Emergence of nontoxic mutants as revealed by single filament analysis in bloom-forming cyanobacteria of the genus *Planktothrix*

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Abstract

Background: Bloom-forming cyanobacteria cause toxic algae outbreaks in lakes and reservoirs. We aimed to explore and quantify mutation events occurring within the large *mcy* gene cluster (55 kbp) encoding microcystin (MC) biosynthesis that inactivate MC net production. For this purpose we developed a workflow to detect mutations in situ occurring anywhere within the large *mcy* gene cluster as amplified from one single filament of the red-pigmented cyanobacterium *Planktothrix rubescens*. From five lakes of the Alps eight hundred *Planktothrix* filaments were isolated and each individual filament was analyzed for mutations affecting the *mcy* genes.

Results: Mutations inactivating MC synthesis were either thorough an insertion element ISPlr1 or the partial deletion of *mcy* genes. Neutral mutations not affecting MC biosynthesis occurred within two intergenic spacer regions, either through the insertion of a Holliday-junction resolvase RusA or ISPlr1. Altogether, the insertions affected a few *mcy* genes only and their location was correlated with regions similar to repetitive extragenic palindromic DNA sequences (REPs). Taking all of the filaments together, the mutations leading to the inactivation of MC synthesis were more rare (0.5–6.9 %), when compared with the neutral mutations (7.5–20.6 %). On a spatial-temporal scale the ratio of MC synthesis-inactivating vs. neutral mutations was variable, e.g., the filament abundance carrying partial deletion of *mcyD* (5.2–19.4 %) and/or *mcyHA* (0–7.3 %) exceeded the abundance of neutral mutations.

Conclusions: It is concluded that insertion events occurring within the *Planktothrix* *mcy* gene cluster are predictable due to their correlation with REPs. The frequency of occurrence of the REPs within the *mcy* gene cluster of *Planktothrix* relates to the rather common mutation of *mcy* genes in *Planktothrix*. Spatial-temporal variable conditions may favor the emergence of partial *mcy* deletion mutants in *Planktothrix*, in particular a higher proportion of genotypes resulting in inactivation of MC synthesis might be caused by increased ISPlr1 activity.

Keywords: cyanohab, microcystin, microevolution, mobile elements, single-colony PCR, in situ observation

Background

The filamentous, bloom-forming cyanobacterium *Planktothrix*, occurs in the pelagic zone of lakes and reservoirs, and is one of the main producers of microcystin (MC) – a hepatotoxin that poses a health threat to humans and livestock [1]. The blooming of *Planktothrix* regularly leads to toxic algae outbreaks, e.g., in December 2013 the water distributed in Užice in Serbia was banned for drinking and food preparation affecting thousands of people because of the intense bloom of toxic *P. rubescens* in Lake Vrutci, which served as the source of water supply. There is a documented history on these relatively sudden mass appearances of toxic *P. rubescens*, particularly in reservoirs [2–4], which is of relevance not at least due to their ongoing construction [5].

MC is synthesized by a nonribosomal peptide synthetase (NRPS) encoded by the *mcy* gene cluster containing nine to ten genes that have been elucidated from three abundant
MC-producing genera Microcystis, Planktothrix and Anabaena [6–8], Planktothrix [9] and Anabaena [10] have been reported to contain mutations within the mcy gene cluster. In Planktothrix the mcy gene cluster is affected by (i) recombination events affecting enzymatic domains [11, 12], and (ii) by inactivation, e.g., due to the partial deletion of mcy genes [13] or the insertion of transposable elements [9]. The latter mutations that lead to nontoxic subpopulations have been found to co-occur with the toxic subpopulation in nature [13, 14]. Individuals carrying mutations inactivating the mcy genes could not be differentiated from those carrying the original mcy gene cluster by phylogenetic analysis using housekeeping genes [15, 16]. This result implies that the respective genotypes carrying those mutations in the mcy gene cluster are evolutionary relatively young and/or possibly the selective pressure is not high enough to favor their phylogenetic fixation. Previously, the specific mutant mcy genotypes were found to be distributed among twelve lakes of the Alps (Austria, Germany, Switzerland) and to occur consistently irrespective of the total population density [14]. Only recently, through an almost 30-year observation period, a gradual increase of one mcy mutant genotype carrying a 1.8 kbp deletion of the mcy gene cluster was discovered [17].

Currently, our understanding on the factors leading to the mutations within the mcy gene cluster is low, both with regard to molecular factors as well as the ecological factors influencing the activity of mutagenic elements such as transposons. In general, physiological stress conditions have been observed to favor transposase activity [18]. Since mutations often lead to the inactivation of MC synthesis, the emergence of nontoxic genotypes in Planktothrix populations would decrease the MC production, which is potentially relevant since P. rubescens populations have been described with the on average highest MC content in nature [19].

We aimed to develop a workflow to detect mutations in situ occurring within the large mcy gene cluster as amplified from one single P. rubescens filament. Such results can pave the way to understand the regulation of the occurrence of mutations, especially those inactivating MC synthesis. In addition several mutations within the mcy gene cluster have been characterized previously using isolated clonal strains thus, enabling to differentiate between mutations resulting in inactivation of MC synthesis and neutral mutations not affecting MC synthesis [9, 11, 13, 14]. Since the entire mcy gene cluster spans > 55,000 bp, a sustainable technique is required that is able to amplify fragments covering the entire mcy gene cluster. This would make it possible to analyze (i) a population for all mutations affecting MC synthesis in real-time, (ii) the phylogenetic divergence of those mutants when compared with genotypes still containing the original mcy gene cluster using housekeeping genes, (iii) the abundance of each of the discovered mcy mutants on a spatial and temporal scale.

Results
Influence of filament length on PCR results
In total, 914 filaments of red-pigmented P. rubescens were isolated from five lakes in the Alps (Table 1) and analyzed for mutations affecting the mcy genes (Fig. 1). Taking together all filaments, 87.5 % (800 out of 914) showed a PCR product when amplifying the intergenic spacer region (IGS) between psaA and psaB. A minimum of hundred positive filaments from each of the five populations were sampled.

Due to the observed variability in filament length (Table 1) it was important to know whether the shorter filaments contained sufficient DNA template for PCR. The minimum filament length (88 µm) that was recorded corresponded to 29 cells (Additional file 1). Taking all filaments together, the filaments that were found PCR positive differed marginally in length (average 477 ± 7 SE) from the filaments that were found PCR negative (447 ± 21), (Mann–Whitney Rank Sum Test, p = 0.04, Student’s t-test, p = 0.17, Additional file 1). When comparing the proportion of PCR positive filaments between populations rather the opposite was observed, i.e., the on average longest filaments isolated from Lake Wörthersee showed the lowest proportion of PCR positive samples (73.5 %). Thus the influence of DNA template amount on PCR result variability between populations was considered of minor importance.

Mutations within the microcystin synthesis gene cluster
All Planktothrix filaments that were found to be PCR positive also contained the mcy gene cluster. An example for PCR amplification of the entire mcy gene cluster from one single filament is shown in Fig. 2a. Sixteen primer pairs were used to amplify mcy gene fragments of 3.5 kbp without interruption.

Among all of the filaments, 20 % were indistinguishable from the reference mcy gene cluster described from P. agardhii NIVA-CYA126/8 (Access No. AJ441056), while 80 % showed polymorphisms in PCR product size (Fig. 2b, c). Among these, one smaller PCR product (59 %) was due to the functional recombination of the mcyAA1 adenylation domain, i.e., the replacement of the mcyA variant containing a gene of the N-methyltransferase at pos. 34,656 – 37,592 (AJ441056) by the shorter mcyA variant lacking the N-methyltransferase as described [11].

Four different polymorphisms with increased amplicon size were located within the IGS of mcyTD (20.6 %), within mcyD (0.5 %), within the IGS of mcyEG (7.5 %), and within mcyA (0.6 %), (Fig. 3a). The increase in amplicon size located between mcyT and mcyD (1194 bp) at pos. 1294 of...
| Filament number | Positive Entry PCR (%) | Characteristics of filaments | Mutations within the mcy gene cluster (%) | Inactivation of MC synthesis |
|-----------------|-----------------------|-----------------------------|------------------------------------------|-----------------------------|
|                 |                       | Average (min, max) length (μm) | Average (min, max) cell number<sup>a</sup> | MC synthesis not inactivated | mcyTD insertion | mcyEG insertion | Short mcyA variant | mcyD insertion | mcyA insertion | mcyD deletion | mcyHA deletion |
| Mondsee (AT)     |                       |                             |                                          |                             |                |
| Mar 2012        | 105                   | 95.2                        | 1356 (484, 2574)                         | 435 (155, 825)              | 5.7            | 3.4            | 81.1             | 0              | 0              | 5.2            | 0              |
| Jun 2012        | 108                   | 92.6                        | 1251 (506, 2222)                         | 401 (162, 712)              | 2.0            | 2.0            | 67.7             | 2.0            | 1.0            | 7.2            | 3.0            |
| Sep 2012        | 102                   | 98                          | 1653 (990, 2618)                         | 530 (317, 839)              | 27.0           | 1.0            | 44.8             | 0              | 0              | 19.4           | 7.3            |
| Apr 2013        | 107                   | 93.4                        | 1392 (748, 2354)                         | 446 (240, 754)              | 13.1           | 0              | 53.3             | 1.0            | 0              | 18.0           | 7.1            |
| Other lakes     |                       |                              |                                          |                             |                |
| Wörthersee (AT) | 136                   | 73.5                        | 2200 (858, 6600)                         | 705 (275, 2115)             | 18.4           | 36.0           | 67.3             | 1.0            | 4.0            | 5.0            | 3.1            |
| Zürichsee (CH)  | 121                   | 82.6                        | 1032 (440, 1738)                         | 331 (141, 557)              | 25.3           | 9.0            | 45.0             | 0              | 0              | 1.0            | 1.0            |
| Hallwilersee (CH)| 120                   | 83.3                        | 1555 (704, 2772)                         | 498 (226, 888)              | 42.6           | 5.3            | 82.4             | 0              | 0              | 0              | 0              |
| Ammersee (DE)   | 115                   | 87                          | 1444 (638, 2420)                         | 463 (204, 775)              | 37.6           | 4.2            | 60.6             | 0              | 0              | 0              | 0              |
| Total           | 914                   | 87.5                        | 1479 (440, 6600)                         | 474 (141, 2115)             | 20.6           | 7.5            | 59               | 0.5            | 0.6            | 6.9            | 2.6            |

<sup>a</sup>estimated from linear regression curve (Additional file 1)
the reference mcy gene cluster (AJ441056) was due to an inserted sequence coding for an ORF (pos. 997–742) homologous to crossover junction endodeoxyribonuclease RusA (85 aa, 100 % similarity on the amino acid level, P. agardhii NIVA-CYA15, WP_027250107), and a second ORF (pos. 742–58) which was a hypothetical protein distantly related to archaeal Holliday junction resolvase, (227 aa, 100 % similarity, P. agardhii NIVA-CYA15, WP_027250106). From the sequences obtained (n = 14) no variability of the insertion site could be found and only one orientation was observed. The other three insertions were caused by the IS element ISPlr1 (1423 bp), i.e., within mcyD (at pos. 11,908 of the mcy gene cluster, AJ441056), within the IGS of mcyEG (at pos. 23,809), and within mcyA (at pos. 41,274), of which the insertion site and the orientation were described previously [9].

Deletions were detected within mcyD (6.9 %) and another deletion affected both mcyH and mcyA (2.6 %). The deletion within mcyD comprised 1665 bp (from pos. 2891 to 4555 of AJ441056), while the deletion occurring within mcyHA (1924 bp, from pos. 33,334 to 35,257 of the mcy gene cluster, AM990462.1) was described previously [9]. In summary, MC biosynthesis-inactivating mutations were observed only within a few genes (mcyD, H, A), while the other six genes part of the mcy gene cluster were not affected. For both housekeeping gene loci, rbcXL (seven genotypes, n = 64) and cpcBA (nine genotypes, n = 58), the one genotype representing the largest number of individuals (rbcXL, n = 50; cpcBA, n = 40) was comprised both of individuals without mutations or either carrying insertions (mcyD, mcyTD, mcyEG or mcyA) or partial deletions (mcyD and mcyHA), Table 2. Consequently, no phylogenetic diversification of specific mcy mutation genotypes was observed.

Direct repeats and repetitive sequences within mutations

The sequencing of the ISPlr1 elements inserted into the mcy gene cluster (n = 17) revealed identical inverted repeats (IRL: 5′-CAGGGCTGTTTCA -3′ and IRR: 5′-TGAACAGCCTG-3′). The ISPlr1 elements also showed similarity with regard to the direct repeat (DR) sequence (10 bp). In total, five DR sequences were found, i.e., for the mcyD gene (5′-CCCGTGCACG-3′ or
5′-CGTGACGGG-3′), for the mcyEG-IGS region (5′-GGTTTGGGG-3′ or 5′-GGCTGTTCCC-3′), and for the mcyA gene (5′-CCCCAAACCC-3′). Within the entire reference mcy gene cluster of *P. agardhii* NIVA-CYA126/8 (AJ441056), seven GC rich repetitive regions (RRs), which were 43–45 bp in length, were identified. These RRs contained the above mentioned DR sequences and were similar to the repetitive extragenic palindromic DNA sequences (REPs) described in Proteobacteria and Actinobacteria [20] and were predicted to form DNA stem-loop hybridization [21]. RR 1 (within mcyD), RR 2 (within mcyEG-IGS), and RR 3 (within mcyA), all showed insertion by ISPlr1. RR 4 was located at the 5′ end of the mcyTD insertion (the putative resolvase), while RR 5 was located within the recombination leading to the short mcyA variant (Fig. 3). RR 6 was found 121 bp downstream of mcyJ, and RR 7 was located 622 bp upstream of mcyT. For RR 6 no mutation was observed (Additional file 2). The same IRR sequence of ISPlr1 (5′-TGAAACAGCCCTG-3′) was also observed next to RR 7. In summary, within the *Planktothrix mcy* gene cluster the insertion of ISPlr1 elements was not randomly distributed but correlated with repetitive DNA described as REPs from bacteria in general.

**Abundance of mutations**

One fifth of the population (*n* = 162, 20 %) did not show any detectable mutation in comparison with the reference mcy gene cluster (AJ441056). Most frequently, the filaments contained one mutation (*n* = 492, 62 %), while filaments carrying two mutations (*n* = 142, 18 %) or three mutations (*n* = 4, 0.5 %) were rare. In general, the recombination resulting in the short mcyA variant constituted the dominant part of the population (45–82 %, Table 1). The second most abundant filament number was found to contain the mcyTD insertion (2–43 %), while 0–36 % carried the mcyEG insertion. Within IS element caused insertions, the number of ISPlr1 elements inserted into the IGS of mcyEG exceeded the number of ISPlr1 elements inserted into mcyDo mcyA considerably. Taking all filaments together, the mutations leading to the inactivation of MC synthesis were more rare (0–7 %), when compared with the abundance of the MC synthesis neutral mutations (7.5–20.6 %).

The filaments carrying either the long mcyA variant or the short mcyA variant contained a rather similar proportion of all the other mutations (Fig. 4). Only the mcyHA deletion (0–7 %) was perfectly linked to the mcyA short variant genotype. Filaments carrying a deletion in mcyHA (*n* = 21) also often had a deletion in mcyD (*n* = 15). In contrast, filaments carrying the mcyD partial deletion (*n* = 55) were comprised not only of mcyHA deletion, but also of undeleted mcyA short variant (*n* = 8) and long mcyA variant (*n* = 32). Of all the filaments carrying the mcyTD insertion (*n* = 165) only 6 filaments contained a second mutation (deletion in mcyD, insertion into mcyEG, mcyHA deletion, and insertion into mcyA). The filaments carrying the insertion of

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**Fig. 3 a** Schematic view of *Planktothrix mcy* gene cluster and location of mutations found in individual filaments and location of repetitive regions 1–7 (in green). Taking all filaments together the relative frequency of each mutation is given in parentheses. ISPlr1, *P. rubescens* IS element containing the conserved DDE domain for DNA transposition [9]; **b** Alignment of repetitive sequence regions within the *P. agardhii* NIVA-CYA126/8 mcy gene cluster (ASAK00000000). The framed boxes indicate the short directly repeated sequences (DR) of 10 bp in length resulting in insertion of transposable element ISPlr1. Bold letters indicate palindromic sequences.
Table 2 Overview of sequences obtained from individual *P. rubescens* filaments during this study

| Locus  | Title                                                                 | Length (bp) | Access No. (Isolated filaments) |
|--------|----------------------------------------------------------------------|-------------|----------------------------------|
| mcyTD  | Crossover junction endodeoxyribonuclease RusA and putative Holliday junction resolvase | 1194        | KP315862 (H22); KP315863 (A11); KP315864 (H11, H31, W22, Z14, Z24, Z51, Z72); |
| rbcLX  | ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, and ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcX) gene | 383-390     | KP315874 (Z75, M78, W22, A11); KP315875 (M41); KP315876 (H39); KP315877 (A20); KP315878 (M27, H81); H96, W36, Z66; KP315879 (A24); KP315880 (M2, M7, M19, M21, M40, M49, M52, M65, M88; M85, W3, W13, W17, W29, W35, W39, W53, W82, Z4, Z5, Z7, Z14, Z18, Z24, Z52, Z59, Z96, H19, H20, H31, H32, H49, H84, H95, A11, A13, A18, A30, A32, A45, W11, W18, W23, W34, W40, W47, W63, W65, A36, W87, W93); |
| cpcBA  | partial cpcB gene for phycocyanin beta subunit and partial cpcA gene for phycocyanin alpha subunit | 470         | KP315865 (M2, M52, Z4, Z18, H96, H49, A32); KP315866 (M21); KP315867 (W29); KP315868 (W18); KP315869 (W4); KP315870 (M27, M40, M85, W3, W53, Z52, Z96, H19, H20, H32, H39, A13, A45, M49, M70, W39, W13, W82, Z5, Z7, Z14, Z24, Z52, Z59, H31, H81, H94, H95, A1D, A11, A18, A30, M19, M41, M49, W11, W40, W87, W93, W65, Z66); KP315871 (A34); KP315872 (M65, M78, W35, W23, W63); KP315873 (M74); |
| mcyD   | partially deleted mcyD gene of the MC synthetase                        | 1964        | KP710231 (M14, M19, M41, M74, W40, W47, W49, W81, W87, W93, Z66); |
| mcyA   | partial mcyA gene of MC synthetase, short mcyA1 variant, 5′end + 3′end of recombination site | 447, 428    | KP315861 (A16, A33, H9, H59, MNewSp9, MNewSp92, W29, W98, Z21, Z94); |

*The recombination of the short mcyA variant was indicated by italic font (M19, M41 and M74 was unknown), A Ammersee, H Hallwilersee, M Mondsee (March 2012), W Wörthersee, Z Zürichsee, MNewSp Mondsee (Apr 2013). filament carrying mcyTD insertion; mcyD deletion; mcyD insertion; mcyEG insertion; mcyA insertion; mcyHA deletion.*
mcyEG \( (n = 60) \) comprised only two filaments with another mutation (insertion of mcyTD and mcyEG). Taking into account the probability that one filament carrying an insertion is hit by another insertion/deletion is quite low (only 18 % of all filaments carried two mutations), the co-occurrence between mcyHA and mcyD deletions was considered remarkable.

When considering each population separately, the ratio of the proportion of neutral MC synthesis mutations vs. MC synthesis-inactivating mutations was more variable. Particularly, in Lake Mondsee, the number of mcyD and mcyHA deletions was equal to or exceeded the number of MC synthesis neutral mutations (Table 1). On average, the mcyD and mcyHA partial deletions were more abundant than mcyD and mcyA insertions. In contrast, in Lake Zürichsee, Hallwilersee, and Ammersee rather few mcy gene cluster mutations resulting in the inactivation of MC synthesis were observed. In summary, although the MC synthesis-inactivating mutations were never found to be dominant, spatial and seasonal variability in their proportion occurred.

**Discussion**

**Single filament genetics**

In this study, using the short-term sonification of filaments, large DNA fragments up to 6 kbp were amplified from single filaments. Assuming a genome size of 5 mega base pairs and four chromosome copies per cell [22], it was calculated that the shortest filaments contained 2.8 pg (440 μm) and the longest filament (6600 μm) contained 42 pg of DNA resulting in 126–1890 mcy gene copies (Additional file 1). The influence of length variation among filaments on the PCR result variability was small, suggesting the DNA template amount to be of minor influence. Indeed, it was observed that longer filaments have a stronger tendency to stick to the walls of the reaction tube, and without immersion into PCR buffer they were lost from the PCR analysis. However, we cannot exclude a positive relationship between filament length and the proportion of PCR positive filaments if populations with on average shorter filaments (<1000 μm) would have been included.

This study proposes an alternative method to metagenomics approaches with the aim to elucidate genetic variation on an individual basis. Since in general planktonic cyanobacteria grow clonal, resulting in the formation of macroscopic colonies or filaments, single isolated colonies and filaments can be considered a single genotype [23]. Walsby and co-workers were the first to use DNA obtained from heat-burst single filaments stored in PCR buffer and up to four different PCR amplifications of one or several gene loci were achieved [24]. So far, single colony/filament PCR techniques were applied to amplify relatively short DNA regions (a few hundreds of base pairs). In contrast, genomic approaches on the single cell level have revealed high genetic diversity within populations, e.g., of high-light adapted ecotypes of Prochlorococcus [25]. Since even within the cyanobacteria a significant cultivation bias toward a few genera exists, combining single colony isolation with more advanced molecular biological methods holds great potential to analyze the genetic and ecophysiological diversification of cyanobacteria at the individual level.

**Location of mcy gene cluster mutations**

All the mutations arising from insertions into the mcy gene cluster were not randomly distributed but correlated with palindromic RRs of high genetic similarity (Fig. 3). The insertion of ISPlr1 was never found located outside such RRs, suggesting that the insertion of ISPlr1 depends on this type of inverted repeat sequence motif recognition that might be assisted by DNA stem-loop formation. Fewer et al. [10] described non-autonomous mobile miniature inverted-repeat transposable elements (MITEs) to insert into mcyD of *Anabaena* isolated from the Baltic Sea. In this study, the inverted RR observed for *Planktothrix* did not show the terminal inverted repeats that are typical for transposable elements [26]. Rather, these RR are similar to REPs that are characterized by a short stretch of high G-C content and have been associated with insertion of transposable elements in many
bacteria previously [27]. For the cyanobacteria, REPsis have long been used to aid taxonomic classification [28], but their role for genomic mutation and reorganization has been less explored. Notably, the short mcyA variant and rusA gene inserted into mcyTD also contained this RR, suggesting that the insertion of DNA fragments other than IS elements might be favored as well. The RR sequence reported in this study could not be found within mcy gene clusters sequenced from other cyanobacteria, i.e., Anabaena strain 90 [8] or Nodularia [29] using standard BLASTn search algorithm. Only for Microcystis the short motif 5′-CCCCAAACCCC-3′ occurred once within mcyE (strains PCC7806 and K-139, [6]; [30]) or twice in mcyE and mcyD (strain NIES 843, [31]). The absence of these Planktothrix specific RR in other cyanobacteria fits to the overall conclusion that the mcy gene cluster evolved from a cyanobacterial ancestor that occurred two billion years ago and then diversified according to the speciation of the different genera [32]. Consequently, the presence of these RR sequences within the mcy gene cluster of Planktothrix can explain why the insertion of transposable elements into mcy genes are the most common in Planktothrix populations and more rare for Microcystis and Anabaena [33].

Emergence of the mcy gene cluster mutations

In general, no congruency between phylogenetic diversification and a certain mutation was observed (Table 2). A straightforward explanation is that all of these mutations do not provide sufficient selective advantage to lead to their phylogenetic fixation. Still, the question remains as to why those mutants are maintained at relatively high frequency in nature. All of these mutations have been found distributed across the lakes of the Alps, and even the partial deletion of mcyD that has been discovered in this study has been detected in all study lakes (R.K. unpublished results). One possible explanation is that the same mutations emerge repeatedly and independently and die off regularly. This speculation is supported through the seasonal variation in abundance of the mutations in Lake Mondsee. It has been argued that the preservation of a certain ability of evolvability is of selective advantage for the total population that has been used as a kind of insurance of genome flexibility in unstable environments [34]. We have preliminary evidence that the activity of ISPlr1 also can be observed in vivo using the single filaments of Planktothrix strain No110 carrying ISPlr1 insertion within mcyD and mcyEG-IGS ([9]; C.Q., G.C., R.K. unpublished results). It is tempting to speculate that the varying abundance of MC synthesis mutations is based on variable ISPlr1 activity, which itself might be regulated by environmental conditions.

Abundance of microcystin synthesis inactivating mutations

The mutations described from single filaments have also been found among isolated clonal strains ([9], R.K., unpublished results). Thus, mutations that inactivate MC synthesis could be differentiated from MC synthesis neutral mutations by strain analysis as described previously [11, 13]. In particular strains carrying the mcyEG-IGS insertion (strains SAG6.89, CCAP1459/21, 1459/16, No31/1, 72, 82), as well as strains carrying the insertion between mcyT and mcyD (CCAP1459/24, No21/2, 64, 241), all produce MC [35]. In contrast strains carrying the ISPlr1 insertion in mcyD (No110, 139, 145, 161, 166, 169, 170, 178) or mcyA (No40), and strains with a partial deletion of mcyHA (No62, 65) or mcyD (No130, 137, 194) did not produce MC [9, 13, 35]. Taking all of the filaments together, the number of ISPlr1 inserted into mcyEG-IGS exceeded the number of ISPlr1 by far that was inserted directly into mcyA or mcyD. Since the former does not lead to the inactivation of MC synthesis, while the latter does, it might be speculated that selective pressure is preventing a high frequency of inactive mcy genotypes. Frequency-dependent selective pressure has been reported for the coexistence of genotypes in Pseudomonads either carrying plasmids providing mercury detoxification or plasmids free of mercury detoxification genes at a certain range of mercury concentration [36]. Taking into account that MC provides protection against grazers and parasites [37], such frequency dependent selective pressure would be a reasonable scenario that keeps functional mcy genotypes as the dominant part of the Planktothrix sp. population in Lake Mondsee since its first observation in 2002 [13].

On the other hand, among the population from Lake Mondsee, the number of mcyD and mcyHA deletions outweighed the number of mcyEG-IGS ISPlr1 genotypes at least at two sampling dates. At present, it is not known as to whether the imprecise excision of ISPlr1 can cause the partial deletions observed within mcyD and mcyHA. For many years, transposons Tn5, Tn7, Tn10 and bacteriophage Mu have been described to excise imprecisely from their point of insertion with the concomitant creation of deletions [38]. Among the 55 filaments carrying the partial deletion of mcyD, none carried an insertion of ISPlr1 into mcyD. In contrast, the partial deletion of mcyHA was only observed subsequent to the recombination of the short mcyA variant. This mcyA short variant, however, carried the same RR sequence as has been found at the ISPlr1 insertion sites, and it seems possible that an imprecise excision of ISPlr1 finally leads to both partial deletions in mcyD and mcyA. Accordingly, Vasas et al. [39] reported the occurrence of a partial deletion within the mcyEG-IGS region of a P. rubescens strain isolated from a gravel pit pond in Hungary. In the future, more quantitative data
will be needed to find out whether short-term shifts in the abundance of mutations occur and what the factors are that are leading to it.

Conclusions
By observing recombination processes within the mcy gene cluster of hundreds of single filaments in real-time, we conclude that insertion events occurring within the mcy gene cluster of Planktothrix are predictable due to the dependence on repetitive nucleotide sequence motifs, which represent REPs that are described as IS element insertion sites from other bacterial phyla. The higher abundance of those REPs within the mcy gene cluster of Planktothrix but not in other genera Microcystis and Anabaena can explain the more frequent mutation of the mcy gene cluster in Planktothrix when compared with other genera. Although the MC synthesis inactive genotypes were not observed to be dominant, we found evidence that under certain conditions a higher proportion of MC synthesis inactive mcy genotypes occurred, which might be caused by the increased activity of ISPlr1.

Methods
Study area and sampling
Five lakes located in the Alps, Mondsee (47°49’N, 13°22’E), Wörthersee (46°37’N, 14°07’E), Zürichsee (47°15’N, 08°38’E), Hallwilersee (47°17’N, 08°12’E), Ammersee (47°59’N, 11°07’E) were sampled for red-pigmented P. rubescens filaments by pulling a plankton net (30 μm mesh size) from a depth of 20 m to the surface from a boat in the middle of the lake (Table 1). The lakes are deep, physically stratified, and have been shown to inhabit red-pigmented Planktothrix during the summer either seasonally (Ammersee) or perennially (Mondsee, Wörthersee, Zürichsee, Hallwilersee). No specific permissions were required for these locations/activities, as in all three countries (Austria, Germany, Switzerland) water quality analyses are free of permission in public waterbodies. In addition the field study did not involve endangered species.

Filament isolation
Individual P. rubescens filaments were picked randomly from diluted samples under a dissecting microscope as described [13]. Each filament was washed three times by subsequent transfers between drops of BG11 medium [40], measured in length and finally transferred to a 0.5 ml Eppendorf tube containing 10 μl of sterile Millipore water and stored at −20 °C.

DNA extraction
Single Planktothrix filaments were ultrasonified using a sonifier cell disruptor equipped with a microtip (Branson, Danbury, Connecticut, USA) under optimized conditions (see Additional file 3). The microtip was washed with 10 % (v/v) H₂O₂ between individual samples. The quality of the obtained DNA fragments was tested with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) following manufacturer’s instructions. In a pilot experiment filaments were isolated from P. rubescens strain No3 and were sonified in different concentrations of 100, 50 and 10 filaments μl⁻¹ (in 10 μl, 15 % ultrasound strength, 1 s) to analyze the fragment size distribution of the obtained DNA. Independent from filament concentration a major peak of DNA elution was observed at 6000 bp, indicating that DNA templates of appropriate size for subsequent PCR amplification were available. As expected, DNA concentration and height of the major peak increased linearly with the number of sonified filaments (Additional file 3). In addition, the variable intensity of sonification (10, 15, 20, 25, 30, 35 and 40 % of total output, 100 filaments in 10 μl, 1 s) revealed an overall increase in DNA concentration. In contrast, the size of the DNA and the major peak height decreased indicating the increased fragmentation of the DNA (Additional file 3). Analogously, the DNA concentration increased with longer sonification time (1, 20, 30, 90, 180, 270 and 450 s, 100 filaments, in 100 μl, 15 % ultrasound strength), (Additional file 3). However, both DNA size and the major peak height decreased suggesting that DNA became increasingly fragmented with sonification time. Thus, in order to guarantee maximum DNA size, a low sonification intensity (15 %) and a short sonification time (1 s) were used during further analyses.

DNA storage time
To analyze the storage time of extracted DNA isolated from individual filaments, DNA was both stored at −20 °C in Millipore water and in Phire Hot Start Polymerase Plant PCR dilution buffer (Finnzymes, Espoo, Finland). No PCR products were obtained from DNA extracted from filaments stored in Millipore water for more than two days (Additional file 4). In contrast, DNA extracted from filaments stored in Millipore water for more than two days (Additional file 4). In contrast, DNA extracted from filaments stored in Millipore water for more than two days (Additional file 4). In contrast, DNA extracted from Millipore stored in PCR dilution buffer consistently revealed PCR products during a storage time until 9 months (data not shown). Consequently, DNA stored in PCR dilution buffer was used for all further application.

In general, 16 primer pairs (Fmcyamplify fragments of 3.5 kbp of the mcy gene cluster without interruption (see below). Pilot tests showed that using the Phire Hot start II DNA polymerase (Finnzymes, Espoo, Finland) following manufacturer’s instructions, PCR products ranging from 500 bp to 9 kbp could be obtained from one single filament. In order to determine the maximum of PCR assays possible for each filament sample, DNA extracted from individual filaments was diluted with Phire Hot Start Polymerase
PCR buffer 1-, 2-, 4-, 8-, and 16-fold. Using both the PC-IGS and 16S rDNA primer pairs (see below), PCR amplification was achieved until 16-fold dilution (Additional file 5).

**PCR amplification**

In order to confirm the presence of *Planktothrix* DNA after the sonification of each individual filament, the IGS between psaA and psaB was amplified (Additional file 6). The PCR was performed using Dream Taq polymerase (Thermo Scientific, Fermentas, St. Leon Rot, Germany) in 10 μl, containing 1 μl of Dream Taq PCR buffer (10×), 0.4 μl of MgCl₂ (50 mM), 0.3 μl of dNTPs (10 mM each), 0.3 μl of each primer (10 pmol μl⁻¹), 0.05 μl of polymerase, 6.65 μl sterile Millipore water and 1.0 μl DNA template. The PCR thermal cycling protocol included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles (denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s), and a final elongation step at 72 °C for 1 min.

In order to detect all the potential deletions and insertions within the entire mcy gene cluster, 16 primer pairs (Fmcy1 - 16) were designed and used to amplify fragments of 3.5 kbp without interruption (Additional file 6). Individual filament DNA samples were diluted 5-fold resulting in 50 μl of DNA template per filament in total. The PCR amplifications were performed in reaction mixtures of 10 μl, containing 2 μl of PCR reaction buffer (5×), 0.2 μl of dNTPs (Kapa Biosystems, Woburn, MA, USA), 0.5 μl of each primer (10 pmol μl⁻¹), 0.2 μl of Phire Hot start II DNA polymerase, 5.6 μl sterile Millipore water and 1 μl DNA template. The PCR thermal cycling protocol included an initial denaturation step at 98 °C for 30 s, followed by 40 cycles (denaturation at 98 °C for 5 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s) and final elongation (72 °C, 1 min). The mutations were detected via the size difference of the PCR products during ethidium bromide stained gel electrophoresis as compared to the PCR products obtained from the reference strain *P. agardhii* NIVA-CYA126/8 (AJ441056) [7]. If no PCR product was obtained, shorter PCR products (1.75 kbp, 35 s annealing) were amplified by combining a primer from the set Fmcy1 - 16 with the corresponding primer from the set Fmcy1m1 - 16 (Additional file 6), which were located in the middle of each 3.5 kbp fragment. In order to identify insertions (i.e., the mcyTD insertion and mcyEG insertion), specific primer pairs were used, which consisted of one primer binding to a locus within the *mcy* gene cluster and the second primer binding to a locus within the insert. For the sequencing of selected PCR products, gene fragments were amplified using the 3′-5′ exonuclease-containing Phusion polymerase (Finnzymes, Espoo, Finland). The PCR amplifications were performed in reaction mixtures of 25 μl, containing 5 μl of Phusion GC reaction buffer, 0.5 μl of dNTPs (10 mM each, Kapa), 1.25 μl of each primer (10 pmol μl⁻¹), 0.25 μl of Phusion DNA polymerase, 15.8 μl sterile Millipore water and 1 μl DNA template. The PCR thermal cycling protocol was identical to the Phire Hot start II DNA polymerase.

**Phylogenetic analysis**

In order to assign individual filaments to *Planktothrix* phylogenetic groups, the IGS of cpcBA (the intergenic spacer region between phycocyanin B and phycocyanin A protein) and rbcLX (the intergenic spacer region between the large subunit of the ribulose bisphosphate carboxylase/oxygenase and rbcX) were amplified and sequenced (Additional file 6). The oligonucleotides used to amplify the cpcBA and rbcLX locus were designed previously [13], [16]. The PCR thermal cycling protocol included an initial denaturation step at 98 °C for 30 s, followed by 40 cycles (98 °C for 5 s, 66 °C for 20 s, 72 °C for 30 s) and final elongation (72 °C, 1 min). PCR products were purified and sequenced by standard automated fluorescence techniques (Applied Biosystems, Weiterstadt, Germany). All sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers KP315865 - 73 (cpcBA, 475 bp), KP315874 - 80 (rbcLX, 390 bp), KP315862 - 64 (the insertion into the intergenic region of mcyTD, 1194 bp), KP315861 (the flanking region of the inserted short mcyA variant, 447 and 428 bp), KP710231 (the partial deletion of mcyD, 1964 bp), (Table 2).

**Availability of supporting data**

For nucleotide acid sequences submitted to the DDBJ/EMBL/GenBank databases under accession numbers KP315861-80, KP710231 see text. The data supporting the results of this article are included in Additional files 1, 2, 3, 4, 5, 6 and 7.

**Additional files**

Additional file 1: Relationship between *Planktothrix* filament length and cell number and length of *Planktothrix* filaments as compared between samples found PCR negative or PCR positive. (DOCX 17 kb)

Additional file 2: Summary of repetitive regions (RR) and associated mutations through ISPlr1 and RusA occurring within the *Planktothrix* mcy gene cluster. (DOCX 13 kb)

Additional file 3: Measures of genomic DNA isolated from *Planktothrix* filaments using a cell disruptor sonifier. (DOCX 21 kb)

Additional file 4: Effect of storage time (~20 °C) of DNA extracted from sonified *Planktothrix* filaments in Millipore water on PCR amplification of the PC-IGS region. (DOCX 766 kb)

Additional file 5: Amplification of PCR products from DNA extracted from one single *Planktothrix* filament and diluted 1-, 2-, 4-, 8-, and 16-fold. (DOCX 916 kb)
Oligonucleotides used in the present study. 

Additional file 7: Occurrence of mutations in individual Planktothrix filaments (Excel spreadsheet). (XLSX 74 kb)

Abbreviations
MC: Microcystin; REPs: repetitive extragenic palindromic DNA sequences; NRPS: nonribosomal peptide synthetase; IGS: intergenic spacer region; IS element: Insertion sequence element; ISPIR: IS element in P. rubescens; IRL: IRR, inverse repeat left, inverse repeat right; DR: direct repeat; RRs: GC rich repetitive regions; MITE: non-autonomous mobile miniature inverted-repeat transposable element.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
CQ, GC and RK carried out the molecular genetic studies, RK and LD participated in the design of the study and performed the statistical and sequence analysis. CQ, LD and RK have drafted the manuscript and RK, GC revised the manuscript. All authors read and approved the final manuscript.

Authors’ information
This article is constituting part of the written thesis of CQ in order to fulfill the requirements for the PhD granted by the Northwest A & F University, Yangling, Shaanxi Province, P.R. China.

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