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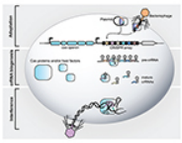

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RESEARCH PAPER

CRISPR-Cas systems in multicellular cyanobacteria

Shengwei Hou [a], Manuel Brenes-Álvarez[b], Viktoria Reimann[a], Omer S. Alkhnbashi[c], Rolf Backofen [c,d,e], Alicia M. Muro-Pastor [c], and Wolfgang R. Hess [a,f]

[a]Faculty of Biology, Genetics and Experimental Bioinformatics, University of Freiburg, Freiburg, Germany; [b]Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Seville, Spain; [c]Bioinformatics group, Department of Computer Science, University of Freiburg, Freiburg, Germany; [d]Center for Biological Systems Analysis (ZBSA), University of Freiburg, Freiburg, Germany; [e]BIOSS Centre for Biological Signaling Studies, University of Freiburg, Freiburg, Germany; [f]Freiburg Institute for Advanced Studies, University of Freiburg, Freiburg, Germany

ABSTRACT

Novel CRISPR-Cas systems possess substantial potential for genome editing and manipulation of gene expression. The types and numbers of CRISPR-Cas systems vary substantially between different organisms. Some filamentous cyanobacteria harbor > 40 different putative CRISPR repeat-spacer cassettes, while the number of *cas* gene instances is much lower. Here we addressed the types and diversity of CRISPR-Cas systems and of CRISPR-like repeat-spacer arrays in 171 publicly available genomes of multicellular cyanobacteria. The number of 1328 repeat-spacer arrays exceeded the total of 391 encoded Cas1 proteins suggesting a tendency for fragmentation or the involvement of alternative adaptation factors. The model cyanobacterium *Anabaena* sp. PCC 7120 contains only three *cas1* genes but hosts three Class 1, possibly one Class 2 and five orphan repeat-spacer arrays, all of which exhibit crRNA-typical expression patterns suggesting active transcription, maturation and incorporation into CRISPR complexes. The CRISPR-Cas system within the element interrupting the *Anabaena* sp. PCC 7120 *fdxN* gene, as well as analogous arrangements in other strains, occupy the genetic elements that become excised during the differentiation-related programmed site-specific recombination. This fact indicates the propensity of these elements for the integration of CRISPR-*cas* systems and points to a previously not recognized connection. The gene *all3613* resembling a possible Class 2 effector protein is linked to a short repeat-spacer array and a single tRNA gene, similar to its homologs in other cyanobacteria. The diversity and presence of numerous CRISPR-Cas systems in DNA elements that are programmed for homologous recombination make filamentous cyanobacteria a prolific resource for their study.

Abbreviations: Cas: CRISPR associated sequences; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; C2c: Class 2 candidate; SDR: small dispersed repeat; TSS: transcriptional start site; UTR: untranslated region.

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CRISPR; cyanobacteria; heterocyst; nitrogen fixation; programmed DNA recombination

Introduction

Genetic tools based on CRISPR-Cas technology are currently the most popular technology for the manipulation of gene expression and genome editing. In most of these approaches, the CRISPR-Cas Type II enzyme Cas9 is used together with a guide RNA to target specific regions in chromosomal DNA [1]. In addition, large potential exists for alternative CRISPR-Cas systems and novel applications, e.g., in the markerless generation of point mutations using Type I and Type III CRISPR-Cas systems for genome editing [2] or the use of the Type V-A Cas12a (previously known as Cpf1) for the rapid engineering of markerless knock-ins, knock-outs and point mutations [3,4]. Such facts underline that the search for additional types of CRISPR-Cas modules can lead to productive innovation.

Currently, six major types of CRISPR-Cas systems are known, which belong to two major classes and can be further subdivided into multiple subtypes [5]. The functions of the diverse genes and gene products involved in these systems can be classified into three primary functions: adaptation, processing and interference [6]. During adaptation, CRISPR-associated (Cas) proteins excise the protospacer sequence from an invader DNA directly or after reverse transcription of RNA into cDNA [7] and insert it into the first repeat of the CRISPR locus. The CRISPR RNAs (crRNAs) are transcribed from the repeat-spacer array in the form of a long precursor (pre-crRNA) that is processed into the individual crRNAs each consisting of a single spacer sequence and part of the adjoining repeat sequences. During the interference stage, sequences on either invading DNA elements or their transcripts become recognized by crRNAs as guides for the Cas protein complexes that cleave the targeted nucleic acid.

CRISPR-Cas systems have been classified into two classes with regard to the complexity of the effector ribonucleoprotein complexes. Class 1 systems consist of several different subunits, whereas Class 2 systems utilize a single modularized

CONTACT Wolfgang R. Hess wolfgang.hess@biologie.uni-freiburg.de Faculty of Biology, Genetics and Experimental Bioinformatics, University of Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany

The underlying research materials for this article can be accessed at https://figshare.com/articles/CRISPR-Cas_Systems_in_Multicellular_Cyanobacteria/6807191/1
Supplemental date for this article can be accessed here.

large protein, such as Cas9, Cas12a or Cas13a [5]. Proteins implicated in adaptation are the endonuclease Cas1, Cas2 and, in some systems, Cas4, which facilitates the integration of PAM-compatible spacers [8,9]. The PAM (protospacer adjacent motif) is a short sequence motif in the target DNA that flanks the crRNA-DNA duplex and is crucial for avoiding self-cleavage [10,11]. Despite the impressive general variation in gene content and sequence diversity among different types of CRISPR-Cas systems, all systems have been assumed until very recently to possess a single Cas1 protein, which is less diverse than other Cas proteins and therefore has served as a marker for CRISPR loci. However, this notion has been challenged by recent observations of C2c (Class 2 candidate) systems lacking the *cas1* gene, as they apparently only contain a CRISPR array and single gene encoding a large protein with no sequence similarity to Cas12a, Cas12b, Cas13a, or Cas9 [5]. Additionally, it was speculated that these systems might rely on an adaptation module (*cas1-cas2*) encoded elsewhere in the genome [12,13]. Therefore, the detection of putative novel CRISPR systems is not trivial: the numbers and types of CRISPR-Cas systems vary greatly, even between closely related strains, the similarity between Cas proteins can be very remote, and the existence of direct sequence repeats may also relate to different (non-CRISPR) genetic elements.

Cyanobacteria are the only bacteria that perform oxygenic photosynthesis. They occur in widely different environments as long as there is at least some light. Many cyanobacteria are also able to convert atmospheric nitrogen, N_2, into organic biomass, hence sustaining a diazotrophic lifestyle. This process, called N_2 fixation, is catalyzed by nitrogenase, an enzyme that can be irreversibly damaged by oxygen [14]. The need to protect the oxygen-sensitive nitrogenase from photosynthetically produced oxygen has probably driven the evolution of heterocysts, a type of differentiated cells providing a microoxic environment compatible with N_2 fixation in some filamentous cyanobacteria. The evolution of this specialized cell type has driven the division of cellular functions and processes between heterocysts vegetative cells along the filaments [15]. Heterocysts transfer fixed nitrogen to the neighboring vegetative cells whereas vegetative cells provide heterocysts with photosynthetically fixed carbon in return. Hence, heterocyst-forming cyanobacteria are true multicellular organisms. Heterocysts are formed in a complex differentiation process that includes the programmed site-specific deletion of large genetic elements that interrupt the reading frames of critical genes by homologous recombination between direct repeats [15–17].

It has been suggested that the Cas9 effector proteins of Class 2 CRISPR-Cas systems evolved from a type of TnpB-like transposase with an HNH nuclease insert that is particularly abundant in cyanobacteria [12]. Type I and Type III (Class 1) CRISPR-Cas systems are frequent in cyanobacteria [18]; however, no proteins with sequence similarity to the hitherto characterized Class 2 effectors such as Cas12a, Cas12b, Cas13a or Cas9 have been identified thus far. Therefore, novel Class 2 CRISPR systems could exist in some cyanobacteria, a view consistent with multiple instances of CRISPR-Cas candidate systems classified as subtype V-U [5,13].

The genomes of multicellular cyanobacteria are complex (up to 12.29 Mb, > 10,000 annotated genes) and rich in the number of transposable elements and transposase genes including some encoding TnpB-like transposases. Therefore, we scanned 171 publicly available genomes of multicellular cyanobacteria for the presence of CRISPR-like repeat-spacer cassettes (Table S1). We report a high number of CRISPR-Cas candidate systems, including some with likely Class 2 effector proteins that are associated with a repeat-spacer array that is almost invariably adjacent to a tRNA gene. We then focus on *Anabaena* (*Nostoc)* sp. PCC 7120 (from here: *Anabaena* 7120) in greater detail and demonstrate crRNA-typical expression patterns for three Class 1, one Class 2 and five orphan repeat-spacer arrays in this well-established model for filamentous cyanobacteria.

Results

CRISPR-Cas systems are frequent in multicellular cyanobacteria

When searching for arrangements of direct repeats that match the criteria of CRISPR repeat-spacer arrays with relaxed parameters, some cyanobacteria, e.g., *Tolypothrix bouteillei* VB521301, host many, up to 44 widely different CRISPR-Cas systems (Table 1, see Table S1 for the full results). Together with Cas2, the Cas1 DNA-specific endonuclease makes up the core machinery of the CRISPR adaptation process. Therefore Cas1 is, with very few exceptions [5], almost universally conserved among different types of CRISPR systems. The here studied genomes of 171 filamentous cyanobacteria contain altogether 391 *cas1* genes with different domain composition. Forty of the deduced Cas1 proteins are fused to a reverse transcriptase (RT) domain and 31 to a Cas4 protein domain. Such gene fusions are in line with findings that Cas4 promotes the integration of spacers from invading DNA with the correct PAM [8,9] and that the fused RT domains facilitate the direct CRISPR spacer acquisition from RNA [7]. Phylogenetic analysis of our set of Cas1 proteins yielded seven distinct clusters (Figure 1 and Figure S1). Three of these clusters consist of free-standing Cas1 sequences, whereas two clusters each contain Cas1-Cas4 or Cas1-RT fusions. The three clusters of Cas1 proteins encoded by free-standing genes each contain one of the three Cas1 proteins of *Synechocystis* sp. PCC 6803. We included them in this analysis because the unicellular *Synechocystis* sp. PCC 6803 is the best-studied model for CRISPR-Cas systems in cyanobacteria [8,19–23]. The clustering of the three *Synechocystis* sp. PCC 6803 Cas1 sequences suggests that they match well to the three major groups of Cas1 proteins lacking other fused domains of filamentous cyanobacteria. The set of 391 Cas1 proteins is available in Supplemental Dataset 1.

We noticed a striking discrepancy in the number of *cas1* genes and the number of repeat-spacer arrays. There are 5 *cas1* genes and 44 repeat-spacer instances in *Tolypothrix bouteillei* VB521301, 12 *cas1* genes and 29 repeat-spacer arrays in *Scytonema hofmannii* sp. PCC 7110, 5 and 15 in *Aphanizomenon flos-aquae* NIES-81, 3 and 14 in *Rivularia* sp. PCC 7116, 1 and 6 in *Nostoc punctiforme* PCC 73102, and 3 and 11 in *Anabaena* 7120 (Table 1), respectively. The genome sequences of aforementioned organisms are complete or in draft state with rigorous quality control [24], excluding assembly artefacts as a possible source of

Table 1. GenBank assembly accession numbers and Cas1 protein features of selected cyanobacterial strains. Morphological subsections were assigned according to Rippka et al. [53]. Cas1 gene types were defined as free-standing *cas1*, *cas4_cas1* or RT_*cas1* fusions. Accession numbers labelled by an asterisk refer to the JGI genome portal, all other refer to Genbank.

Cyanobacterial strains	Subsection	Habitat	Accession	Number of Cas1 genes	Domain types of Cas1	Number of CRISPR cassettes	Identified C2c5 homologs
Anabaena sp. PCC 7120	IV	Freshwater	GCA_000009705.1	3	RT_Cas1, 2x Cas1	11	All3613, Alr2691
Anabaena cylindrica sp. PCC 7122	IV	Freshwater	GCA_000317695.1	5	RT_Cas1, 4x Cas1	13	Anacy_2856, Anacy_0603
Calothrix sp. HK-06	IV	Terrestrial	GCA_001904745.1	1	Cas1	10	0
Calothrix sp. PCC 7507	IV	Terrestrial	GCA_000316575.1	9	Cas4_Cas1, RT_Cas1, 7x Cas1	10	0
Calothrix desertica sp. 7102	IV	Terrestrial	2509887024*	6	Cas4_Cas1, RT_Cas1, 4x Cas1	14	0
Fischerella major NIES-592	V	Hot spring	GCA_001904645.1	2	RT_Cas1, Cas1	4	0
Nostoc sp. PCC 7107	IV	Freshwater	GCA_000316625.1	3	RT_Cas1, 2x Cas1	14	Nos7107_4709
Nostoc calcicola FACHB-389	IV	Terrestrial	GCA_001904715.1	2	2x Cas1	19	0
Nostoc punctiforme PCC 73102	IV	Terrestrial/ symbiontic	GCA_000020025.1	1	Cas1	6	Npun_R5656
Limnothrix rosea IAM M-220	III	Marine	GCA_001904615.1	3	RT_Cas1, 2x Cas1	9	0
Phormidium ambiguum IAM M-71	III	Freshwater	GCA_001904725.1	8	RT_Cas1, 7x Cas1	14	0
Rivularia sp. PCC 7116	IV	Marine	GCA_000316665.1	3	Cas1, 2x Cas1	14	0
Scytonema hofmannii sp. PCC 7110	IV	Terrestrial (limestone)	GCA_000346485.2	12	2x Cas4_Cas1, 2x RT_Cas1, 8x Cas1	29	WA1_24145
Tolypothrix sp. PCC 7601	IV	Terrestrial	GCA_000300115.1	1	Cas1	19	FDUTEX481_03012, FDUTEX481_08898
Tolypothrix bouteillei VB521301	IV	Stone surface	GCA_000760695.2	5	Cas4_Cas1, RT_Cas1, 3x Cas1	44	0

* JGI Taxon ID

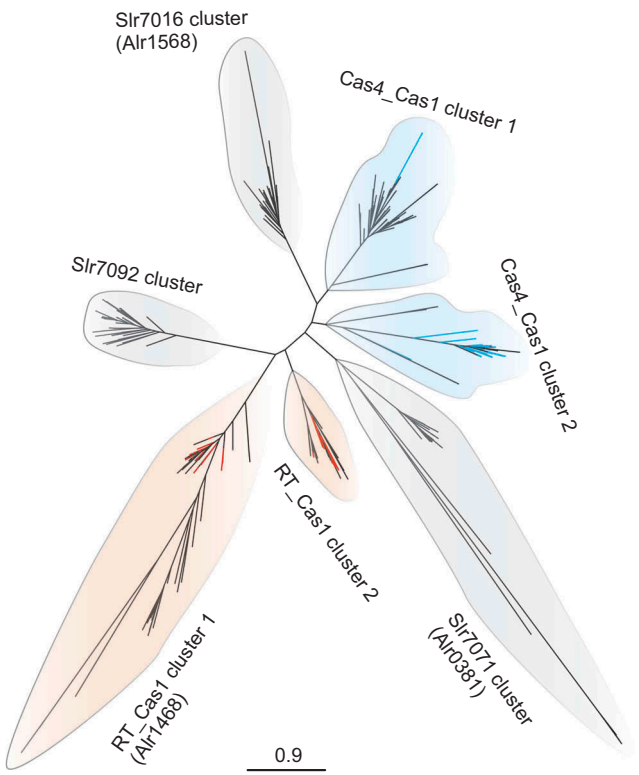

Figure 1. Phylogenetic analysis of 391 Cas1 sequences from 171 filamentous cyanobacteria. The three Cas1 protein sequences from *Synechocystis* sp. PCC 6803 were included in this unrooted phylogenetic tree, each representing a Cas1 cluster (grey shaded clusters) of 7 major clusters. Dark blue branches within light blue shaded clusters represent Cas1 proteins fused with a Cas4 domain, while red branches within light pink shaded clusters represent Cas1 proteins fused with a RV_1 domain. The positions of Cas1 proteins from *Anabaena* 7120 are given in brackets. The detailed Cas1 tree with sequence names and bootstrap values is presented in Figure S1, the protein sequences can be found in Supplemental Dataset 1.

overestimation. To look further into this obvious discrepancy, we filtered the available sequences (Table S1) for completion of sequencing, yielding 36 finished genome sequences. From these, we chose three representative examples for which we performed a detailed re-annotation of the CRISPR-Cas systems (available upon request). *Calothrix* sp. PCC 7507 possesses 10 repeat-spacer arrays, 9 *cas1* genes and four identifiable CRISPR-*cas* loci (Figure 2). However, three *cas1* genes are fragmented and constitute pseudogenes. The remaining six include two gene copies encoding a Cas1-Cas4 and a Cas1-RT fusion (Figure 2). *Rivularia* sp. PCC 7116 and *Nostoc* sp. PCC 7107 both have 14 separate instances of repeat-spacer arrays but possess only three *cas1* genes. Hence, in all these cases the numbers of repeat-spacer arrays is larger than the number of *cas1* gene copies. Moreover, the two instances of genes encoding Cas1-RT fusions (in *Calothrix* sp. PCC 7507 and in *Nostoc* sp. PCC 7107) co-locate with an additional free-standing *cas1* gene and are part or very close to subtype III-D systems (Figure 2). From these observations we conclude that genes encoding Cas1-RT fusions may become integrated in addition to existing *cas1-cas2* adaptation modules and that the number of repeat-spacer arrays regularly exceeds the number of *cas1* gene copies.

Unicellular cyanobacteria do not share this feature, e.g., the model cyanobacteria *Synechocystis* sp. PCC 6803 and PCC 6714 each harbor three different *cas1* genes, matching the number of three different repeat-spacer arrays [19,20]. Therefore, the *cas1*-lacking systems in multicellular cyanobacteria might rely on adaptation modules encoded elsewhere in the genome [12,13] or depend on other mechanisms for recombination.

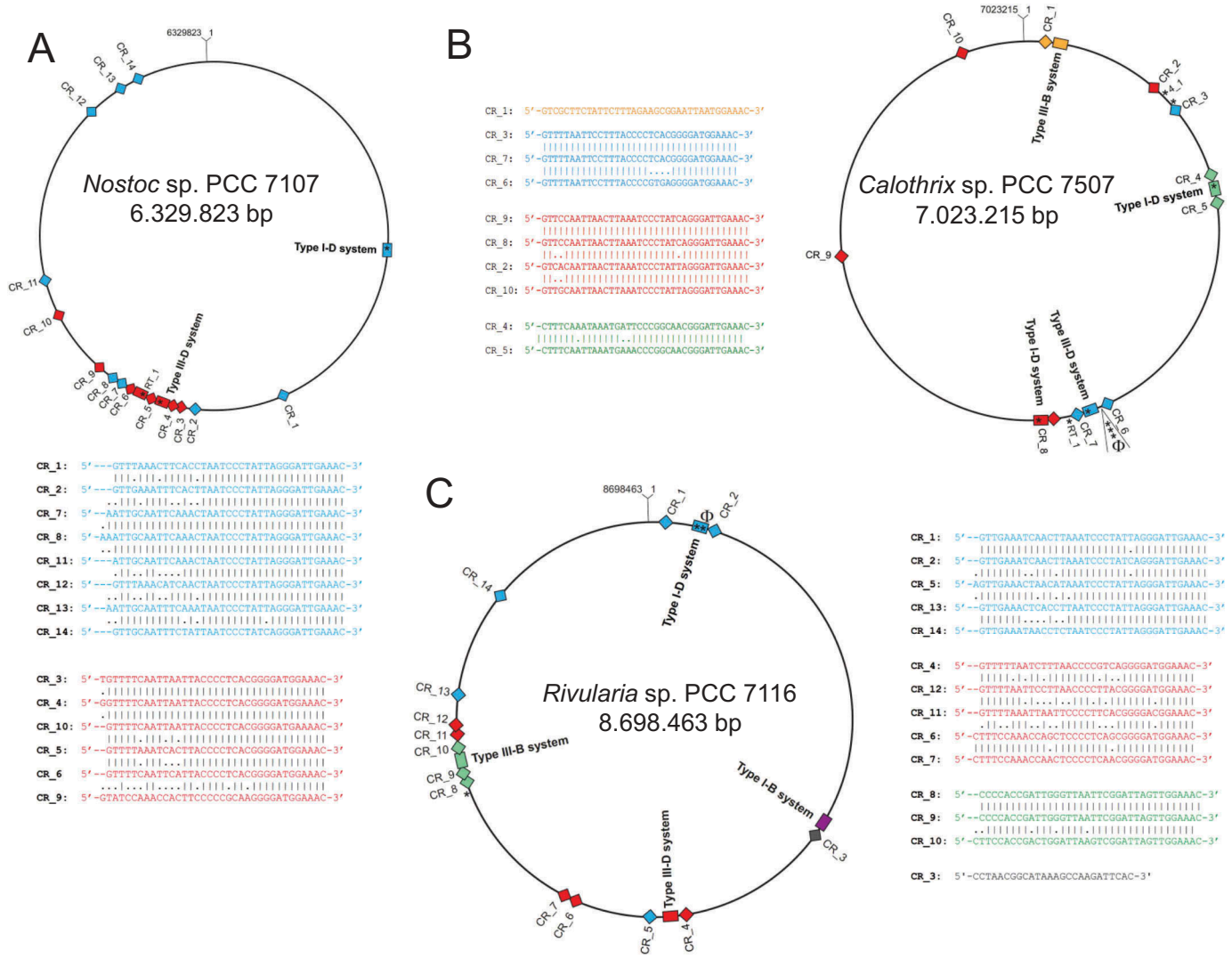

Figure 2. CRISPR-Cas systems and repeat-spacer arrays in three representative species, (A) *Nostoc* sp. PCC 7107, (B) *Calothrix* sp. PCC 7507 and (C) *Rivularia* sp. PCC 7116. The location of *cas1* genes is indicated by asterisks, an added 'RT_1' or '1_4' indicates RT or Cas4 fusions. Pseudogenes are labelled by a Φ symbol.

In the model *Anabaena* 7120, five DNA recombinase proteins are involved in the recombination in heterocyst differentiation. XisA mediates the excision of the 11 kb element from the *nifD* gene [25–27], the three-subunit enzyme encoded by the *xisF, xisH* and *xisI* genes excises the 55 kb element from the *fdxN* gene [28–30] (see Figure 3 for their location), while the XisC recombinase deletes the 10.5 kb element from the *hupL* gene [31,32]. The XisI recombinase has recently been identified as a candidate protein for an anti-phage defense system based on its pfam08869 domain [33]. Moreover, there are at least eight additional recombinase genes in the genome of *Anabaena* 7120 (*all3124, alr0083, alr0084, alr2075, alr3224, alr3645, asl0560* and *asl0561*). The involvement of host-encoded factors such as IHF (integration host factor) in Cas1-Cas2 mediated adaptation has been reported for some types of CRISPR systems [34–36]. Therefore, it is tempting to speculate that one or several of the cyanobacterial recombinases are involved in CRISPR adaptation by functionally replacing the Cas1-Cas2 integrase complex that normally is facilitating the site-specific integration of new spacers into the CRISPR array [37,38].

CRISPR-Cas systems are present in genetic elements that are excised during cell differentiation by homologous recombination

The appearance of fusions between the Cas1 protein and an RT domain is typical of certain types of CRISPR-Cas systems in cyanobacteria [39]. We observed that the presence of genes encoding RT_Cas1 fusions is frequently linked to the occurrence of two separate CRISPR repeat-spacer units framing the *cas1*-RT gene on the forward and reverse DNA strands (e.g., in *Anabaena* 7120, Figure 3). This suggests that an unknown DNA recombination event is involved in the evolution of some of the cassettes that contain these genes. The *cas1*-RT gene in *Anabaena* 7120 is not fused to a *cas6* gene encoding the maturation endoribonuclease activity as observed in some other bacteria, e.g., *Marinomonas* [7,39].

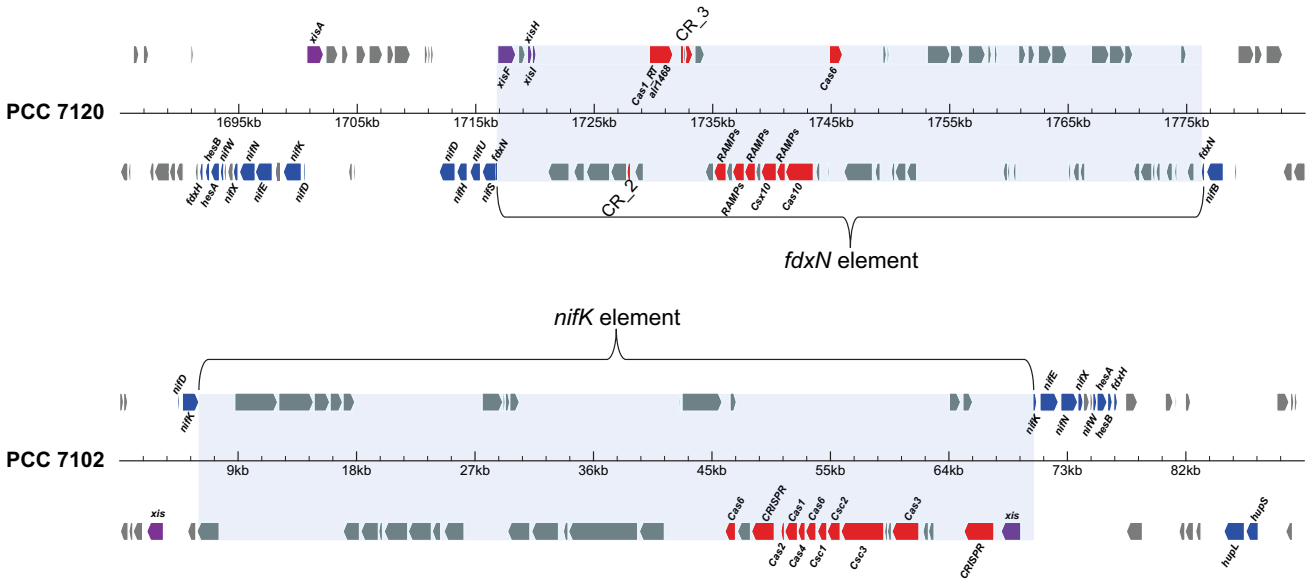

Figure 3. Examples of CRISPR-Cas systems that are encoded on genetic elements that are excised during cell differentiation into nitrogen-fixing heterocysts. The upper example is in the model organism *Anabaena* 7120, in which a CRISPR-Cas system is present within the *fdxN* element. The gene *alr1468* encoding a reverse transcriptase-Cas1 fusion protein is annotated; details for the repeat spacer arrays CR_2 and CR_3 can be found in Table 2. The lower example presents the *nifK* element in *Calothrix desertica* sp. PCC 7102. CRISPR repeat-spacer arrays are labelled 'CRISPR'. Note that the *Anabaena* 7120 element contains a *cas1*-RT fusion gene that is framed by split instances of the repeat-spacer arrays CR_2 and CR_3. Recombinase genes are labeled *xis* and *xisAFHI* and colored purple, *cas* genes are colored red, genes related to nitrogen fixation are colored blue, all other genes are in grey.

Heterocyst differentiation includes the deletion of large genetic elements that interrupt the reading frames of critical genes. In different cyanobacteria, there are altogether more than ten different genes known that can be interrupted by such elements. Some of the frequently interrupted genes are the *nifH, nifD* and *nifK* (encoding nitrogenase Fe protein and subunits alpha and beta), *hupL* (encoding a subunit of heterocyst-specific uptake hydrogenase), *fdxN* (heterocyst ferredoxin) and *hglE* (encoding heterocyst glycolipid synthase) genes [25,28–32]. We observed that in several cases, CRISPR-*cas* systems are associated with these genetic elements that are precisely excised from the genome during the differentiation of heterocyst cells. In *Anabaena* 7120, this is the case for the *fdxN* element (Figure 3). Similar CRISPR arrangements can be found in the *nifK* elements of *Calothrix* sp. 7102 (Figure 3), *Calothrix* sp. HK-06 and *Calothrix* sp. 7103, *nifD* element of *Tolypothrix* PCC 7601, and *nifH* and *hglE* elements of *Calothrix* sp. PCC 6303. The fact that different types of CRISPR-Cas systems are present in different elements suggests that they evolved independently from each other. Thus, these elements constitute a preferred site for the integration and hosting of mobile CRISPR-Cas cassettes, as was observed previously for certain types of mobile genetic elements [5]. It should be noted that the CRISPR-Cas cassettes together with the elements in which they reside are eliminated during heterocyst differentiation. These facts point further to a previously not recognized connection between CRISPR-Cas cassettes and the genetic mechanisms involved in heterocyst differentiation.

Characteristics of CRISPR-Cas systems in the model Anabaena *7120*

Based on the number of *cas1* genes, there are three different Class 1 CRISPR-Cas systems in *Anabaena* 7120 (Table 1). However, a search for interspaced direct repeats showed that there are at least 11 CRISPR and CRISPR-like repeat-spacer cassettes (designated here CR_1 to CR_11) in *Anabaena* 7120, with more than 100 spacers (Table 2, Figure 4), all located in the 6,413,771-bp chromosome, whereas the six plasmids are free of such cassettes. The presence of multiple small dispersed repeat (SDR) sequences was previously reported for *Nostoc punctiforme* and related cyanobacteria [40]; however, these SDR sequences are different from the CRISPR repeats presented here. The repeat-spacer cassettes CR_1 to CR_11 could be fragmented versions of a lower number of functional CRISPR-Cas systems, pseudogenized versions or belong to novel types of such systems. To test their transcription as an indicator of functionality, we isolated RNA from four different cultures and hybridized specific single-stranded RNA probes after gel electrophoretic separation and blotting. The observed lengths of mature crRNAs correlated well with the theoretically expected lengths of ~ 44 nt (± 5 nt) for double processing (e.g. CR_4, 7, 8, 11 in Figure 5) or ~ 73 nt (± 5 nt) in case of single processing (e.g. CR_1, 2, 3 in Figure 5). Hence, the results showed that all of the elements are transcribed and exhibit the typical pattern of precursor accumulation, processing intermediates and accumulated crRNAs (Figure 5). Thus, they are likely part of functional CRISPR-Cas systems. We included RNA from cultures grown for a nitrogen starvation time course that would be long enough to trigger heterocyst differentiation because of the affiliation of CRISPR-Cas systems, such as CR_2 and CR_3, with elements directly affected by this process. However, remarkable nitrogen-dependent differences in crRNA accumulation were not detected over the here applied time course of 32 h (Figure 5), indicating these CRISPR

Table 2. Predicted repeat-spacer arrays in *Anabaena* 7120. Each array has been numbered (ID), followed by the nucleotide positions of the TSS and the repeat-spacer arrays in the chromosome, the orientation (O) on the forward (+) or reverse (–) strand, the repeat sequence (DR), the number of repeats, with the total number including imperfect repeats in brackets (#), the length of spacers (L) the structural motif family (M), sequence family (F) and super family (S) as classified by the CRISPRmap algorithm [49], followed by the subtype and remarks (?, unknown). For the location within the chromosome of *Anabaena* 7120 see also Figure 4.

ID	TSS	Start	End	O	transcribed leader	DR	#	L	M	F	S	Subtype, remarks
CR_1	445445	445573	447786	+	128	GTTACTTACCATCACTTCCCCGCAAGGGGATGGAAAC	28 (29)	33–48	4	8	E	subtype I-A, no cas6
CR_2	1728349	1728071	1727817	–	278	GTTTTAATTCCTTTACCCCTCACGGGGATGGAAAC	3 (4)	37–40	4	9	E	subtype III-D; CR_2
CR_3	1731975	1732269	1733321	+	294	GTTTTAATTCCTTTACCCCTCACGGGGATGGAAAC	9 (15)	34–40	4	9	E	and CR_3 belong to one element; RT_cas1 fusion
CR_4	1836427	1836813	1837723	+	386	GTTTCTATTAACACAAATCCCTATCAGGGATTGAAAC	13	33–41	8	9	E	subtype I-D
CR_5	2179566	2179167	2178606	–	399	GTTTTTAATCTCTTACCCCTCACGGGGATGGAAAC	8 (11)	39–42	4	2	E	?
CR_6/7	3518141	3518084	3516820	–	57	GTTTTAACTAACAAAAATCCCTATCAGGGATTGAAAC	13 (16)	31–44	8	9	E	134 nt MITE insertion in repeat 9
CR_8	3836504	3840120	3840737	+	3616	GTTGCAACACCATATAATCCCTATTAGGGATTGAAAC	9	33–44	8	9	E	?
CR_9	4362990	4362,577	4362255	–	413	CGTTGCAACCCTCCTTCCAGTAATGGGAGGGTTGAAAG	3 (5)	32–35	?	?	?	C2c5, All3613 effector
CR_10	5647342	5,647145	5646379	–	197	CTTTCCGATCACATCACCCCGAAAGGGGATGGAAAC	10 (11)	32–45	-	18	C	?
CR_11	5654075	5654133	5654384	+	58	GTTAAAACCCTCTAAAATCCCTATCAGGGATTGAAAC	3	34–36	8	9	E	?

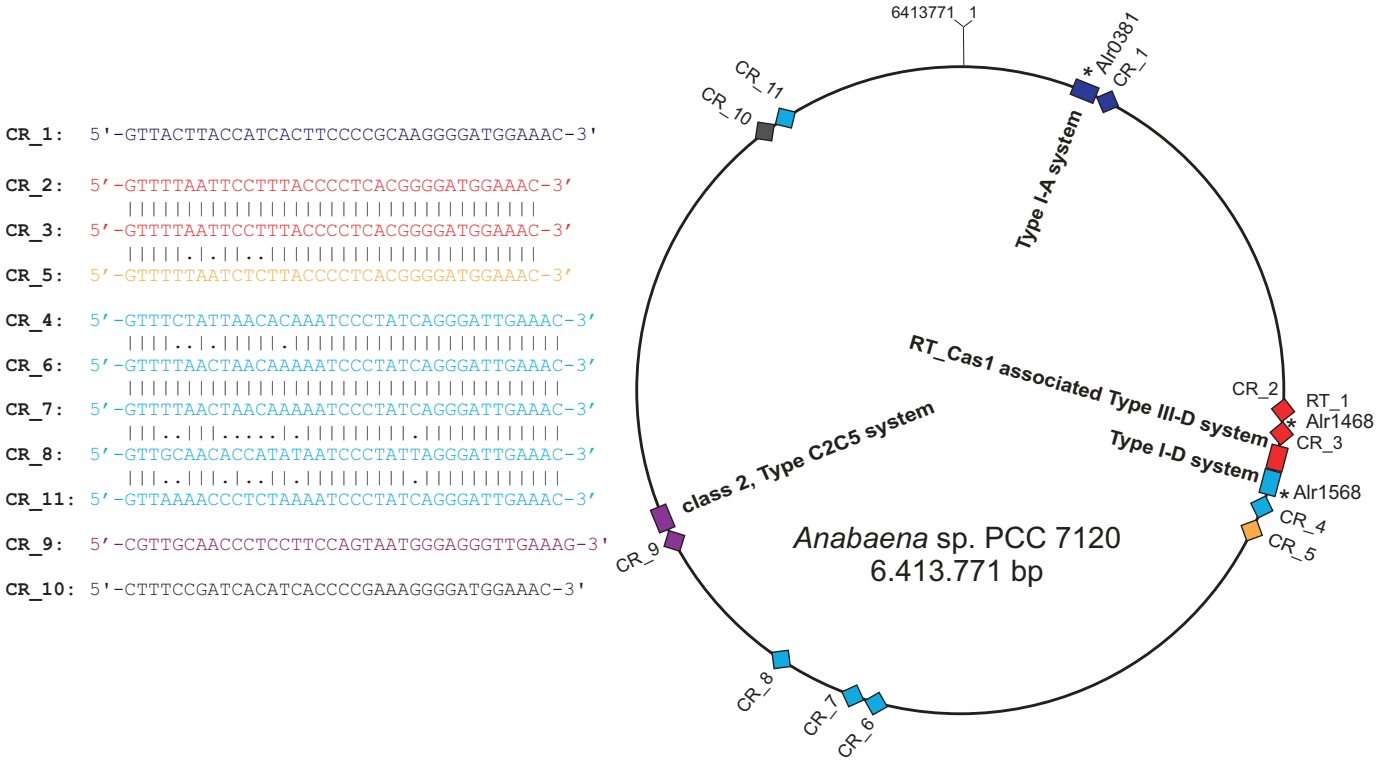

Figure 4. CRISPR-Cas systems in *Anabaena* 7120. Left: Alignments of CRISPR direct repeats. Right: Schematic distribution of CRISPR-*cas* systems, their subtype annotation and the location of repeat-spacer arrays in the chromosome of *Anabaena* 7120. The location of *cas1* genes is indicated by asterisks and the respective gene IDs. For further details, see also Table 2.

cassettes were actively transcribed in the vegetative cells independent of nitrogen availability.

Organisms possessing CRISPR-Cas systems become immune to phage or other invading DNA by the insertion of DNA sequences (spacers) into the leader-repeat junction (i.e., at the 5′ end of the repeat-spacer array) in a site-specific process called adaptation. The leader region, especially its 3′ end, is indispensable for this adaptation [41–45]. Therefore, it must contain sequence determinants important for adaptation. However, the lengths of CRISPR leaders vary greatly in size, from 47 nt in some bacteria to several hundred nt in some hyperthermophilic archaea. Moreover, they possess long regions of low complexity sequence, show only limited sequence conservation [46] and therefore are difficult to predict [47]. Using differential RNA-seq, we previously experimentally defined a genome-wide map of more than 10,000 transcriptional start sites (TSS) of *Anabaena* 7120 at single-nucleotide resolution [48].Therefore, it is possible to precisely map the first transcribed nucleotide and to infer the length of the transcribed part of the leader when the element was expressed. This was possible for all

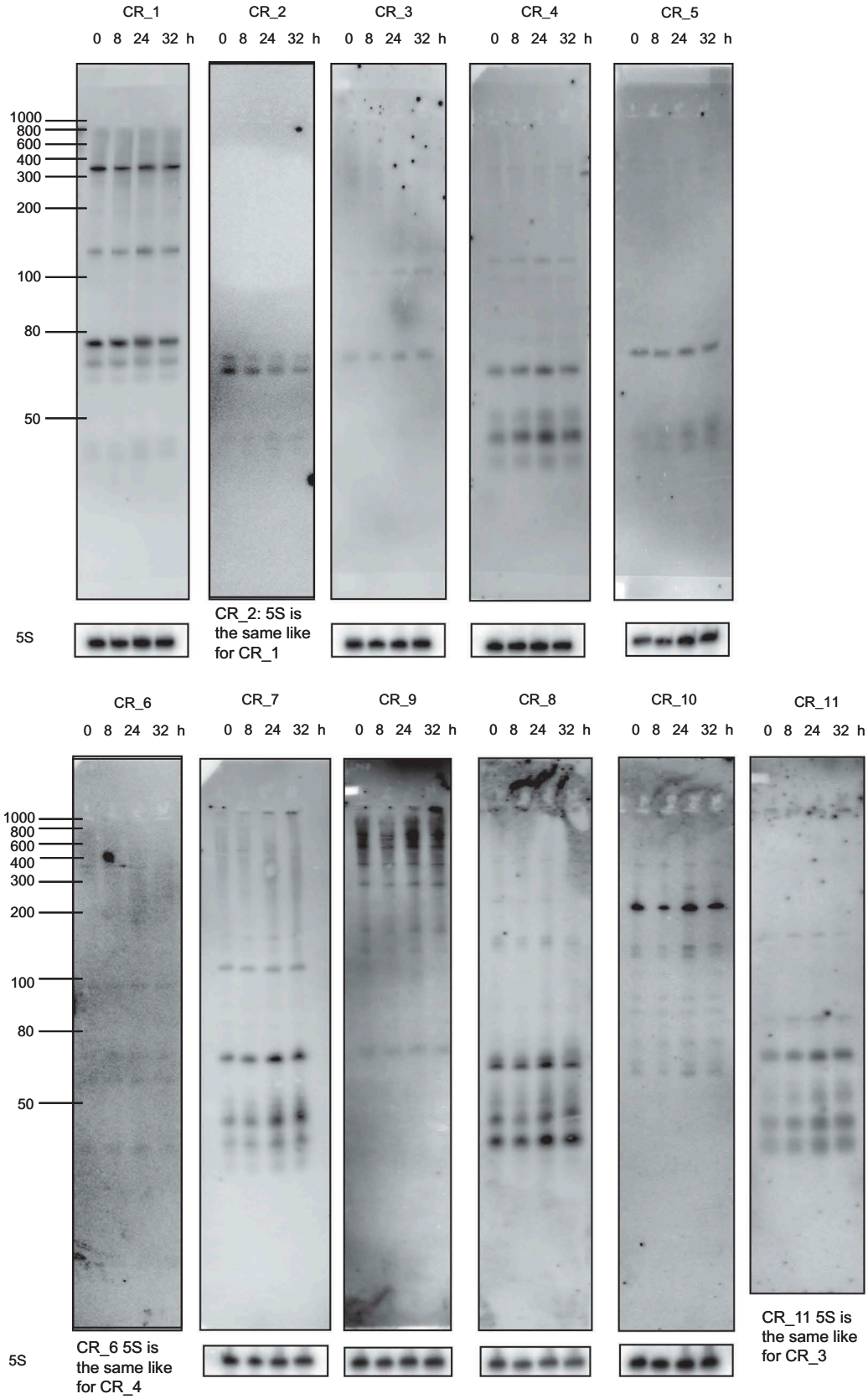

Figure 5. Expression of crRNAs from repeat-spacer arrays in *Anabaena* 7120. Total RNA was isolated from cultures grown under standard conditions for 8, 24 and 32 h after the removal of nitrogen, separated by electrophoresis on denaturing 15% PAA gels and transferred to nylon membranes. Single-stranded specific RNA probes were used for Northern hybridization. A control hybridization against 5S rRNA was performed to control for equal loading (the following membranes were used twice: for CR_3 was re-hybridized with the CR_11 probe, CR_1 with the CR_2 and CR_4 with the CR_6 probe later). The size of marker fragments is given on the left.

11 repeat-spacer cassettes. The length of the transcribed leaders varied from 57 to 3,616 nt (Table 2).

When judged by the association with known *cas1* genes, the arrays CR_1 to CR_4 represent classical Class 1 CRISPR elements (the two repeat-spacer arrays CR_2 and CR_3 frame the RT_*cas1* gene and belong to the same element, as depicted in Figures 3 and 4). Thus, there are at least three distinct Class 1 systems and one Class 2 (CR_9) system. The repeats CR_6 and CR_7 can be joined because they are only separated by the insertion of a 134 nt long miniature inverted repeat element (MITE) in repeat 9 of an originally contiguous array, yielding a total of five orphan repeat-spacer arrays.

Some repeats might be unified according to the similarities among their sequences, lumping CR_5 together with CR_2 and CR_3 in one group and CR_4, CR_6, CR_7, CR_8 and CR_11 in another (Figure 4), leaving CR_1, CR_9 and CR_10 separate. This is consistent with their assignment to distinct structural motif, sequence and super families as classified by the CRISPRmap algorithm [49] (Table 2). However, this unification might be an oversimplification. Hence, even when judged in a very restrictive way, there are at least five different types of arrays in total.

Novel CRISPR-Cas systems have substantial potential for genome editing and manipulation of gene expression. Therefore, it is interesting that one of the remaining systems, CR_9, is associated with a gene encoding All3613, a relatively large protein of unknown function. This protein is significantly similar in its C-terminal region to a subset of TnpB proteins encoded by transposons of the IS605 family, a feature typically associated with the Class 2 effector proteins Cas12b (C2c1) and C2c3 [12]. Among the studied 171 genomes of filamentous cyanobacteria, we found 86 All3613 homologs with a bit score ≥ 100, of which 29 were associated with a CRISPR array. This percentage is higher than expected by chance, supporting the idea that All3613 represents a novel type of CRISPR effector. This view was further supported when All3613 was analyzed by the HHpred algorithm [50], identifying a ~ 200 residues long similarity of the C terminus to the Cas12a (Cpf1) proteins of *Lachnospiraceae* bacterium ND2006 (probability 98.82, E-value $3.3e^{-10}$), *Acidaminococcus* sp. BV3L6 (probability 98.58, E-value $2.2e^{-9}$) and of *Francisella tularensis* subsp. novicida (probability 98.57, E-value $7.7e^{-9}$). All three proteins have been well characterized as single RNA-guided Type V effector proteins [4,51,52]. Nevertheless, proteins such as All3613 are with 648 amino acids substantially shorter than these effectors (e.g., Cas12a (Cpf1) of *Lachnospiraceae* bacterium ND2006 is 1231 residues long). Therefore, it is important that All3613 as well as many of its homologs are directly adjacent to a repeat-spacer cassette and that this cassette is expressed (Figure 5). A likely paralogous gene with *all3613* is *alr2691*, encoding a protein that in a pairwise alignment exhibits 40% identical and 60% similar amino acid residues with All3613 (bit score of 447). However, *alr2691* is not connected to a repeat-spacer array anywhere close in the genome.

The number of direct repeats in the CRISPR arrays associated with *all3613* homologs in cyanobacteria was relatively low, with a maximum count of 13, mean count of 6, and median count of 5, pointing to a possibly inefficient insertion process of new spacers. We observed that the majority of these CRISPR arrays (24/29) were adjacent to a tRNA gene, for example, *trnT*(CGT) in *Anabaena* 7120, *trnV*(GAC) in *Nostoc punctiforme* PCC 73102, *trnA*(CGC) in *Aphanizomenon flos-aquae* NIES 81 and *trnA*(GGC) and *trnR*(CCG) in two instances in *Tolypothrix* sp. PCC 7601 (Figure 6). Hence, All3613 or its homologues in other cyanobacteria might constitute effector protein candidates for a novel type of CRISPR system or some kind of mobile genetic element. It cannot be excluded from consideration that the immediately adjacent tRNA genes served as integration sites of the respective cassettes. But the association with multiple different tRNA genes is puzzling in this regard (Figure 6). Although none of these putative Class 2 systems would be directly associated with a *cas1* gene, the assignment of these regions as uncharacterized CRISPR system would be consistent with biocomputational analyses, which suggested that homologs of All3613 (Ava_2196 in *Trichormus variabilis* ATCC 29413 (previously called *Anabaena variabilis* sp. PCC 8801) and protein WP_027402996.1 in *Aphanizomenon flos-aquae* NIES 81) might constitute the core subunits of a Class 2 system called C2c5 [5,13]. Because All3613 and its homologs are shorter than the characterized Class 2 single effectors we looked for the possible synteny with other genes. Except the tRNA genes following the arrays, we only identified a gene encoding a MerR-type transcriptional regulator that is frequently located directly adjacent to the *all3613/c2c5* gene (Figure 6).

Summary and perspective

Certain filamentous cyanobacteria appear to be a rich source of CRISPR-Cas systems. In the case of *Anabaena* 7120, we show expression of the separate instances of repeat-spacer arrays by Northern hybridization with a pattern typical of the accumulation of crRNAs. We report that different types of CRISPR-Cas systems are encoded in different types of the genetic elements that are recombined during the differentiation of heterocysts, suggesting their independent evolution. All3613 might be a possible effector protein of the C2c5 type Class 2 CRISPR-Cas systems or belong to a novel genetic element.

Material and methods

The wild type strain of *Anabaena* 7120 was bubbled with an air/CO_2 mixture (1% v/v) and grown photoautotrophically at 30°C in $BG11_0C$ medium [53] lacking $NaNO_3$ but containing 6 mM NH_4Cl, 10 mM $NaHCO_3$ and 12 mM tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid-NaOH buffer (pH 7.5) until exponential phase. In order to induce nitrogen deficiency, cells grown in the presence of ammonium were collected by filtration, washed with and resuspended in nitrogen-free $BG11_0C$. Four RNA samples were isolated from cells taken at 0h, 8h, 24h and 32h after removing combined nitrogen from the media.

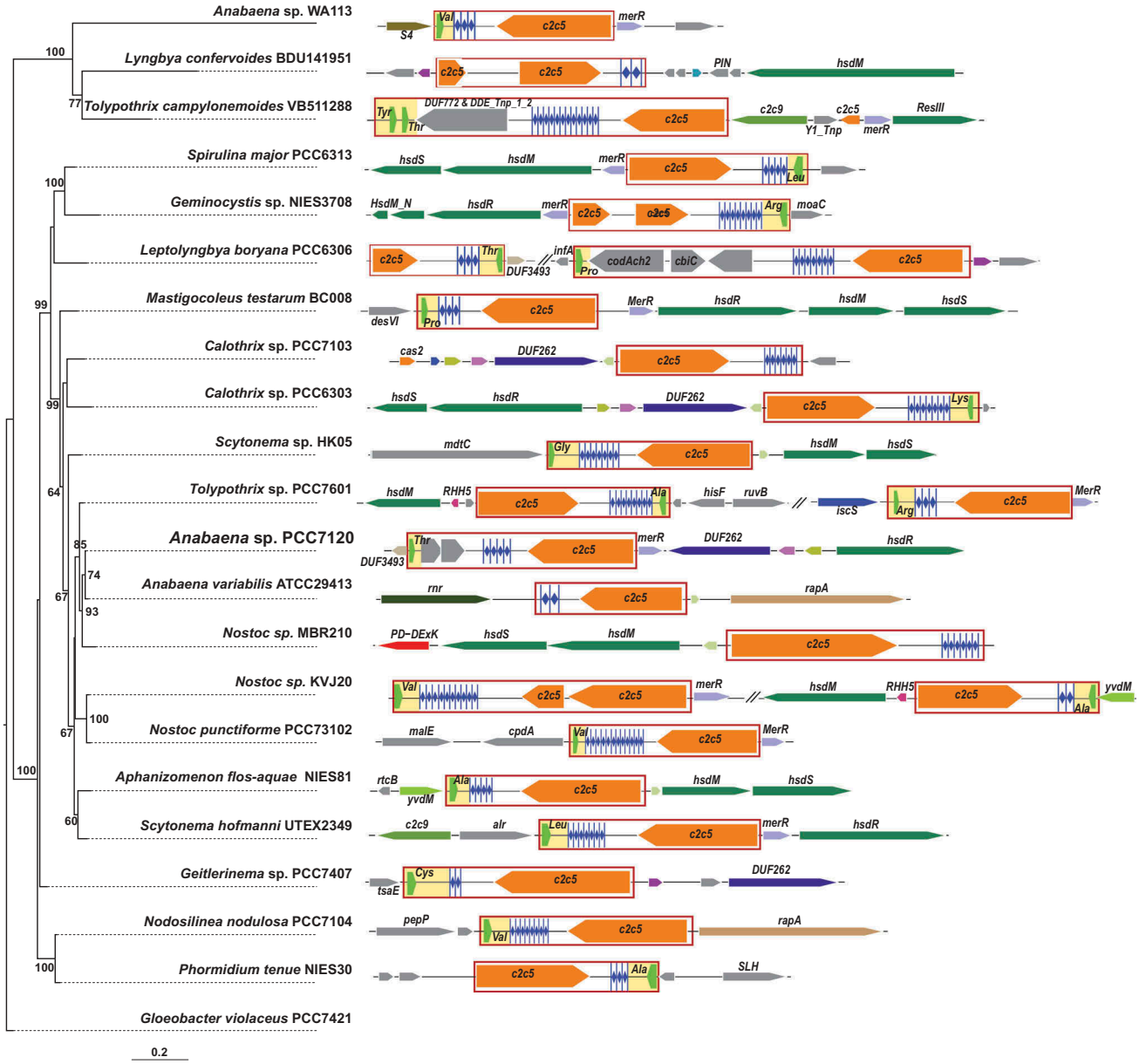

Figure 6. Synteny of Class 2 candidate systems. On the left the phylogenetic relationships are drawn based on 16S rRNA sequences, on the right the arrangements of putative CRISPR-*cas* systems are depicted that have an *all3613* homolog next to the array and lack any known genes for adaptation or other known *cas* gene. Note the frequent presence of different tRNA genes adjacent to the repeat-spacer arrays. The units consisting of the *all3613* homolog, the repeat-spacer array and tRNA gene (if present) are boxed. Known and putative *cas* genes are colored orange. The different tRNA genes are colored in light green and shaded in yellow for visualization, and their cognate amino acid is indicated. Numbers on the phylogenetic tree are bootstrap values and given if ≥ 60. The position of the model *Anabaena* 7120 is highlighted by larger fonts. 16S rDNA sequence from *Gloeobacter violaceus* PCC7421 was used as an outgroup to root this tree.

Total RNA was isolated using hot phenol as described [54] with some modifications. Hot phenol was added to the cells immediately after addition of lysis buffer and incubation was carried out at 65°C for 5 min. Further extractions were carried out with hot phenol, phenol:chloroform (1:1) and chloroform, followed by RNA precipitation by addition of one volume of isopropanol. CRISPR-related transcript accumulation was analyzed by Northern hybridization using single-stranded radioactively labelled RNA probes transcribed *in vitro* from PCR-generated templates (see Table S2 for primers) as described [55]. The oligonucleotide for the detection of 5S rRNA was ^{32}P labelled using polynucleotide kinase (Thermo Fisher) and γ-ATP.

Phylogenetic analysis

The maximum likelihood tree was constructed based on 391 Cas1 proteins from 171 sequenced filamentous cyanobacteria and 3 Cas1 proteins from *Synechocystis* sp. PCC 6803. These Cas1 protein sequences were separately aligned with Clustal Omega v1.2.4 [56] and MAFFT E-INS-i v7.313 [57] with default parameters. The resulting alignments were merged using TrimAl v1.4.rev15 [58] with a minimum consistency score 0.5 (ct = 0.5) and only columns with a gap percentage < 50% were kept (gt = 0.5) for further phylogenetic analysis. ProtTest v3.4 [59] was used to find the best amino acid replacement model with best tree search operation of NNI and SPR (-s BEST) and empirical frequency estimation (-F).

Based on Bayesian information criterion (BIC), the estimated best model LG+ G (-m PROTGAMMALG) was chosen to infer the maximum likelihood tree using RAxML v8.1.20 [60] with 20 best-scoring maximum likelihood searches and 1000 fast bootstrap searches (-f a -# 1000) from a random seed 12345 (-p 12345). The final phylogenetic tree was visualized using FigTree v1.4.2 (available at: http://tree.bio.ed.ac.uk/software/figtree/) and iTOL v4 online server (available at: http://itol.embl.de/[61]). The 16S rRNA gene tree was constructed based on a MAFFT E-INS-i v7.313 [57] alignment using RAxML v8.1.20 [60] with GTR nucleotide substitution model and GAMMA model of rate heterogeneity (-m GTRGAMMA). The 16S rDNA sequence of *Gloeobacter violaceus* PCC 7421 was used as an outgroup to root the 16S rDNA tree. 1000 fast bootstrap searches were done with the same setting as used in the Cas1 tree (-f a -# 1000 -p 12345).

Genome annotation and identification of CRISPR-Cas containing interruption elements

The genome sequences of filamentous cyanobacteria used in this study were downloaded from NCBI on March 25th, 2017 using phyloutils v1.0 (available at: https://github.com/housw/phyloutils). To keep the genome annotations consistent, all the genomes used in this study were re-annotated using Prokka v1.12-beta [62] with phyloutils wrapper. CRISPR cassettes were predicted using MinCED v0.2.0 with default parameters (MinCED is available at https://github.com/ctSkennerton/minced). Protein domains were predicted using pfam_scan.pl v1.6 (available at ftp://ftp.ebi.ac.uk/pub/databases/Pfam/Tools/) against Pfam release 30.0 [63] with an E-value cutoff of $1e^{-5}$. Interruption elements were scanned against all genomes using a *xis* gene-anchored algorithm with modifications [64], which required to determine the *xis* genes as a first step. In brief, for each genome, the *xis* genes were identified by searching all protein sequences against the previously identified Xis proteins [64] using blastP with an E-value cutoff of $1e^{-20}$. Then, the DNA sequences 3 kb upstream and 3 kb downstream of identified *xis* genes were extracted to check partial coding regions against all full-length protein sequences of *Anabaena* sp. PCC 7120 using blastX with default parameters. The extracted *xis*-containing regions were extended accordingly to cover the full length of surrounding overlapping genes. When a partial hit was identified, the target reference protein sequence was used as query to search against the whole genome sequence to find the other parts using tblastN. After that, all the partial coding regions were translated and aligned against the reference proteins to compose the full-length proteins. If successful, the excised regions were further checked for CRISPR cassettes. Motifs, families and super families of CRISPR direct repeats were identified using the CRISPRmap [49] online server (http://rna.informatik.uni-freiburg.de/CRISPRmap/Input.jsp).

Author contributions

SH and OSA performed bioinformatics analyses, MBA and VR provided the experimental data, SH, OSA, MBA, RB, AMP and WRH analyzed data and WRH drafted the manuscript with contributions from all authors.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Shengwei Hou http://orcid.org/0000-0002-4474-7443
Rolf Backofen http://orcid.org/0000-0001-8231-3323
Alicia M. Muro-Pastor http://orcid.org/0000-0003-2503-6336
Wolfgang R. Hess http://orcid.org/0000-0002-5340-3423

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