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A multiplex qPCR targeting hepato- and neurotoxigenic cyanobacteria of global significance

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ABSTRACT

Toxic bloom-forming cyanobacteria are a global health hazard. These photosynthetic microorganisms produce a suite of secondary metabolite toxins including hepatotoxins such as microcystin, nodularin and cylindrospermopsin and neurotoxins such as saxitoxin. These toxins can threaten the safety of drinking water supplies and in the case of saxitoxin, can accumulate to dangerous levels in shellfish, affecting the seafood industry. Several molecular methods have been described for the detection and quantification of toxigenic cyanobacteria, however, to date there is no method for the simultaneous detection and quantification of hepatotoxin and neurotoxin producing genera. This paper describes the development and validation of a quadruplex quantitative-PCR (qPCR) assay capable of detecting and quantifying toxin genes from the microcystin, nodularin, cylindrospermopsin and saxitoxin biosynthesis pathways. The primers and probes employed in this assay were designed from conserved regions within toxin biosynthesis genes from most of the representative cyanobacterial genera. The qPCR assay was optimized to reliably determine the copy number of cyanotoxin biosynthesis genes, as well as an internal cyanobacteria 16S rDNA control, in a single reaction. Amplification efficiency and reproducibility were similar among the cyanotoxin genes, while the sensitivity of the reaction for the toxin genes ranged from 10^2 to 10^6 gene copies per reaction. This multiplex qPCR assay is a powerful tool for detecting and quantifying potentially toxic cyanobacteria in laboratory and field samples. Such technology will enable water quality and food safety authorities to better forecast, evaluate and reduce the impact of future harmful algal bloom events.

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1. Introduction

Cyanobacteria, or blue–green algae, are notorious for their proliferation into toxic blooms (Chorus and Bartram, 1999; Codd et al., 1999; Duy et al., 2000). Cyanotoxins are produced by many strains of cyanobacteria spanning multiple genera, however, as toxicity is not uniform among strains or morphotypes, conventional bacteriological classification methods are unable to accurately predict toxicity. Despite the challenges faced by pioneering researchers in this field, it has long since been realized that early detection methods for toxic cyanobacteria are critical as the consumption of cyanotoxins can lead to a myriad of serious health effects and can be fatal in high doses (Falconer, 2005; Sivonen and Jones, 1999). Direct measurements of cyanotoxins in water samples using bioassays and chemo-analytical methods have been reported (Etheridge et al., 2006; Falconer, 2005; Welker et al., 2002; Yuan et al., 2006). However, these methods are often laborious and costly or require specialized equipment. Furthermore, they are only applicable once the toxins are already present in the water above a certain detection threshold. Molecular methods, on the other hand, are able to detect toxigenic cyanobacteria before they produce and release their toxins into a water body. Thus, molecular methods may be employed as an early warning system capable of predicting the composition and potential toxicity of a bloom before it becomes problematic.

Current molecular methods targeting cyanotoxin biosynthesis genes include PCR and qPCR (for review see Pearson and Neilan, 2008). Both techniques are highly sensitive and can be tailored according to desired specificity. However, qPCR has the added advantage of being able to quantify the genetic target. In practical terms, this means it is possible to determine the concentration of toxigenic cyanobacteria in a bloom, be it a complex or unialgal sample. Most of the qPCR methods described to date are uniplex, that is, they utilize a single primer pair that targets an individual toxin gene. Primers can be designed to be highly specific (e.g. to target a toxin gene from a single species) or broad-range (e.g. to target multiple species producing the same toxin), however, uniplex reactions are limited to a single genetic target (Al-Tebrineh et al., 2011, in press; Koskenniemi et al., 2007; Vaitomaa et al., 2003). Multiplex qPCRs, on the other hand, can be tailored to target

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multiple toxin genes from a number of toxigenic species in a single reaction. Despite the great potential of this method for water quality testing to date, no multiplex qPCR assays have been described for the simultaneous detection and quantification of cyanotoxin biosynthesis genes, spanning multiple toxin classes from the known producing genera.

Rapid assessment of the distribution and concentration of toxic cyanobacterial species in environmental samples is critical, particularly in areas where complex bloom populations are known to occur. We report here a novel multiplex qPCR assay targeting four different cyanotoxin gene clusters: *mcy* (microcystin), *nda* (nodularin), *cyr* (cylindrospermopsin), and *sxt* (saxitoxin). This assay, which utilizes TaqMan technology, has been designed to target all the major microcystin, nodularin, cylindrospermopsin and saxitoxin-producing cyanobacteria. In addition, we have incorporated an internal control based on a conserved region of the 16S rRNA gene present in toxic and non-toxic cyanobacterial species.

2. Materials and methods

2.1. Cyanobacterial strains and culturing

The cyanobacterial strains used in this study are listed in the supplementary data (Table S1). Strains were either provided as lyophilized pellets or grown as batch cultures in the laboratory. *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis* and *Lyngbya* cultures were grown in Jaworski's medium (Thompson et al., 1988). *Anabaena*, *Microcystis*, and *Planktothrix* cultures were grown in BG11 medium (Rippka et al., 1979). *Nodularia* cultures were grown in artificial seawater medium (ASM) (Provasoli et al., 1957). All cultures were grown at room temperature (25 °C) under an 8:12 h light:dark cycle with a light intensity of 10 mmol m^{-2} s^{-1} of photons.

2.2. Microscopic cell counts

Microscopic cell counts were conducted for all reference cultures (*Microcystis aeruginosa* PCC7806, *Cylindrospermopsis raciborskii* AWT205 and *Anabaena circinalis* AWQC131C) using a light microscope and a KOVA Glasstic Slide with a counting chamber and grid (Hycor Biomedical).

2.3. DNA extraction

Genomic DNA was extracted as follows: Cells were lyzed mechanically via micro-bead beating using a Fast Prep® FP120 instrument (Savant) at a speed of 5.5 for 30 s. DNA was then extracted using the PowerPlant™ DNA isolation kit (GeneWorks) according to the manufacturer's instructions. DNA concentration and purity (A_{260}/A_{280}) was determined using a Nanodrop® ND-1000 spectrophotometer (Biolab). DNA extracts with an A_{260}/A_{280} of 1.7–2.0 were considered sufficiently pure for amplification experiments.

2.4. Primer and TaqMan probe design

TaqMan probes (double dye probes, Sigma Genosys) were used for the qPCR. These probes have a fluorophore incorporated at the 5′ and a quencher at the 3′ end. Primer and probe sets were designed to allow amplification of gene targets from the biosynthesis genes of toxic species determined thus far. Cyanotoxin biosynthesis gene sequences were obtained from The National Centre for Biotechnology Information (NCBI) database. Separate nucleotide alignments were performed for the *mcy*/*nda*, *sxt* and *cyr* sequences using ClustalX version 1.83. For the design of the microcystin/nodularin synthetase primers, the entire *mcy*/*nda* gene cluster was examined manually for potential target regions. Conserved regions with at least 90% sequence identity among 17 strains belonging to four genera were further examined using Primer Express Software V2.0 from Applied Biosystems and BLASTn (NCBI). For the design of the cylindrospermopsin synthetase primers, amidinotransferase sequences from the available *cyr* (and *aoa*) gene clusters, including eight sequences from three genera, were aligned. For the design of the saxitoxin synthetase primers, all available *sxtA* sequences (encoding a unique enzyme with methyl transferase, acetyl transferase, acyl carrier protein, and aminotransferase domains) from four different genera of cyanobacteria were aligned. Regions with 100% sequence identity within each of the *cyr*/*aoa* and *sxt* alignments were assessed as potential genetic target regions as previously described for *mcy*/*nda* genes. The targets ultimately selected from each toxin gene cluster were within the following toxin biosynthesis genes: aminotransferase (AMT) domains located on the *mcyE*/*ndaF* modules of the microcystin and nodularin synthetases, respectively; the amidinotransferase (*cyrA*) gene involved in cylindrospermopsin synthesis; and the aspartate aminotransferase domain of *sxtA* for saxitoxin synthesis (Table 1). These genes encode key enzymes within their respective toxin biosynthesis pathways, responsible for the initial steps in forming each toxin's structural backbone. PCRs targeting these genes are therefore unlikely to produce false negative results. False positive results, however, may be encountered if a frame shift mutation has occurred at a non-primer locus elsewhere within these biosynthesis gene clusters.

Table 1
Primers and TaqMan probes used in this study.

Target gene	Primer/probe name	Primer/probe sequence[a]	Size[b] (bp)	T_m (°C)	Concentration[c] (μM)	Reference
16S rRNA	16SF	AGCCACACTGGGACTGAGACA	80	59	0.2	(Al-Tebrineh et al., 2010)
	16SR	TCGCCCATTGCGGAAA		59	0.2	
	16SP	FAM-CCTACGGGAGGCAGCAGTGGG-BHQ1		69	0.2	
mcyE/*ndaF*	mcyF + flap	AATAAATCATAATTTAGAACSGGVGATTTAGG	128	63	1.2	This study
	mcyR + flap	AATAAATCATAACGRBTVADTTGRTATTCAATTTCT		64	1.2	
	mcyP	CY5-AATCAAGTTAAGGTVAATGGYTATCG-BHQ1		64	1.2	
cyrA	cyrF	GTCTGCCCACGTGATGTTATGAT	71	61	0.64	This study
	cyrR	CGTGACCGCCGTGACA		60	0.64	
	cyrP	CY3-CCTTTGGGAACGAAATTCTCGAAGCAACT-BHQ2		69	0.4	
sxtA	sxtF	GGAGTGGATTTCAACACCAGAA	148	60	0.68	This study
	sxtR	GTTTCCCAGACTCGTTTCAGG		61	0.68	
	sxtP	Texas Red-TGCCGATTTAGAAGAAAGTATCCTCTCAG-BHQ2		67	0.4	

[a] Fluorophore and quencher dyes (underlined) attached to 5′ and 3′ ends of TaqMan probes, respectively.
[b] Expected size of PCR amplicon generated by forward and reverse primers.
[c] Concentration of each primer/probe used per 25 μl reaction. Flap indicates primer with short 5′AT-rich overhangs (non-complementary to target sequence).

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