The cylindrospermopsin gene cluster of *Aphanizomenon* sp. strain 10E6: organization and recombination

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Cylindrospermopsin (CYN), a potent hepatotoxin, occurs in freshwaters worldwide. Several cyanobacterial species produce the toxin, but the producing species vary between geographical regions. *Aphanizomenon flos-aquae*, a common algae species in temperate fresh and brackish waters, is one of the three well-documented CYN producers in European waters. So far, no genetic information on the CYN genes of this species has been available. Here, we describe the complete CYN gene cluster, including flanking regions from the German *Aphanizomenon* sp. strain 10E6 using a full genome sequencing approach by 454 pyrosequencing and bioinformatic identification of the gene cluster. In addition, we have sequenced a ~7 kb fragment covering the genes *cyrC* (partially), *cyrA* and *cyrB* (partially) of the same gene cluster in the CYN-producing *Aphanizomenon* sp. strains 10E9 and 22D11. Comparisons with the orthologous gene clusters of the Australian *Cylindrospermopsis raciborskii* strains AWT205 and CS505 and the partial gene cluster of the Israeli *Aphanizomenon ovalisporum* strain ILC-146 revealed a high gene sequence similarity, but also extensive rearrangements of gene order. The high sequence similarity (generally higher than that of 16S rRNA gene fragments from the same strains), atypical GC-content and signs of transposase activities support the suggestion that the CYN genes have been horizontally transferred.

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INTRODUCTION

Cylindrospermopsin (CYN) is a potent hepatotoxin produced by a number of cyanobacteria species. It was first characterized from an Australian strain of *Cylindrospermopsis raciborskii* (Ohtani *et al.*, 1992) and was retrospectively suggested as the causative agent of the Palm Island mystery disease. This disease, which occurred in 1979 on Palm Island, Queensland, Australia, caused a sudden severe hepatitis-like illness in 138 children and some adults. It was traced back to a bloom of *C. raciborskii* in the main drinking water reservoir of the island (Bourke *et al.*, 1983).

Since its first characterization from the Australian *C. raciborskii* strain, CYN has been detected in freshwater

bodies around the world, including New Zealand (Stirling & Quilliam, 2001), South, Central and North America (Berry & Lind, 2009; Burns *et al.*, 2002; Carmichael *et al.*, 2001), Asia (Chonudomkul *et al.*, 2004; Li *et al.*, 2001a) and Europe (e.g. Fastner *et al.*, 2003). In addition to *C. raciborskii*, the cyanobacterial species *Umezakia natans* (Harada *et al.*, 1994), *Aphanizomenon* (*Aph.*) *ovalisporum* (Banker *et al.*, 1997; Shaw *et al.*, 1999; Wörmer *et al.*, 2008; Yilmaz *et al.*, 2008), *Raphidiopsis curvata* (Li *et al.*, 2001b), *Aphanizomenon flos-aquae* (Preußel *et al.*, 2006), *Anabaena* (*A.*) *lapponica* (Spoof *et al.*, 2006) and *Lyngbya wollei* (Seifert *et al.*, 2007) have been shown to synthesize the toxin.

CYN (C₁₅H₂₁N₅O₇S) is a highly water-soluble alkaloid with a tri-cyclic guanidine group and an uracil ring attached at C-7 (Ohtani *et al.*, 1992). Isotope-labelled precursor feeding experiments have shown that CYN is most likely synthesized by the interplay of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) complexes and that guanidinoacetate is the starter unit for its biosynthesis (Burgoyne *et al.*, 2000). PKS and NRPS genes putatively involved in the biosynthesis of CYN have been independently identified in *C. raciborskii* (Schembri

Abbreviations: CYN cylindrospermopsin; HGT, horizontal gene transfer; IGS, intergenic spacer; ML, maximum likelihood; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

The GenBank/EMBL/DBJ accession number for the contig containing the *cyr* gene cluster is GQ385961 and for the *hyp* gene cluster is HM131805.

Six supplementary tables, showing primer sequences and sequence identity data, and two supplementary figures, showing ML analyses of *cyrB/aoaB* and *cyrA/aoaA* genes, are available with the online version of this paper.

et al., 2001) and *Aph. ovalisporum* (Shalev-Alon *et al.*, 2002). Further, an amidinotransferase gene, whose gene product is suggested to catalyse the synthesis of the guanidinoacetate starter unit, has been characterized in *Aph. ovalisporum* (Shalev-Alon *et al.*, 2002).

The entire putative CYN gene cluster was first characterized from the Australian *C. raciborskii* strain AWT205 (Mihali *et al.*, 2008). The cluster spans 43 kb and contains 15 ORFs, including four PKSs, one PKS/ NRPS hybrid and one amidinotransferase. The first three steps in the CYN biosynthesis involve an amidinotransferase, a PKS/NRPS hybrid and a PKS. In *C. raciborskii*, these three ORFs have been denoted *cyrA*, *cyrB* and *cyrC*, respectively, and correspond to the orthologues denoted *aoaA*, *aoaB* and *aoaC* in *Aph. ovalisporum* (Mihali *et al.*, 2008). Recently, a second CYN gene cluster has been described from the Australian *C. raciborskii* strain CS-505. The two *C. raciborskii* gene clusters show perfect synteny and very high sequence conservation (Stucken *et al.*, 2010).

Aph. flos-aquae and *Aphanizomenon gracile* are common cyanobacterial species of temperate fresh and brackish waters and have the capacity to form mass developments. In Germany, CYN-producing and -non-producing strains of these species have been reported to co-occur, and CYN-producing strains of both phenotypes have been isolated (Preußel *et al.*, 2006; Preußel, 2007; Stüken *et al.*, 2009). Together with *Aph. ovalisporum* and *A. lapponica* isolates from Spain and Finland, respectively (Spoof *et al.*, 2006; Wörmer *et al.*, 2008), these CYN-producing *Aphanizomenon* sp. isolates are the only confirmed CYN-producing species in Europe. *C. raciborskii* does occur in the same water bodies (Fastner *et al.*, 2003, 2007; Rücker *et al.*, 2007), but so far, CYN-producing *C. raciborskii* strains have only been isolated from Australian and Asian waters (e.g. Berger *et al.*, 2006; Bernard *et al.*, 2003; Fastner *et al.*, 2003; Saker *et al.*, 2003; Yilmaz *et al.*, 2008).

Recently, a number of studies have reported the co-occurrence of CYN and *Aphanizomenon* sp. in European water bodies (Bláhová *et al.*, 2008; Brient *et al.*, 2009; Kokocinski *et al.*, 2009; Messineo *et al.*, 2009). However, no genetic data on the CYN genes of *Aphanizomenon* sp. are currently available, thus rendering studies on the evolution and distribution of this gene cluster in *Aphanizomenon* sp. impossible.

We have sequenced and characterized the entire CYN gene cluster in *Aphanizomenon* sp. strain 10E6 (Preußel *et al.*, 2006) using a massive parallel (454) pyrosequencing approach. In addition, we have sequenced a ~7 kb fragment of the same gene cluster in *Aphanizomenon* sp. strains 10E9 and 22D11 covering the entire *cyrA* and partial *cyrB* and *cyrC* sequences. To study the evolution and processes associated with the distribution of this gene cluster, we have compared the *Aphanizomenon* sp. 10E6 gene cluster and its flanking regions with the orthologous gene clusters of *C. raciborskii* strains AWT205 and CS-505 and to the partial *aoa* gene cluster of *Aph. ovalisporum*

strain ILC-146. Further, we have phylogenetically analysed all available *cyr* and *aoa* gene sequences and have compared the results with a phylogeny of the cyanobacterial RNA polymerase gene *rpoC1*. Our data show extensive recombination, signs of transposase activities, atypical GC content and possible horizontal gene transfer events.

METHODS

Strain cultivation and DNA isolation. *Aphanizomenon* sp. strains 06D11, 10E6, 10E9, 14E6, 16D11 and 22D11 were provided by Karina Preußel (Federal Environmental Agency, Berlin, Germany) and cultivated in Z8 medium, at 20 °C, at a photon flux density of 65 $\mu\text{mol of photons m}^{-2} \text{ s}^{-1}$ and under 12h/12h light:dark cycles. Strains were not axenic. Strain 10E6 has been placed in the NIVA culture collection, Oslo, Norway, and is available under the strain number NIVA-CYA626. Prior to genomic DNA isolation, cells were filtered over 3 μm membrane filters (Millipore), resuspended in culture medium (sterile, diluted to 25 % of original concentration) and centrifuged (30 min at 16 100 g). The supernatant was discarded, and the pellet was resuspended in 1 $\times$ TE-buffer and frozen at -20 °C. The DNA isolation was performed as follows: the cells were thawed under vortexing, lysozyme (5 mg ml^{-1}) was added (incubation 50 °C, 30 min), followed by incubation with RNaseA (Omega Bio-Tek; 50 °C, 30 min) and then Proteinase K (2 mg ml^{-1} ; 60 °C, 60 min). Subsequently, 1 vol. chloroform–isoamyl alcohol [24:1 (v/v)] was added, and the sample was mixed and centrifuged (5 min at 16 100 g). The upper layer was transferred and the chloroform–isoamyl alcohol procedure was repeated twice. Sodium acetate (3 M; pH 5.2, 0.3 M final concentration) and 0.7 vol. 2-propanol were added; the solution was mixed, incubated (room temperature, 30 min) and centrifuged (30 min at 16 100 g). The DNA pellet was washed twice with ethanol (70 %), dried at room temperature and dissolved in TE buffer.

Identification of the CYN biosynthesis gene cluster of *Aphanizomenon* sp. 10E6. Whole genome shotgun sequencing was carried out at the Ultra-high Throughput Sequencing Platform (UTSP) at the Centre for Ecological and Evolutionary Synthesis (CEES), Dept of Biology, University of Oslo, using a GS FLX instrument (Roche). Library preparation, amplification and pyrosequencing were performed according to the manufacturer's instructions, using GLS FLX standard chemistry. One full plate was used for shotgun sequencing and a half plate was used for 3 kb paired-end sequencing. In addition, a half plate of 3 kb paired-end sequencing was performed at 454 Life Sciences (Branford). In paired-end sequencing, both ends of the DNA fragment are sequenced. This information can be used to sort and orientate contigs, which is important in reconstructing the original genome sequence. In shotgun sequencing, contigs are defined as a set of overlapping DNA segments derived from a single genetic source. A series of contigs that are sorted into the right order and orientation, but not necessarily connected in one continuous stretch of sequence, are referred to as scaffold (<http://454.com/glossary/index.asp>). The newbler assembler (gsAssembler) from Roche/454 (version 2.00.22) was used to assemble the reads. The assembly parameters were set to a minimum alignment identity of 98 % with a minimum alignment overlap of 60 bp. To identify the CYN gene cluster, all contigs were compared to the (partial) *aoa* genes from *Aph. ovalisporum* ILC-146 (AF395828), the *cyr* genes from *C. raciborskii* AWT205 (EU140798) and the (partial) *cyrA–C* sequences obtained from the *Aphanizomenon* sp. strains (see below) using the BLASTN and BLASTX algorithms (Altschul *et al.*, 1997) on the University of Oslo Biportal (<http://www.biportal.uio.no>).

Table 1. Cyanobacterial strains for which CYN synthesis and the presence *cyrA/aoaA* and/or *cyrB/aoaB* genes (or fragments thereof) have been investigated

+, CYN-producing/gene present; –, CYN-non-producing/gene absent. ND, no data; PS, present study. References (given in parentheses) are as follows: 1, Preußel (2007); 2, Preußel *et al.* (2006); 3, Haande *et al.* (2008); 4, Stucken *et al.* (2010); 5, Hawkins *et al.* (1997); 6, Schembri *et al.* (2001); 7, Rasmussen *et al.* (2008); 8, Saker & Griffiths (2000); 9, Fastner *et al.* (2003); 10, Fergusson & Saint (2003); 11, Pollingher *et al.* (1998); 12, Shalev-Alon *et al.* (2002); 13, Ballot *et al.* (2010); 14, Kellmann *et al.* (2006); 15, Neilan *et al.* (2003); 16, Harada *et al.* (1994); 17, Berger *et al.* (2006); 18, Yilmaz *et al.* (2008); 19, Bernard *et al.* (2009); 20, Wilson *et al.* (2000); 21, Lagos *et al.* (1999); 22, Valério *et al.* (2005); 23, Saker *et al.* (1999).

Organism	Country	CYN	<i>cyrB/aoaB</i>	<i>cyrA/aoaA</i>
<i>Anabaena</i>				
<i>Anabaena bergii</i>				
Three strains	Germany	– (13)	– (13)	ND
ANA360A	Australia	– (7)	– (7)	– (7)
ANA360D	Australia	– (10)	– (10)	ND
ANA360H	Australia	– (7)	+ (7)	– (7)
ANA366A	Australia	– (10)	– (10)	– (7)
<i>Anabaena circinalis</i> , ANA118C	Australia	– (6)	– (6)	ND
<i>Anabaena crassa</i> , one strain	Germany	– (13)	– (13)	ND
<i>Anabaena flos-aquae</i> , two strains	Germany	– (13)	– (13)	ND
<i>Anabaena lemmermannii</i> , three strains	Germany	– (13)	– (13)	ND
<i>Anabaena planctonica</i> , 22 strains	Germany	– (13)	– (13)	ND
<i>Anabaena</i> sp., one strain	Germany	– (13)	– (13)	ND
<i>Anabaenopsis</i>				
<i>Anabaenopsis elenkinii</i> , two strains	Germany	– (13)	– (13)	ND
<i>Aphanizomenon</i>				
<i>Aphanizomenon</i> cf. <i>klebahnii</i>				
NIVA-CYA 364–365	Hungary	– (PS)	– (PS)	– (PS)
<i>Aphanizomenon flos-aquae</i>				
Eighteen strains	Germany	– (13)	– (13)	ND
NIVA-CYA 142	Canada	– (PS)	– (PS)	– (PS)
<i>Aphanizomenon gracile</i>				
Thirty-four strains	Germany	– (13)	– (13)	ND
NIVA-CYA 103	Norway	– (PS)	– (PS)	– (PS)
NIVA-CYA 338	Hungary	– (PS)	– (PS)	– (PS)
<i>Aphanizomenon issatschenkoi</i>				
Six strains	Germany	– (13)	– (13)	ND
APH016B	Australia	– (24)	– (23)	– (23)
CAWBG02	New Zealand	– (23)	+ (23)	+ (23)
<i>Aphanizomenon ovalisporum</i>				
ANA283A*	Australia	+ (6)	+ (6)	+ (7)
ANA366B*	Australia	+ (10)	+ (10)	ND
APH031E, APH031G, APH035C	Australia	+ (7)	+ (10)	+ (7)
APH033C, APH035F	Australia	+ (10)	+ (10)	+ (7)
FAS-AP1	USA	+ (18)	+ (18)	ND
ILC-164	Israel	+ (11)	+ (12)	+ (12)
MS1	Israel	+ (22)	+ (22)	+ (22)
<i>Aphanizomenon</i> sp.				
06D11, 14E6, 16D11	Germany	– (1)	– (1)	– (PS)
10E6, 10E9, 22D11	Germany	+ (2)	+ (2)	+ (PS)
NIVA-CYA 638–641	Norway	– (PS)	– (PS)	– (PS)
<i>Cylindrospermopsis</i>				
<i>Cylindrospermopsis curvispora</i>				
Two strains	Senegal	– (17)	– (17)	ND
<i>Cylindrospermopsis raciborskii</i>				
Nineteen strains	Senegal	– (17)	– (17)	ND
4799	Portugal	– (15)	– (14)	– (14)
AQS	Australia	+ (22)	+ (22)	+ (22)
AWT205	Australia	+ (5)	+ (6)	+ (14)

Table 1. cont.

Organism	Country	CYN	<i>cyrB/aoaB</i>	<i>cyrA/aoaA</i>
Brazil1	Brazil	– (10)	– (10)	ND
Caia	Portugal	– (15)	– (14)	– (14)
CR3	Australia	+	– (3)	ND
CS-505	Australia	+	+	+
Cyli-19, -31, -42, -53, -94	Germany	– (9)	– (10)	– (7)
Cyli-29	Germany	– (9)	– [+] (10) [(7)]	– (7)
Cyli-75	Germany	– (9)	– [+] (10) [(7)]	+
CYP003A	Australia	– (6)	– (6)	– (7)
CYP003B, CYP025C	Australia	+	+	ND
CYP003K, CYP020A, CYP020B, CYP023A, CYP023E, CYP025B, CYP026J	Australia	+	+	+
CYP005E	Australia	+	+	+
CYP014A, CYP015A	Australia	– (6)	– (6)	– (7)
CYP018A	Australia	– (7)	+	– (7)
CYP023B, CYP023D	Australia	+	+	+
CYP023C	Australia	+	+	+
FAS-C1, FAS-C2, FL-E, FL-I	USA	– (18)	– (18)	ND
FL-D, -F, -G, -I	USA	– (18)	– (14)	– (14)
GERM1, GERM2	Germany	– (15)	– (14)	– (14)
Goon	Australia	+	+	+
HUNG1	Hungary	– (14)	+	– (14)
HUNG2	Hungary	– (14)	– (14)	– (14)
LJ	Australia	– (8)	– (22)	– (22)
LMECYA132–135, 168	Portugal	– (22)	– (22)	– (22)
Marau1 and Marau2	Portugal	– (15)	– (14)	– (14)
NIVA-CYA 225, 352–354, 399	Hungary	– (ps)	– (ps)	– (ps)
NIVA-CYA 506–511	Uganda	– (3)	– (3)	ND
PMC99.06	Mexico	– (17)	– (17)	ND
QHSS/NR/CYL/03	Australia	+	+	ND
SDC	Australia	+	+	+
T3	Brazil	– (21)	– (14)	– (14)
VOLL1, VOLL2	Germany	– (15)	– (14)	– (14)
Microcystis				
<i>Microcystis aeruginosa</i> PCC7806	The Netherlands	– (6)	– (6)	ND
<i>Microcystis viridis</i> NIES 102	Japan	– (7)	– (7)	– (7)
Nodularia				
<i>Nodularia spumigena</i> CS-590	Australia	– (7)	– (7)	– (7)
<i>Nodularia spumigena</i> PCC73104	Canada	– (6)	– (6)	ND
Limnithrix				
<i>Limnithrix</i> sp. AC0233	Australia	– (19)	– (19)	– (19)
Raphidiopsis				
<i>Raphidiopsis brookii</i> D9	Brazil	– (4)	– (4)	– (4)
Umezakia				
<i>Umezakia natans</i> TAC101	Japan	+	+	+

*Originally identified as *A. bergii*, reclassified as *Aph. ovalisporum* (Stüken *et al.*, 2009).

ORFs were identified and annotated using the NCBI ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>).

Amplification of *cyr* genes. Based on the *aoa* and *cyr* genes of *Aph. ovalisporum* ILC-146 (AF395828) and *C. raciborskii* AWT205 (EU140798), primers were designed that amplified the entire *cyrA* and partial *cyrC* (~1000 bp) and *cyrB* (~4200 bp) gene sequences in *Aphanizomenon* species. PCRs were carried out on the CYN-producing strains 10E6, 10E9 and 22D11 (Preußel *et al.*, 2006) and the non-CYN-producing strain 14E6 (Preußel, 2007). Reactions were carried out in

50 µl volumes containing 1 unit 10 × BD Advantage 2 PCR buffer (BD Biosciences), 250 µM of each dNTP, 25 pmol of each primer (Supplementary Table S1, available with the online version of this paper), 0.5 units 50 × BD Advantage 2 Polymerase Mix (BD Biosciences) and 10–50 ng DNA. Cycling conditions were: 95 °C for 60 s, 30 × (94 °C for 30 s, 65 °C for 60 s, 68 °C for a variable time, see Supplementary Table S1), 70 °C for 720 s, 8 °C hold. PCR products were visualized on 1% agarose gels stained with ethidium bromide. PCR products were cleaned with ExoSAP-IT (USB Corporation) and then sequenced directly. Sequencing primers are listed in Supplementary Table S2. Sequences were

analysed and assembled using the software programs Sequencher version 4.8 and CLC DNA Workbench version 5.0.2.

Further, 18 *Aphanizomenon* and *Cylindrospermopsis* strains obtained from the Norwegian Institute for Water Research (NIVA) culture collection and six *Aphanizomenon* sp. strains received from K. Preußel (Table 1) were investigated for the presence of *cyrA* and *cyrB* gene fragments. PCRs were carried out in 20 µl reactions using the Qiagen HotStarTaq Plus PCR kit. The *cyrB* and *cyrA* fragments were amplified using the primer pairs M13 and M14 (Schembri *et al.*, 2001), and *cylatF* and *cylatR* (Kellmann *et al.*, 2006), respectively.

CYN ELISA. The 18 NIVA strains (Table 1) were tested for CYN synthesis with the CYN ELISA from Abraxis (Abraix LLC). *Aphanizomenon* sp. strains 10E6 and 14E6 were included as positive and negative controls, respectively. The ELISA was carried out according to the manufacturer's instructions. Prior to the test, 2 ml culture material from each strain was frozen and thawed three times to break the cells and release the toxin into the medium. After 30 s centrifugation at 16 100 g, the supernatants were directly used in the ELISA test. The colour reaction was evaluated at 450 nm using the FLUOstar Optima microplate reader from BMG LABTECH.

Phylogenetic and G+C-content analyses. All available sequences of *cyr* and *aoa* genes, as well as *rpoC1* gene sequences from cyanobacterial strains of the genera *Aphanizomenon*, *Anabaena*, *Cylindrospermopsis* and *Raphidiopsis* were retrieved from GenBank. Alignments were created using the software program CLC DNA Workbench version 5.0.2 and checked manually. Identical sequences were removed and maximum-likelihood (ML) analyses were carried out using the software program RAxML version 7.0.4 (Stamatakis, 2006) on the University of Oslo Biportal. Evolutionary models were determined using the software TREEFINDER (Jobb, 2008). One hundred bootstrap replicates were generated for each analysis.

Further, bacterial amidinotransferase gene sequences were obtained from GenBank using the non-redundant database and the BLASTP algorithm (Altschul *et al.*, 1997). The amidinotransferase genes *cyrA* and *aoaA* from *C. raciborskii* AWT205 (EU140798) and *Aph. ovalisporum* ILC-146 (AF395828) were used as query sequences. The sequences were aligned using the multiple alignment program MAFFT with the E-INS-I option (Katoh & Toh, 2008). The alignments were checked manually, identical sequences were removed and ML analyses were carried out as described above.

G + C-content analyses were carried out using the program Isochore from the European Molecular Biology Open Software Suite (Rice *et al.*, 2000). The window size was set to 5000 and the shift to 500.

RESULTS

Identification of the CYN biosynthesis gene cluster of *Aphanizomenon* sp. 10E6

The pyrosequencing approach generated 929 136 shotgun and 229 474 paired-end reads (with 275.2 million bases of raw data). The reads assembled into 35 scaffolds, 12 of which had a maximum coverage of 9, the remaining 23 had a minimum coverage of 14. The 12 low-coverage scaffolds are likely to stem from contaminating organisms (Nederbragt *et al.*, 2010). The high-coverage scaffolds had an average coverage of 36 times and a cumulative length of 6.18 Mb. They contained 890 contigs with a GC-content of 0.38 ± 0.03 . Raw and assembled datasets are available from the authors on request.

BLAST database comparisons identified three contigs with highly similar sequences to the *cyr/aoa* genes of *C. raciborskii*, *Aphanizomenon* sp. and *Aph. ovalisporum* (contigs 393–395), which were located next to each other on scaffold 7. The gaps between contigs 393–395 as well as the gaps to the neighbouring contigs were closed by PCR and Sanger sequencing. The resulting contig containing the *cyr* gene cluster had a length of 57 112 bp, contained 25 ORFs and is available under the GenBank accession number GQ385961.

Comparisons of the *cyr* gene cluster of *Aphanizomenon* sp. strain 10E6 and the *cyr* gene cluster characterized from *C. raciborskii* strain AWT205 (Mihali *et al.*, 2008) revealed that 11 ORFs identified in strain 10E6 were clear orthologues of genes *cyrA–K* in strain AWT205 (Table 2). The sequence similarity of these 11 genes was between 95.0 % (*cyrK*) and 99.7 % (*cyrB*) nucleotide identity (Table 2). No clear orthologues for the genes *cyrL–O* were found in *Aphanizomenon* sp. strain 10E6. In AWT205, *cyrL* and *cyrM* encode transposases, *cyrN* encodes an adenylylsulphate kinase, which is putatively involved in tailoring reactions resulting in the sulphation of CYN, and *cyrO* encodes a putative regulator (Mihali *et al.*, 2008). In strain 10E6, *orf3* codes for a transposase and vestiges of a transposase are present between *cyrH* and *cyrK*. *Orf4* is a short ORF with high similarity to an adenylylsulphate kinase from the cyanobacterium *Trichodesmium erythraeum* IMS101, and *orf12* encodes a putative transcriptional attenuator. All three ORFs are found in the flanking region of the *cyr* gene cluster in *Aphanizomenon* sp. 10E6 (Table 3). Whether *orf4* and *orf12* are functional and involved in CYN synthesis and regulation remains to be experimentally investigated.

cyr gene cluster organization

The *cyr* gene clusters of the *C. raciborskii* strains AWT205 and CS-505 show perfect synteny (Stucken *et al.*, 2010) and >99.9 % nucleotide identity (22 bp difference; 42 164 bp alignment). The most notable difference between these gene clusters, the partial *aoa* gene cluster of *Aph. ovalisporum* strain ILC-146 and the *cyr* gene cluster of *Aphanizomenon* sp. strain 10E6, is the gene arrangement (Fig. 1). In the *C. raciborskii* strains, *cyrA–J* are transcribed on the direct strand and *cyrK* on the reverse strand. In *Aphanizomenon* sp. 10E6, *cyrA*, *B*, *E* and *K* are transcribed on the direct strand, and *cyrC*, *D* and *F–J* are transcribed on the reverse strand. It appears as if the *cyr* gene cluster has been rearranged in three blocks: the first block contains *cyrA*, *B* and *E*, and is located on the direct strands in *C. raciborskii* and *Aphanizomenon* sp. The second block corresponds to *cyrC*, which is situated downstream of *cyrE* and transcribed on the direct strand in *C. raciborskii*, but which is flipped around and located upstream of *cyrA* in *Aphanizomenon* sp. 10E6. The third block involves *cyrD–K*, which is (apart from *cyrK*) transcribed on the forward strand in *C. raciborskii*, but which is flipped around and

Table 2. Comparisons between the *cyr* and *aoa* genes of *C. raciborskii* AWT205 (EU140798), *Aph. ovalisporum* ILC-146 (AF395828) and *Aphanizomenon* sp. 10E6 (GQ385961, present study)

NA, Not available.

	G + C-content 10E6	Length (bp)			Percentage identity (nucleotide)			Putative function*
		AWT205	10E6	ILC-146	AWT205/ 10E6	10E6/ILC-146	AWT205/ ILC-146	
16S rDNA:		1447	1239	1165	95.0	94.6	93.9	
<i>cyrA/aoaA</i>	0.47	1176	1176	1179	99.3	96.1	96.0	Amidinotransferase
<i>cyrB/aoaB</i>	0.48	8754	8754	5606†	99.7	97.6	97.4	NRPS/PKS A-PCP-KS-AT- DH-MET-KR-ACP
<i>cyrC/aoaC</i>	0.47	5004	5004	4299†	99.5	97.3	97.2	PKS KS-AT-KR-ACP
<i>cyrD</i>	0.47	5631	5556	NA	98.2	NA	NA	PKS KS-AT-DH-KR-ACP
<i>cyrE</i>	0.46	5667	5667	NA	99.6	NA	NA	PKS KS-AT-DH-KR-ACP
<i>cyrF</i>	0.47	4074	4074	NA	98.4	NA	NA	PKS KS-AT-ACP
<i>cyrG</i>	0.48	1437	1437	NA	96.2	NA	NA	Uracil ring formation
<i>cyrH</i>	0.49	1431	1431	NA	97.8	NA	NA	Uracil ring formation
<i>cyrI</i>	0.38	831	831	NA	97.1	NA	NA	Hydroxylation of C-7
<i>cyrJ</i>	0.45	780	786	NA	98.7	NA	NA	Sulphotransferase
<i>cyrK</i>	0.44	1398	1440	NA	95.0	NA	NA	Exporter

*According to Mihali *et al.* (2008).

†Partial gene sequence.

transcribed on the reverse strand (apart from *cyrK*) in *Aphanizomenon* sp. 10E6 (Fig. 1). The gene arrangement of the partial *cyr* gene clusters generated from the *Aphanizomenon* sp. strains 10E9 and 22D11 (GenBank accession GQ385959 and GQ385960) is the same as observed for *Aphanizomenon* sp. 10E6. The nucleotide sequences for the *cyrA* gene (1176 bp) and the partial *cyrB* (996 bp) and *cyrC* (4182 bp) genes were, apart from a

single bp difference in strain 22D11, identical in all three *Aphanizomenon* sp. strains. No PCR products were amplified for the non-CYN-producing *Aphanizomenon* sp. strain 14E6.

The arrangement of the partial *aoa* gene cluster from *Aph. ovalisporum* strain ILC-146 was the same as the *cyr* genes in the other *Aphanizomenon* strains (Fig. 1). The sequence

Table 3. Best BLASTX hits for the putative ORFs flanking the *cyr* gene cluster in *Aphanizomenon* sp. 10E6

There were no hits for ORFs 6 (399 bp), 8 (360 bp) and 9 (348 bp).

ORF	Length (bp)	Hit	Description	Organism	Percentage identity (% positive)
1	903	YP_001864536	RNA-binding S1 domain-containing protein	<i>Nostoc punctiforme</i> PCC 73102	85 (95)
2	2244	YP_324283	Oxidoreductase alpha (molybdopterin) subunit	<i>Anabaena variabilis</i> ATCC 29413	86 (93)
3	1641	ZP_00516180	Similar to transposase	<i>Crocospaera watsonii</i> WH 8501	56 (73)
4	324	YP_721651	Adenylylsulfate kinase	<i>Trichodesmium erythraeum</i> IMS101	65 (80)
5	444	YP_003137528	Hypothetical protein Cyan8802_1794	<i>Cyanothece</i> sp. PCC 8802	58 (76)
7	378	ZP_05485278	DNA-damage-inducible protein D	<i>Streptomyces</i> sp. SPB78	77 (93)
10	402	YP_001868948	Hypothetical protein Npun_F5704	<i>Nostoc punctiforme</i> PCC 73102	71 (82)
11	1077	YP_001868387	Nucleotidyl transferase	<i>Nostoc punctiforme</i> PCC 73102	86 (92)
12	1506	ZP_03764199	Cell envelope-related transcriptional attenuator	<i>Nostoc azollae</i> 0708	77 (88)
13	507	YP_001868389	Pentapeptide repeat-containing protein	<i>Nostoc punctiforme</i> PCC 73102	80 (89)
14	1200	YP_001868391	Integral membrane protein MviN	<i>Nostoc punctiforme</i> PCC 73102	82 (92)

Fig. 1. Comparison between the *cyr* gene clusters of *C. raciborskii* CS-505 and *Aphanizomenon* sp. 10E6 and the partial *aoa* gene cluster of *Aph. ovalisporum* ILC-146. Black, flanking genes; blue, green and yellow, blocks of *cyr* genes that have been rearranged (see text); grey, *cyr* genes without clear homologues in strain 10E6; red, transposases; black triangles with numbers, regions containing transposases or vestiges thereof (see text and Table 4).

similarities between *aoa* and *cyr* genes were slightly less between *Aph. ovalisporum* and *Aphanizomenon* sp. compared with *Aphanizomenon* sp. and *C. raciborskii* (Table 2).

CYN synthesis and presence of *cyr* genes

None of the 18 *Cylindrospermopsis* and *Aphanizomenon* strains tested showed cross-reactivity with the CYN-ELISA, nor were any *cyrA* or *cyrB* fragments amplified from these strains (Table 1). The *cyrA* fragment was amplified for the CYN-producing *Aphanizomenon* sp. strains 10E6, 10E9 and 22D11, but not for the non-CYN-producing strains 06D11, 14E6 and 16D11 (Table 1).

Table 1 lists 226 cyanobacterial strains for which both CYN synthesis and the presence of *cyr/aoa* genes (or fragments thereof) have been investigated. Nine different genera are listed: *Anabaena* (38 strains), *Anabaenopsis* (2 strains), *Aphanizomenon* (90 strains), *Cylindrospermopsis* (89 strains), *Microcystis* (2 strains), *Nodularia* (2 strains), *Limnathrix* (1 strain), *Raphidiopsis* (1 strain) and *Umezakia* (1 strain). Of these, 35 strains have been reported to produce CYN; no CYN was detected in the remaining strains. Fragments of *cyrB* and, if investigated, also of *cyrA*, were amplified in all of the CYN-positive strains. Neither of the fragments was amplified in the majority of CYN-negative strains. However, five CYN-negative strains contained *cyr* gene fragments: one *A. bergii*, one *Aphanizomenon issatschenkoi* and three *C. raciborskii* (Table 1).

Phylogenetic analyses

Phylogenetic analysis of amidinotransferase genes showed that the amidinotransferases involved in CYN synthesis form a well-supported cluster (Fig. 2a). When only the amidinotransferase genes involved in CYN synthesis are analysed, i.e. *aoaA* and *cyrA*, they predominantly cluster according to their taxonomic classification (Fig. 2b). Exceptions are *Aph. issatschenkoi* CAWBG02 and *U. natans* TAC101; the former clusters with *C. raciborskii* and the latter with *Aph. ovalisporum*. Only single *aoaA* sequences of

Aph. issatschenkoi and *U. natans* are publicly available and the *aoaA* sequence of *Aph. issatschenkoi* is the shortest *aoaA* sequence available. When it is removed from the analysis, the alignment length increases from 608 to 1001 bp. As a result, the bootstrap values increase, but the topology of the tree does not change (data not shown). Only a few nucleotide sequences of *cyrB* (*aoaB*) and *cyrC* (*aoaC*) gene fragments were publicly available. Many of these sequences are short, highly similar or identical and/or did not overlap (Supplementary Tables S3 and S4), rendering comprehensive phylogenetic analyses impossible. Preliminary results are shown in Supplementary Figs S1 and S2, and indicate a similar pattern to that observed for *cyrA*.

The *rpoC1* phylogeny (Fig. 2c) has a similar topology to the *cyrA* phylogeny: *Aphanizomenon* sp., *Aph. ovalisporum* and *C. raciborskii* form separate clusters. However, the distances are greater.

Additional sequence features: transposases, flanking regions and G+C-content

One putative transposase protein, denoted *orf3*, was identified adjacent to the *cyr* gene cluster of *Aphanizomenon* sp. 10E6. Further, remnants of a transposase were found between *cyrH* and *cyrK*. Of this 445 bp long intergenic spacer (IGS), 145 bp was identical to the sequence upstream of *cyrH* in *C. raciborskii*, including 46 bp at the 3' end of *cyrL* (Fig. 1, Table 4). In addition to the four transposases and vestiges thereof identified in the two *C. raciborskii* strains (Mihali *et al.*, 2008; Stucken *et al.*, 2010), we found further remnants of a transposase upstream of *cyrD* in both *C. raciborskii* strains. These transposase vestiges had a high similarity to the transposase *cyrM* (denoted gene CRC_01707 in CS-505; Fig. 1, Table 4). No transposases or remnants thereof were found in the partial *aoa* gene cluster of *Aph. ovalisporum* strain ILC-146.

The genes flanking the *cyr* gene cluster in *Aphanizomenon* sp. 10E6 are different to the *hyp/hup* genes flanking the *cyr* gene cluster in *C. raciborskii* AWT205 and CS-505 (Fig. 1,

Fig. 2. Maximum-likelihood analyses of (a) bacterial amidinotransferases (amino acid alignment), (b) all available *cyrA* and *aoaA* genes (nucleotide alignment) and (c) *rpoC1* sequences. GenBank accession numbers of strains in (b) and (c) are listed in Supplementary Tables S5 and S6.

Table 2) (Mihali *et al.*, 2008; Stucken *et al.*, 2010). The *hyp* genes in *Aphanizomenon* sp. 10E6 were located on contig 53, which mapped to scaffold 1, whereas the *cyr* genes were located on scaffold 7 (see above). The gene order was the same as in *R. brookii* D9, but no putative insertion site or ORF was found between *hypF* and *hypC*. Instead, an ORF was present between *hypD* and *hypE* (Fig. 3).

Apart from *cyrI*, all *cyr* genes of *Aphanizomenon* sp. 10E6 have a G+C-content >0.44 (Table 2). Analysis of scaffold 7 showed that the *cyr* genes are located in an area of unusually high G+C-content compared with the rest of the

scaffold (Fig. 4). The same area of high G+C-content was observed on the *C. raciborskii* CS-505 contig 27, which contains the *cyr* genes. No region of high G+C-content was present surrounding or within the region encoding the *hyp/hup* gene cluster of *R. brookii* D9 (Fig. 4).

DISCUSSION

Our results show that the genes involved in CYN synthesis are highly conserved not only between strains of the same species but also between strains of different genera. Gene

Table 4. Transposase regions in the *cyr* gene clusters of *C. raciborskii* and *Aphanizomenon* sp. strains

See Fig. 1 for position in the gene clusters.

Region/gene	Strain	BLASTX hit	Description	Organism	Hit length (aa)	Percentage identity (% positive)
T1 <i>hypF-cyrD</i>	AWT205, CS-505	ZP_06307802	Transposase protein	<i>Cylindrospermopsis raciborskii</i> CS-505	71	93 (93)
T2 <i>cyrL</i>	AWT205, CS-505	YP_002478395	Transposase, IS4 family protein	<i>Cyanothece</i> sp. PCC 7425	214	69 (79)
T3 <i>cyrJ-cyrA</i>	AWT205, CS-505	ZP_06308708	Transposase	<i>Cylindrospermopsis raciborskii</i> CS-505	76	92 (95)
T4 <i>cyrE-cyrC</i>	AWT205, CS-505	ZP_06307257	Transposase	<i>Cylindrospermopsis raciborskii</i> CS-505	33	91 (94)
T5 <i>cyrM</i>	AWT205, CS-505	ZP_06307810	Transposase IS5 family protein	<i>Cylindrospermopsis raciborskii</i> CS-505	79	96 (97)
T6 ORF3	10E6	ZP_00516180	Similar to transposase	<i>Crocospaera watsonii</i> WH 8501	537	56 (73)
T7 <i>cyrH-cyrK</i>	10E6	ACG63861	ProF, transposase	<i>Planktothrix rubescens</i>	33	67 (79)

synteny and nucleotide sequence are almost identical between two *C. raciborskii* strains (Stücken *et al.*, 2010), and in the present study, we found the same degree of conservation between the CYN genes of *Aphanizomenon* sp. 10E6 and the two partial *cyr* gene clusters of *Aphanizomenon* sp. strains 10E9 and 22D11. The synteny

between the *C. raciborskii* and *Aphanizomenon* sp. *cyr* gene clusters differed (Fig. 1) but the nucleotide sequence conservation for all orthologous genes was greater than 95 %. Thus, it was in the range of, or higher than, the similarity of the 16S rRNA genes of the two species (Table 2).

(b)

Hit	Description	Hit length	Percent identity (% positive)
hypA ZP_03766297	Hydrogenase nickel insertion protein HypA [<i>Nostoc azollae</i> ' 0708]	98	87 (94)
hypB ZP_03766298	Hydrogenase accessory protein HypB [<i>Nostoc azollae</i> ' 0708]	261	85 (92)
hypD ZP_03766295	Hydrogenase expression/formation protein HypD [<i>Nostoc azollae</i> ' 0708]	382	89 (95)
hypE ZP_03766296	Hydrogenase expression/formation protein HypE [<i>Nostoc azollae</i> ' 0708]	359	87 (95)
hypF NP_484737	Hydrogenase maturation protein [<i>Nostoc</i> sp. PCC 7120]	785	72 (84)
ORF1 NP_484736	Hypothetical protein alr0693 [<i>Nostoc</i> sp. PCC 7120]	347	81 (90)
ORF2 YP_325097	4-Oxalocrotonate tautomerase [<i>Anabaena variabilis</i> ATCC 29413]	52	81 (94)

Fig. 3. *hyp* gene cluster of *Aphanizomenon* sp. 10E6 and flanking genes. (a) Arrangement of the cluster. (b) Best BLASTX hits. There were no hits for ORF3 and ORF4.

Fig. 4. G+C isochores overlaid with *cyr* gene clusters and their flanking regions (as shown in Fig. 1). Green shading, *cyr* genes; grey shading, *hyp/hup* gene clusters. (a) Contig 27 of *C. raciborskii* CS-505 (ACYA01000027). (b) Contig 46 of *R. brookii* D9, partial sequence (ACYB01000046). (c) Scaffold 7 of *Aphanizomenon* sp. 10E6, gaps removed.

Cyanobacterial toxins are frequently produced by strains of different genera; however, the conservation of genes is usually considerably less than that observed for the CYN genes. Microcystin, for example, is produced by cyanobacteria of the genera *Microcystis*, *Anabaena* and *Planktothrix*. The nucleotide sequence of the different *mcy* genes has been shown to vary between 67 and 81 % (Rouhiainen *et al.*, 2004). Similarly, saxitoxin is produced by several genera of freshwater cyanobacteria and comparisons between the *sxt* genes of *Aphanizomenon*, *Anabaena* and *Cylindrospermopsis* revealed a 53–99 % nucleotide identity of the different orthologous genes (Mihali *et al.*, 2009).

Our phylogenetic analyses showed that the amidinotransferases involved in CYN synthesis are more conserved across different cyanobacterial genera than amidinotransferases of the same genera involved in the synthesis of different cyanotoxins (see Fig. 2a). These results are in line with the high sequence conservation observed. When the CYN gene sequences were analysed alone, they clustered according to their taxonomic affiliation (see Fig. 2b and Supplementary Fig. S1). The tree topology was similar to that observed for *rpoC1* (see Fig. 2c) except the distances

were considerably shorter. In essence, three clusters emerged: one containing all the *Aphanizomenon* sp. strains, the second containing strains of the genera *Cylindrospermopsis* and *Raphidiopsis* and the third containing *Aph. ovalisporum*, *A. bergii* and *U. natans*. The same pattern was also observed for other genes, such as the 16S rDNA gene and *cpcBA*-IGS (e.g. Stüken *et al.*, 2009). The only sequences found to contradict this pattern were partial *cyr/aoa* sequences derived from strains which do not produce CYN (Fig. 2b and Supplementary Fig. S1).

The presence of transposases and vestiges thereof coupled with the different arrangement of genes in the *C. raciborskii* and *Aphanizomenon* sp. *cyr* gene clusters indicate past recombination events within these clusters. The *mcy* and *sxt* gene clusters show similar signs of reorganization. For the *mcy* genes, it has been shown that the operon structure and the order of genes varies between the genera *Planktothrix*, *Anabaena* and *Microcystis* (reviewed by Dittmann & Börner, 2005). In addition, recombination between different domains within the *mcy* gene clusters, point mutations and deletion of gene regions, as well as lack of substrate specificity of A-domains have been shown to contribute to the synthesis of different microcystin

isoforms (Fewer *et al.*, 2008; Mikalsen *et al.*, 2003; Tooming-Klunderud *et al.*, 2008). A deletion within the second module of *mcvA* and the first module of *mcvB* resulted in the synthesis of another cyanotoxin, nodularin (Moffitt & Neilan, 2004; Rantala *et al.*, 2004). The number of genes within the *sxt* gene clusters of *Aphanizomenon*, *Anabaena* and *Cylindrospermopsis* varies, but five regions containing homologous *sxt* genes have been identified in all three genera. The arrangement of these five regions differs in each cluster, and the presence of transposases indicates a prevalent recombination (Mihali *et al.*, 2009). The number, type and conservation of tailoring genes present in the *sxt* gene clusters are thought to determine which saxitoxin analogues are produced. It has been shown that the *sxtI* gene, which encodes an *O*-carbamoyltransferase, is truncated in a *Lynbya wollei* strain. As a result, this strain only produces decarbamoylated analogues of saxitoxin (Kellmann *et al.*, 2008). In contrast with microcystin and saxitoxin, for which numerous chemical variants have been described (e.g. Schantz, 1986; Welker & von Döhren, 2006), only two analogues of cylindrospermopsin are known: 7-epi-CYN and deoxy-CYN (Banker *et al.*, 2000; Norris *et al.*, 1999). The genetic basis of these CYN variants is not known, but it is likely that the dearth of analogues is related to the high sequence conservation.

The *cyr* gene clusters in *C. raciborskii* strains AWT205 and CS-505 are flanked by genes of the hydrogenase gene cluster (Mihali *et al.*, 2008; Stucken *et al.*, 2010). This *hyp/hup* gene cluster is present in the *Aphanizomenon* sp. 10E6 genome (Fig. 3) but is not in close proximity to the *cyr* genes. Instead, the *cyr* genes are flanked by a transposase and an oxidoreductase protein on one side and a series of short ORFs with unknown function on the other side (Fig. 1 and Table 3). G+C-content analysis showed that the genomic region containing the *cyr* genes had an atypically high G+C-content compared with the rest of the scaffold (Fig. 4c) and also compared with the average genomic G+C-content. A similar high G+C-content region which contains the *cyr* genes was observed for *C. raciborskii* CS-505 (Fig. 4a). In contrast, no such region of high G+C-content was observed between the *hyp/hup* genes in *R. brookii* D9 (Fig. 4b), an area that has been proposed to contain the insertion or deletion site of the *cyr* genes in *C. raciborskii* CS-505 and *R. brookii* D9 (Stucken *et al.*, 2010).

Horizontal gene transfers (HGTs) play a prominent role in shaping cyanobacterial genomes (e.g. Zhaxybayeva *et al.*, 2006) and it has been suggested that the *cyr* genes have been transferred between closely related strains (Stucken *et al.*, 2009) as well as between strains of different genera (Kellmann *et al.*, 2006). The atypically high G+C-content of the *cyr* genes, their high sequence conservation, the presence of transposases and vestiges thereof within and at the sides of the *cyr* gene clusters, and the differences in flanking genes in *Aphanizomenon* sp. compared with *C. raciborskii*, support the view that the *cyr* genes have been transferred by HGT. In addition, the incongruent

phylogeny of the *cyr* gene fragments from non-CYN-producing strains corroborates this view. Since the entire gene sequences and flanking regions of these fragments are not available, it can only be speculated that they represent signs of HGT events. In this light, it would also be valuable to sequence the *aoa* gene fragments and flanking regions found in three non-CYN-producing strains, which are thought to represent natural deletion mutants (Rasmussen *et al.*, 2008).

The only results that may contradict HGT come from the phylogenetic analyses of *cyr/aoa* sequences from CYN-producing strains. These sequences cluster similarly to ribosomal and housekeeping genes, possibly indicating that the genes are ancient and have co-evolved. Such co-evolution has been suggested for genes involved in microcystin and nodularin biosynthesis (Rantala *et al.*, 2004). However, the sequence divergences of *rpoC1*, 16S rRNA and *mcv* genes were found to be approximately the same in microcystin producing strains (Rantala *et al.*, 2004), whereas the *cyr* genes described here are more conserved than 16S rRNA and *rpoC1* genes of the same strains (Table 2 and Supplementary Tables S3–S6).

In summary, two main scenarios may explain the current distribution and organization of the CYN gene clusters. The first involves an ancient origin of the CYN genes and implies that they were present in a common ancestor of all CYN-producing genera. The current distribution of CYN-producing and -non-producing strains within the same species is due to gene loss in the non-producing species. The high sequence conservation of the CYN genes may be a result of strong functional constraints and selection mechanisms. In the second scenario, which we favour, the high sequence conservation may also be caused by functional constraints and purifying selection. But in addition, HGT events are likely to have played a prominent role in spreading the CYN genes. The presence of gene fragments in non-CYN-producing strains with contrasting phylogenetic signals suggests that at least parts of the cluster have been transferred. Single transfers of the entire gene cluster into new genera may explain the phylogeny and distribution of CYN genes in CYN-producing taxa. The direction of transfer and the source of the CYN gene cluster remain obscure.

So far, CYN-producing strains of the *Aphanizomenon* sp. group have only been isolated from German waters. Evidence is accumulating, however, to show that strains belonging to this species also synthesize CYN elsewhere. The toxin has been reported in freshwater bodies in Italy (Messineo *et al.*, 2009), the Czech Republic (Bláhová *et al.*, 2009), Poland (Kokocinski *et al.*, 2009) and France (Brient *et al.*, 2009), which contained *Aph. flos-aquae*, *Aph. gracile* or morphologically related species. Further sampling and isolation of strains from these different locations and the analysis of their *cyr* gene clusters and flanking regions will greatly improve our understanding of the evolution of this gene cluster.

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