in this pathway have been characterized and shown to have similarity in all bacterial isolates currently reported to degrade microcystins (2).

Recent work indicated a greater diversity of microbes capable of degrading microcystins and nodularin, with the tentative identification of several novel degradation intermediates (5). In the present study, enrichment was used to isolate bacteria from three Scottish water bodies previously shown to contain microflora capable of microcystin degradation (5). The Biolog MT2 assay was used to screen the ability of the isolated bacteria to metabolize MC-LR, since this had previously been shown to be an effective means of demonstrating metabolism of microcystin by *Paucibacter toxinivorans* (6).

The ability to metabolize MC-LR was determined in the Biolog MT screen, with 10 of the bacterial isolates giving a positive result. We subsequently confirmed that they could all degrade MC-LR in batch degradation studies, as evidenced by liquid chromatography-mass spectrometry (LC-MS) analysis. The microcystin-degrading bacteria were identified by using 16S rRNA gene analysis and investigated to determine the presence of *mlrA*, *mlrB*, *mlrC*, and *mlrD*, the genes previously reported to be involved in the degradation of MC-LR by *Sphingomonas* sp. strain ACM-3962 (2). We report here isolates identified as *Arthrobacter* spp., *Brevibacterium* sp., and *Rhodococcus* sp. which have the ability to degrade MC-LR, although none of the previously characterized *mlr* genes were detected.

Surface water samples were collected in sterile Pyrex glass bottles on 26 September 2007 from Loch Rescobie (Ordinance Survey grid reference number NO 52505159), Forfar Loch (NO 293458), and the River Carron (NO 877857), Scotland, United Kingdom. Samples were stored at 4°C overnight and filtered as previously described (5). Aliquots from each water sample (2 × 500 ml) were processed and analyzed by high-performance LC to determine the presence of naturally occurring microcystins (13). Enrichment and shake flask die-away kinetics were monitored in triplicate for each water type (50 ml in sterile 100-ml Erlenmeyer flasks). To enrich bacteria with the ability to degrade a range of different microcystins, three microcystins, selected for their differing polarities, and the pentapeptide nodularin were added to each water sample. MC-LR, MC-RR, MC-LF, and nodularin (Enzo Life Sciences, Lausen, Switzerland) were resuspended in a small volume (100 μl) of methanol and diluted with Milli-Q to a total concentration of 0.4 mg ml^{-1}. The toxin cocktail was sterilized (0.2-μm Dynaguard filter; Fisher, United Kingdom) and added to each flask under aseptic conditions to give a final concentration of 1 μg $ml^{-1}$of each toxin (i.e., 4 μg ml^{-1} total concentration). All flasks were incubated at 25°C ± 1°C with shaking at 100 rpm. Aliquots (2 ml) were removed from each flask under sterile conditions every 2 days, transferred into 4-ml glass vials, and frozen (−20°C) immediately. Die-away kinetics were monitored for 14 days. The frozen samples were freeze-dried, reconstituted in 200 μl of 50% aqueous methanol, and centrifuged at 15,000 × *g* for 10 minutes. The supernatant (100 μl) was removed for LC-MS analysis (5). Sterile controls (3 × 50 ml) were prepared, incubated, and sampled as described above to confirm whether loss of toxin was a result of microbial activity.

After 14 days of enrichment, 1 ml of sample was removed aseptically from each flask, namely, the Loch Rescobie (R), Forfar Loch (F), and River Carron (C) samples. Serial dilutions (to 10^{-5}) were made using Ringer's solution (Oxoid Ltd., United Kingdom), and 1 ml of each dilution was removed and mixed with 20 to 25 ml of molten LB agar, poured onto sterile petri dishes, and incubated in the dark at 25°C for 5 days. Colonies with differing morphologies were resuspended in liq-

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uid LB medium, and pure cultures were obtained by repeated streaking onto LB agar plates. For the Biolog MT2 assay, a loop of each isolated bacterial strain was transferred to 5 ml of liquid LB medium and incubated overnight in the dark at 25°C. The exponentially growing cultures were then washed twice by centrifugation at 1,000 × *g* for 15 min, the bacterial pellets were resuspended in sterile 0.01 M phosphate-buffered saline, and the cultures were incubated at 25°C for 24 h to deplete residual carbon. The turbidity of all bacterial suspensions was an A_{590} of 0.35. MC-LR was added to Biolog MT2 plates (Technopath, Limerick, Ireland) in triplicate to give final concentrations of 10, 1, 0.1, and 0 μg ml^{-1}. Wells were inoculated with bacterial suspension (150 μl), and plates were incubated at 25°C. Absorbance at 595 nm was recorded by using a Dynex microplate reader (Jencons, Leighton Buzzard, United Kingdom) immediately after inoculation (0 h) and at 3, 6, 15, 18, 24, and 48 h. Metabolism of MC-LR results in the reduction of tetrazolium violet, giving a color reaction that can be quantified spectroscopically (8). Bacterial isolates found by using the Biolog MT screen to metabolize MC-LR were evaluated for their ability to degrade MC-LR. Isolates C1, C3, and C6 (from the River Carron), F3, F7, and F10 (from Forfar Loch), and R1, R4, R6, and R9 (from Loch Rescobie) were grown overnight in LB liquid medium at 25°C. Bacterial isolates were washed and carbon depleted as described above (0.5 ml) and then added to glass universal bottles containing 9 ml of 0.2-μm-filter-sterilized water from their original locations. Aqueous MC-LR (0.5 ml) was added to each bottle under aseptic conditions at a final concentration of 5 μg ml^{-1}. Triplicate samples were prepared for each isolate and incubated at 25°C ± 1°C with shaking at 100 rpm. Aliquots (0.5 ml) were removed at 24-h intervals under sterile conditions, freeze-dried, reconstituted in 200 μl of 50% aqueous methanol, and centrifuged at 15,000 × *g* for 10 min. The supernatant (100 μl) was removed for LC-MS analysis performed as previously described (6). Experiments with sterile controls were performed for each water sample. *Paucibacter toxinivorans* DSMZ-16998 (Braunschweig, Germany) was used as a positive control as it has been reported to degrade MC-LR, MC-YR, and nodularin (16).

To identify selected isolates, total DNA was extracted from the pellet by using an UltraClean DNA isolation kit (Mo Bio Laboratories, CA). Sequencing was performed with a BigDye Terminator cycle sequencing reaction kit (202 instrument; Applied Biosystems, United Kingdom) using 8F, 1492, and various other internal primers (518R and 1087R) on an automated DNA sequencer (ABI, United Kingdom) (7, 17). The quality of the sequence was checked by using the sequence analysis software (ABI), and the products of the forward and reverse primers were aligned using Kodon (Applied Maths, Saint-Martens-Latem, Belgium). The analyzed sequences were compared to DNA sequences in public databases using the BLAST function of NCBI (http://www.ncbi.nlm.nih.gov). Individual isolates were classified according to their similarity to sequences in the database. DNA sequences of all isolates, along with those of related bacteria and some known microcystin-degrading bacteria, were used to construct a phylogenetic tree using MEGA4 (18). The sequences were first aligned using Clustal W, and then a phylogenetic tree was constructed by performing neighbor-joining tree analysis with 1,000 bootstrap replicates. Each microcystin-degrading isolate was assayed to determine if *mlr* genes for the degradation of microcystin could be detected. The PCR method used primers specific for *mlrA*, *mlrB*, *mlrC*, and *mlrD* with conditions as described before (9). A positive control for these genes was used (*Sphingopyxis* sp. strain LH21).

The Biolog MT2 plates, used to screen 31 isolates, were shown to be an effective means of rapidly identifying bacteria with the ability to metabolize MC-LR (Fig. 1). Ten isolates which demonstrated respiration in the presence of MC-LR using the Biolog format were subsequently proven to be microcystin-degrading bacteria in batch studies where MC-LR almost or totally disappeared after 3 days of incubation (Table 1). The results of LC-MS analysis indicated that MC-LR disappeared with no obvious biotransformation or intermediate degradation products. This may be because degradation resulted in only very low concentrations of these compounds and MC-LR is readily utilized by the isolates, as evidenced by respiration in the Biolog assay. Employing the Biolog MT plate enabled rapid (approximately 24 h) selection of bacteria in a high-throughput format (96-well plates) using considerably less microcystin or nodularin, i.e., 5 μg per isolate in the Biolog MT plate compared to 300 μg to follow degradation in die-away kinetics as described herein). Furthermore, following degradation by the latter method requires sample processing and high-performance LC analysis, increasing the time and cost. While the Biolog plates have been widely used for community profiling and bacterial identification, they have yet to be fully exploited in biodegradation studies, where they may facilitate rapid, cost-effective screening of many more bacterial isolates for the ability to utilize a wide range of environmental pollutants.

Interestingly, individual bacterial isolates with the ability to degrade MC-LR were obtained from water taken from the three different sources, including the River Carron, in whose sample no microcystin degradation by the indigenous microbial flora was observed during the enrichment and die-away study. This could be attributed to the low bacterial numbers observed in the river sample, while much higher bacterial numbers were observed in water from both Loch Rescobie and Forfar Loch.

Genetic analysis of the 16S rRNA gene sequence revealed that two isolates from the river water belonged to the genus *Rhodococcus*, while the majority (seven isolates) originating from all three locations were characterized as genus *Arthrobacter*, and only one bacterium, originating from Forfar Loch, belonged to the genus *Brevibacterium* (Fig. 2). All belong to the phylum *Actinobacteria*, whose members are well known for their metabolic diversity and ability to degrade a range of natural and man-made compounds (19). They have been isolated and reported from a range of environmental samples, including fresh and marine water, soil, and sludge. This study reports the ability of several members of the *Actinobacteria* phylum to degrade microcystins. Until recently, only members of genus *Sphingomonas* were reported to be able to degrade microcystin. The gene cluster responsible for microcystin degradation (*mlr*) has been reported for all *Proteobacteria* (14, 16). We used primers specific for *mlrA*, *mlrB*, *mlrC*, and *mlrD* for PCR amplification of these genes from our 10 isolates (9); however, no PCR

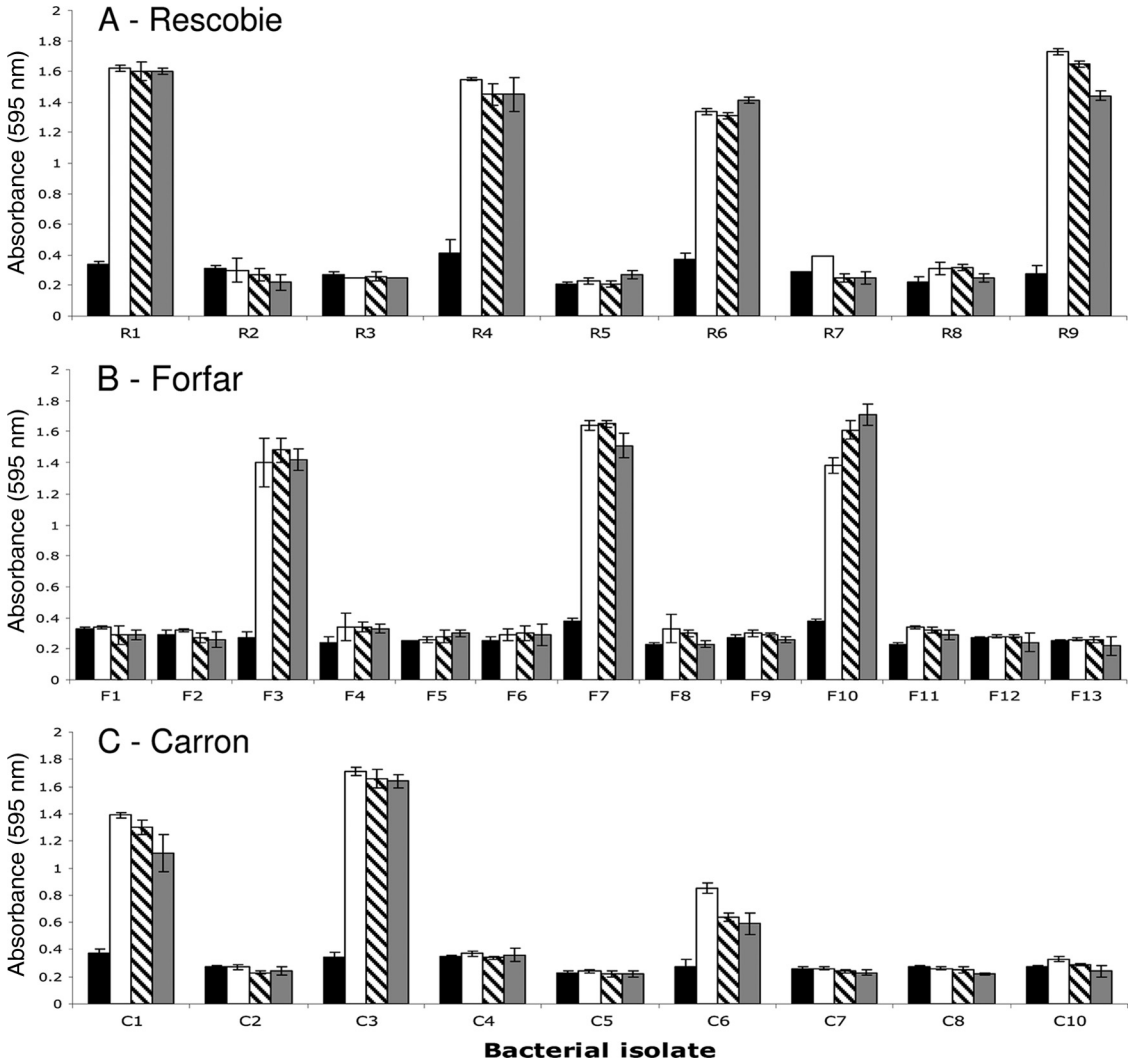

FIG. 1. Results of Biolog screen for MC-LR metabolism by bacteria isolated from Loch Rescobie (A), Forfar Loch (B), and the River Carron (C) after 24 h of incubation. Control samples (black bars) contained no additional carbon source. MC-LR was added as the carbon source at 0.1 (open bars), 1 (hatched bars), and 10 (shaded bars) μg ml^{-1}. Error bars represent 1 standard deviation ($n = 3$).

TABLE 1. Batch degradation of MC-LR by bacterial isolates in source

Bacterial isolate (genus) or control	% (mean ± SD, $n = 3$) MC-LR remaining after[a]	
	2 days	3 days
C1 (*Rhodococcus*)	36 ± 17	1 ± 2
C3 (*Rhodococcus*)	65 ± 4	4 ± 1
C6 (*Arthrobacter*)	41 ± 5	0
F3 (*Brevibacterium*)	77 ± 12	0
F7 (*Arthrobacter*)	23 ± 4	0
F10 (*Arthrobacter*)	72 ± 15	1 ± 0
R1 (*Arthrobacter*)	66 ± 11	0
R4 (*Arthrobacter*)	16 ± 3	0
R6 (*Arthrobacter*)	17 ± 1	0
R9 (*Arthrobacter*)	18 ± 1	0
P. toxinivorans[b]	75 ± 7	20 ± 2
River Carron[c]	92 ± 1	102 ± 2
Forfar Loch[c]	90 ± 6	94 ± 9
Loch Rescobie[c]	100 ± 9	109 ± 15

[a] The original concentration of MC-LR was 5 μg ml^{-1}.
[b] Positive control.
[c] Sterile water sample from source plus MC-LR; negative control.

products were detected, whereas all target genes produced PCR products in the positive control. It is possible that our isolates harbor entirely new genes for microcystin degradation pathways. However, another explanation may be that some homologous genes may be present but the sequences at the primer sites were different.

The present study clearly demonstrates that a greater diversity of bacterial genera can degrade MC-LR, with as-yet-uncharacterized degradation mechanisms since no intermediate products were identified during LC-MS analysis. We also confirm that microcystin degraders can be found in an aquatic environment where previous exposure to these toxins has not occurred (River Carron). Further studies to elucidate the genes involved in microcystin degradation in these novel bacteria, along with studies to determine the degradation pathway, are now being undertaken.

Nucleotide sequence accession numbers. The nucleotide sequences for the 16S rRNA genes have been deposited in

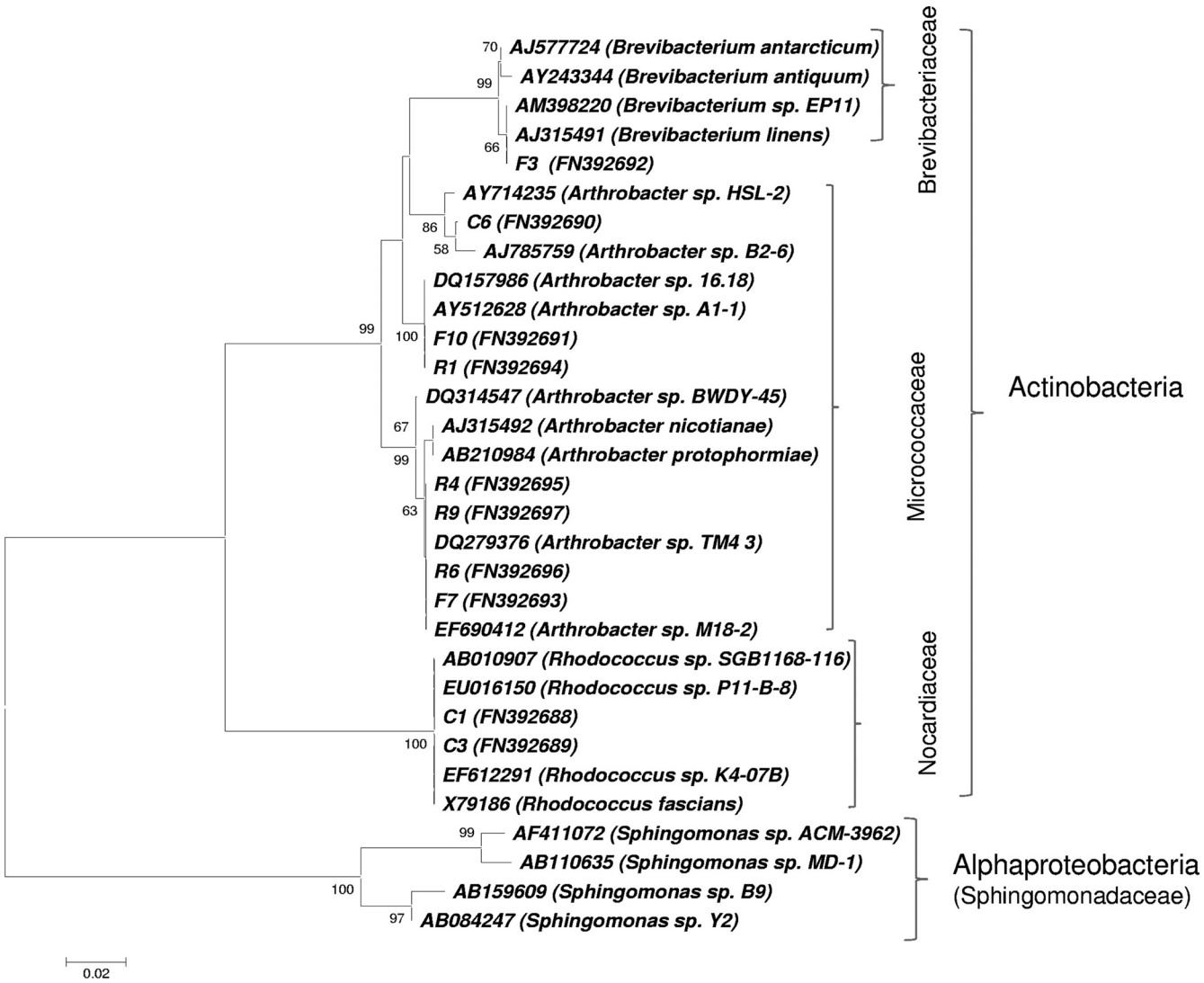

FIG. 2. Neighbor-joining phylogenetic tree showing the similarities of isolated bacteria to other members of the *Actinobacteria* group and to microcystin-degrading *Sphingomonas* spp. Bootstrap support values of >50 are indicated at nodes. The scale bar at the bottom shows the number of nucleotide substitutions per site.

the EMBL database under accession numbers FN392688 to FN392697.

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