



The Hybrid Histidine Kinase HrmK Is an Early-Acting Factor in the Hormogonium Gene Regulatory Network

Esthefani G. Zuniga,^a Natalie M. Figueroa,^a Alfonso Gonzalez,^a Adriana P. Pantoja,^a  Douglas D. Risser^a

^aDepartment of Biology, University of the Pacific, Stockton, California, USA

ABSTRACT Filamentous, heterocyst-forming cyanobacteria belonging to taxonomic subsections IV and V are developmentally complex multicellular organisms capable of differentiating an array of cell and filament types, including motile hormogonia. Hormogonia exhibit gliding motility that facilitates dispersal, phototaxis, and the establishment of nitrogen-fixing symbioses. The gene regulatory network (GRN) governing hormogonium development involves a hierarchical sigma factor cascade, but the factors governing the activation of this cascade are currently undefined. Here, using a forward genetic approach, we identified *hrmK*, a gene encoding a putative hybrid histidine kinase that functions upstream of the sigma factor cascade. The deletion of *hrmK* produced nonmotile filaments that failed to display hormogonium morphology or accumulate hormogonium-specific proteins or polysaccharide. Targeted transcriptional analyses using reverse transcription-quantitative PCR (RT-qPCR) demonstrated that hormogonium-specific genes both within and outside the sigma factor cascade are drastically downregulated in the absence of *hrmK* and that *hrmK* may be subject to indirect, positive autoregulation via *sigJ* and *sigC*. Orthologs of HrmK are ubiquitous among, and exclusive to, heterocyst-forming cyanobacteria. Collectively, these results indicate that *hrmK* functions upstream of the sigma factor cascade to initiate hormogonium development, likely by modulating the phosphorylation state of an unknown protein that may serve as the master regulator of hormogonium development in heterocyst-forming cyanobacteria.

IMPORTANCE Filamentous cyanobacteria are morphologically complex, with several representative species amenable to routine genetic manipulation, making them excellent model organisms for the study of development. Furthermore, two of the developmental alternatives, nitrogen-fixing heterocysts and motile hormogonia, are essential to establish nitrogen-fixing symbioses with plant partners. These symbioses are integral to global nitrogen cycles and could be artificially recreated with crop plants to serve as biofertilizers, but to achieve this goal, detailed understanding and manipulation of the hormogonium and heterocyst gene regulatory networks may be necessary. Here, using the model organism *Nostoc punctiforme*, we identify a previously uncharacterized hybrid histidine kinase that is confined to heterocyst-forming cyanobacteria as the earliest known participant in hormogonium development.

KEYWORDS gliding motility, development, *Nostoc punctiforme*, cell motility, cyanobacteria, developmental biology, histidine kinase, hormogonia, two-component regulatory systems

Filamentous cyanobacteria belonging to taxonomic subsections IV and V are developmentally complex multicellular organisms (1). The vegetative filaments of these bacteria are composed of cells that actively grow and divide while performing oxygenic photosynthesis and can differentiate alternative cell or filament types. Under conditions where reduced nitrogen is limiting, approximately every 10th cell along the filament

Citation Zuniga EG, Figueroa NM, Gonzalez A, Pantoja AP, Risser DD. 2020. The hybrid histidine kinase HrmK is an early-acting factor in the hormogonium gene regulatory network. *J Bacteriol* 202:e00675-19. <https://doi.org/10.1128/JB.00675-19>.

Editor Conrad W. Mullineaux, Queen Mary University of London

Copyright © 2020 American Society for Microbiology. All Rights Reserved.

Address correspondence to Douglas D. Risser, drisser@pacific.edu.

Received 25 October 2019

Accepted 27 November 2019

Accepted manuscript posted online 2 December 2019

Published 11 February 2020

will differentiate into a nitrogen-fixing heterocyst (2). In response to other environmental factors, such as light or phosphate limitation, filaments can differentiate akinetes, metabolically dormant cells analogous to bacterial endospores, which are resistant to cold and desiccation (3). Last, filaments are capable of developing into hormogonia, motile filaments that facilitate the dispersal, phototaxis, and establishment of nitrogen-fixing symbioses (1, 4–6). Of these three developmental alternatives, heterocyst differentiation is by far the most well understood at the molecular level, having been the focus of intense study for several decades (2). In contrast, the genetic control of akinete and hormogonium development is much less defined. However, in recent years, considerable advances have been made by applying molecular approaches to define the hormogonium gene regulatory network (GRN) in the model filamentous cyanobacterium *Nostoc punctiforme* (7).

Initiation of hormogonium development is influenced by a complex assortment of signals that include light quality and quantity, nutrient concentrations, autogenic repressors, and signals from symbiotic partners (8–14). Once initiated, differentiating hormogonia undergo a round of synchronous cell division accompanied by architectural remodeling and filament fragmentation at heterocyst-vegetative cell junctions and necridia (apoptotic cells) to produce the distinctive hormogonium morphology, short filaments of smaller, rod-shaped cells that are devoid of heterocysts and which frequently display tapered filament termini (1). Once differentiated, subsequent cell growth and division are arrested (8), but filaments maintain metabolic activity to power gliding motility. Motility is driven by bipolar, circumferential arrays of type IV pilus motors (15) and is accompanied by the deposition of a hormogonium-specific polysaccharide (HPS) essential for movement (16).

Recent evidence indicates that the GRN promoting hormogonium development involves the activation of a hierarchical sigma factor cascade (7). At the top of this cascade is *sigJ*, which in turn either directly or indirectly activates the transcription of *sigC* and *sigF*, as well as a majority of the genes involved in architectural remodeling, the type 4 pilus (T4P) motor, and hormogonium-specific signal transduction systems. In turn, *sigC* promotes the expression of many genes associated with reductive cell division, while *sigF* has a remarkably limited regulon that consists primarily of *pilA*, which encodes the major pilin of the T4P system. Two signal transduction systems, the Hmp chemotaxis (16, 17) and Hmp partner-switching systems (18), also influence hormogonium development but appear to act downstream of the sigma factor cascade, as their transcription is influenced by *sigJ* and *sigC*, and they are dispensable for the development of morphologically distinct hormogonia. Notably, a substantial subset of hormogonium-specific genes, including several required for HPS synthesis, are still upregulated upon hormogonium induction in the absence of *sigJ*, implying that unidentified components of the hormogonium GRN, including a potential hormogonium master regulator, act upstream of *sigJ* (7).

One likely candidate to serve as the upstream activator is a two-component or phosphorelay system, where a sensor histidine kinase, upon perception of a signal, either directly (two-component), or indirectly through a histidine phosphotransferase protein (Hpt) intermediary (phosphorelay), phosphorylates a response regulator to elicit an appropriate cellular response (19). Response regulators control a variety of functions, including, most commonly, modulation of transcription. Such systems have been shown to serve as master regulators in other bacterial developmental programs, including fruiting body development in *Myxococcus xanthus* (20) and sporulation in *Bacillus subtilis* (21).

In this study, we apply a forward genetic approach to identify HrmK, a putative hybrid histidine kinase required for hormogonium development in *N. punctiforme*. Phenotypic characterization and targeted transcriptional analyses of the *hrmK* deletion strain indicate that *hrmK* acts upstream of the sigma factor cascade in the hormogonium GRN to initiate the developmental program.

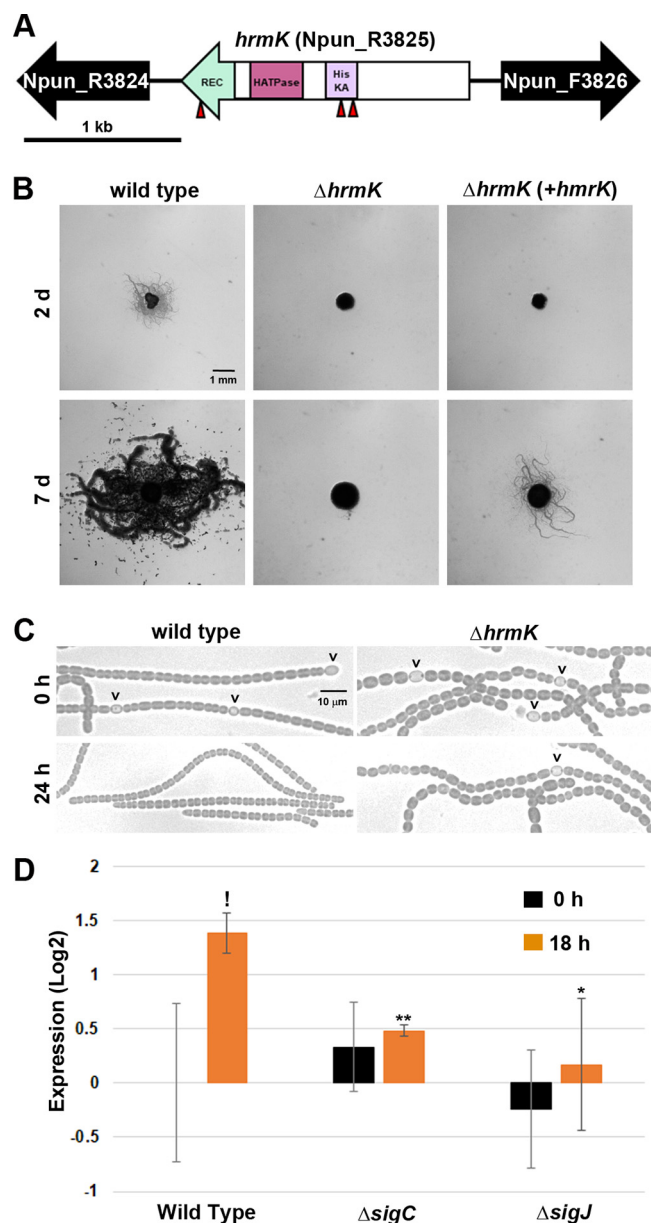


FIG 1 Characterization of *hrmK*. (A) Gene map of the *hrmK* locus. Triangles indicate the sites of transposon insertions. HisKA, His kinase A (phosphoacceptor) domain; HATPase, ATPase domains of histidine kinase; REC, response regulator receiver domain. (B) Plate motility assays of the wild-type strain, $\Delta hrmK$ mutant, and $\Delta hrmK$ mutant with *hrmK* expressed in *trans* from a shuttle vector (+*hrmK*) (as indicated). Images were taken at 2 days and 7 days postinduction. (C) Light micrographs of the filament morphology for the wild-type and $\Delta hrmK$ mutant strains (as indicated) at 0 h and 24 h post-hormogonium induction (as indicated). Carets indicate the presence of heterocysts attached to filaments. (D) RT-qPCR of *hrmK* in the wild-type, $\Delta sigJ$ mutant, and $\Delta sigC$ mutant strains 0 and 18 h after induction for hormogonia. *, $P < 0.05$; **, $P < 0.01$, as determined by two-tailed Student's *t* test between the wild type and each deletion strain at the corresponding time point. !, $P < 0.05$, as determined by two-tailed Student's *t* test between 0 and 18 h for the same strain.

RESULTS

Identification of *hrmK*, encoding a hybrid histidine kinase regulating hormogonium development. During an ongoing study using transposon mutagenesis to identify genes involved in hormogonium development and motility (22), three nonmotile mutants with unique transposon insertions in a gene here designated *hrmK* (hormogonium hybrid histidine kinase, open reading frame Npun_R3825) were identified (Fig. 1A). The gene encodes a putative hybrid histidine kinase (HHK), with the C-terminal portion containing

the phosphoacceptor (pfam00512) and HATPase (pfam02518) domains of a typical histidine kinase, followed by a response regulator receiver domain (REC, pfam00072). The N-terminal portion of HrmK lacks homology to any previously characterized sensory domains (NCBI BLASTP), and based on the absence of predicted transmembrane domains (23), it is unlikely that HrmK is a transmembrane sensor. Genomic context (24) and previously published RNA sequencing (RNA-seq) data (7) indicate that *hrmK* is monocistronic and encodes an orphan HHK with no potential cognate Hpt or response regulator proteins encoded at the same genetic locus.

To confirm that *hrmK* is required for hormogonium development and/or motility, a strain with an in-frame deletion of *hrmK* was constructed and characterized. The deletion of *hrmK* completely abolished motility, as evidenced by the lack of colony spreading in plate motility assays and the absence of motility for individual filaments observed by time-lapse microscopy (Fig. 1B; see also Movie S1 in the supplemental material). The loss of motility in the Δ *hrmK* mutant strain could be complemented by the reintroduction of *hrmK*, along with its 5' intergenic region, in *trans* on a replicative shuttle vector, although there was a substantial delay in the appearance of colony spreading (Fig. 1B).

We routinely employ the supplementation and subsequent removal of 4 mM sucralose from the growth medium to synchronously induce hormogonium development (25). However, higher concentrations of sucralose lead to the formation of aseriate colonies, which become encapsulated in a thick polysaccharide sheath (25). It was noted that the Δ *hrmK* mutant strain was more sensitive to sucralose, forming aseriate colonies in liquid cultures at 4 mM sucralose instead of 6 mM sucralose for the wild type (Fig. S1). We therefore employed supplementation and removal of 2 mM sucralose for induction of hormogonia in the Δ *hrmK* mutant strain to avoid complications that might arise from attempting to induce hormogonia from the aseriate colonies, where washing of the filaments to remove sucralose could be impeded by the presence of the thick polysaccharide sheath.

In liquid cultures prior to induction, filaments of the Δ *hrmK* mutant strain contained cells that were larger and more spherical than those of the wild type (Fig. 1C and S2). Following induction, the Δ *hrmK* mutant strain failed to show any of the characteristics of wild-type hormogonium morphology, which include a reduction in cell size, loss of heterocysts, and the appearance of tapered filament termini (Fig. 1C and S2). This morphological analysis could indicate that *hrmK* functions at an early stage in hormogonium differentiation, given that *sigJ* and *sigC* are currently the only other genes known to be required for the development of morphologically distinct hormogonia (7).

Previous transcriptomic analyses have indicated that the expression of *hrmK* is moderately enhanced in developing hormogonia (7) and that this induction is diminished in the absence of *sigJ* or *sigC*. To confirm this transcriptional profile, the *hrmK* transcript was quantified by reverse transcription-quantitative PCR (RT-qPCR) at 0 and 18 h postinduction in the wild-type, Δ *sigJ* mutant, and Δ *sigC* mutant strains. The results confirmed hormogonium-specific induction of *hrmK* that is both *sigJ* and *sigC* dependent (Fig. 1D).

***hrmK* is required for the accumulation of hormogonium-specific proteins and polysaccharide.** The absence of obvious hormogonium morphology does not necessarily preclude the activation of a substantial portion of the hormogonium GRN. For example, both *sigC* and *sigJ* are required for the development of morphologically distinct hormogonia, but only the deletion of *sigJ* completely prevents hormogonium-specific production of the major pilin PilA or the methyl-accepting chemotaxis-like protein HmpD (7). To further probe which portions of the hormogonium GRN may be disrupted in the Δ *hrmK* mutant strain, immunological and lectin-based assays were employed to measure the accumulation of PilA, HmpD, and HPS. The results from immunoblot analysis demonstrated that unlike the wild-type strain, the Δ *hrmK* mutant strain failed to accumulate detectable levels of either PilA or HmpD following hormogonium induction (Fig. 2A). When staining for accumulated extracellular HPS, heterocyst-specific staining with *Ulex europaeus* agglutinin (UEA)-fluorescein was de-

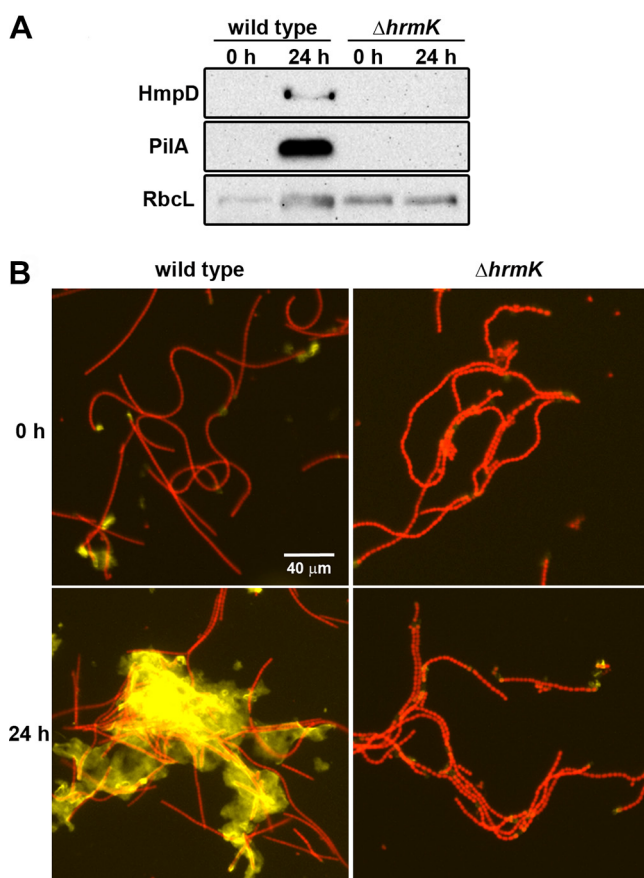


FIG 2 Effect of *hrmK* on accumulation of HmpD, PilA, and HPS. (A) Immunoblot analysis of HmpD, PilA, and RbcL in the wild-type and $\Delta hrmK$ mutant strains (as indicated) 0 and 24 h after hormogonium induction. RbcL is the large subunit of RuBisCO and serves as a protein loading control. (B) Fluorescent lectin staining of HPS in the wild-type and $\Delta hrmK$ mutant strains (as indicated). Depicted are merged images of fluorescence micrographs acquired using a 10 \times lens objective from cellular autofluorescence (red) and HPS (yellow) (strains as indicated) 0 and 24 h after hormogonium induction.

tected in both the wild-type and $\Delta hrmK$ mutant strains prior to hormogonium induction, due to cross-reaction of UEA-fluorescein with heterocyst envelope polysaccharide, as previously reported (Fig. 2B) (26). However, upon hormogonium induction, the wild-type strain accumulated large amounts of HPS loosely associated with the filaments, while no obvious increase was observed for the $\Delta hrmK$ mutant strain (Fig. 2B).

***hrmK* acts upstream of *sigJ* in the hormogonium GRN.** The phenotype of the $\Delta hrmK$ mutant strain is essentially indistinguishable from that of a *sigJ* deletion strain (7), indicating that *hrmK* is an early-acting factor in the hormogonium GRN, possibly either immediately upstream or downstream of *sigJ*. To distinguish between these two possibilities and to provide additional supporting evidence to integrate *hrmK* into a model of the network, RT-qPCR was used to quantify the expression of each of the three hormogonium-specific sigma factors (*sigJ*, *sigC*, and *sigF*), a downstream target for each (*pilB*, *hfq*, and *pilA*, respectively), as well as *hpsE*, a gene induced in hormogonia independently of the sigma factor cascade (Fig. 3). In the wild-type strain, the expression of each gene was upregulated upon hormogonium induction, as previously reported (7). In the $\Delta hrmK$ mutant strain, the expression of both *sigJ* and *sigF* and their downstream targets *pilB* and *pilA*, respectively, is drastically reduced compared to that of the wild type prior to induction and is no longer enhanced postinduction. The transcription of *sigC* and its downstream target *hfq* was also substantially reduced in the $\Delta hrmK$ mutant strain compared to that in the wild type prior to induction. A moderate increase in the expression of *sigC* and *hfq* was observed in the $\Delta hrmK$ mutant

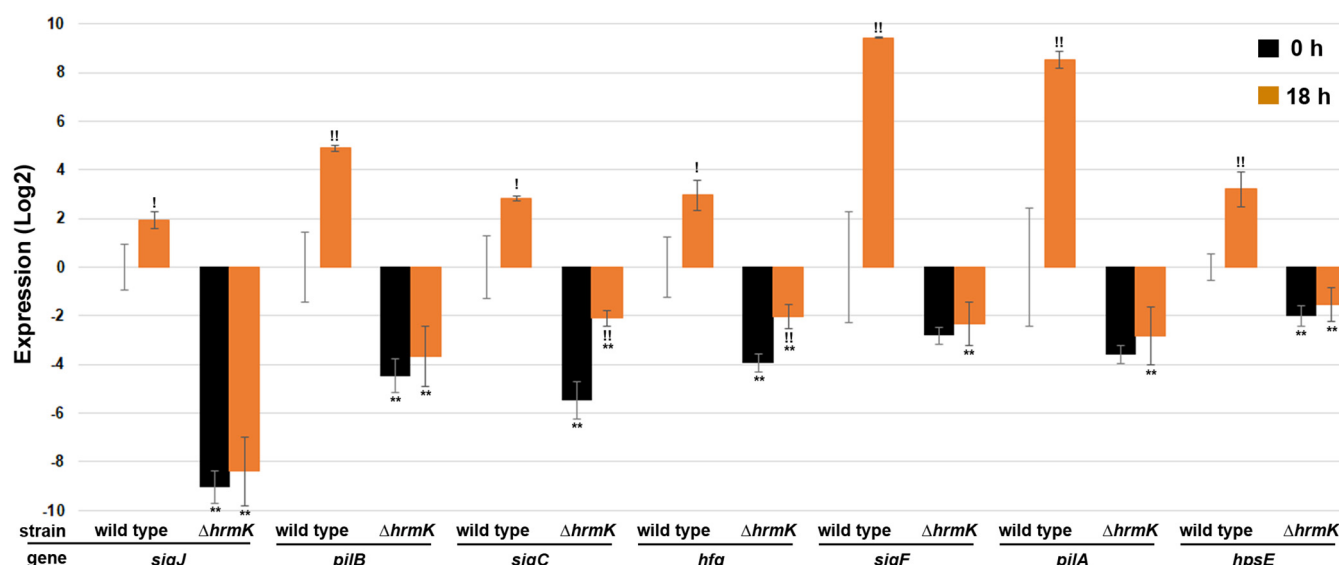


FIG 3 RT-qPCR of hormogonium-specific gene expression (as indicated) in the wild-type and $\Delta hrmK$ mutant strains 0 and 18 h after induction for hormogonia. *, $P < 0.05$; **, $P < 0.01$, as determined by two-tailed Student's t test between the wild-type and $\Delta hrmK$ mutant strains at the corresponding time point. !, $P < 0.05$; !!, $P < 0.01$, as determined by two-tailed Student's t test between 0 and 18 h for the same strain.

strain postinduction, but transcript levels remained well below those observed either before or following induction in the wild-type strain. Finally, *hpsE* expression was also reduced in the $\Delta hrmK$ mutant strain and was not differentially upregulated following induction. These data imply that *hrmK* acts upstream of *sigJ* and promotes the expression of other hormogonium-specific genes independent of the sigma factor cascade.

Evolutionary conservation of *hrmK*. Orthologs of genes essential for hormogonium development and motility in *N. punctiforme* are conserved in a large number of cyanobacteria. In some cases, they are widely distributed among both filamentous and unicellular species, such as those encoding the hormogonium-specific sigma factors (7), the T4P system (26), and the Hmp chemotaxis-like system (26). In others, the genes appear to be confined to filamentous cyanobacteria, as is the case for the Hmp partner-switching system (18) and several genes associated with HPS synthesis (16, 26). In contrast, *hrmK* is ubiquitous and exclusive to the heterocyst-forming cyanobacteria in taxonomic subsections IV and V (Fig. 4). Of the 24 heterocyst-forming cyanobacteria analyzed, only two fail to encode an ortholog of HrmK. Conversely, only 2 orthologs were detected in the 101 non-heterocyst-forming strains, one in a filamentous cyanobacterium (*Leptolyngbya boryana* sp. strain PCC 6306), and the other in a unicellular strain closely related to heterocyst formers (*Gloeocapsa* sp. strain PCC 7428). Homologous proteins identified outside the cyanobacterial lineage (NCBI BLASTP) only share sequence similarity within the C-terminal signaling domain, indicating that HrmK is unlikely to have been acquired via recent horizontal gene transfer. Therefore, it appears that HrmK is an HHK with a unique sensory domain that is confined primarily to heterocyst-forming cyanobacteria.

DISCUSSION

Figure 5 presents a working model of the hormogonium GRN, where *hrmK* indirectly promotes the transcription of both *sigJ* (to activate the hormogonium sigma factor cascade) and other hormogonium-specific genes independent of this cascade. This model is supported by the absence of hormogonium morphology, failure to accumulate hormogonium-specific proteins and polysaccharide, and reduced transcription of hormogonium-specific genes in the $\Delta hrmK$ mutant strain. Critically, the transcription of *hpsE* is diminished in the $\Delta hrmK$ mutant strain but not in any of the sigma factor deletion strains previously analyzed (7), providing key data indicating that *hrmK* acts upstream of the sigma factor cascade. Furthermore, the fact that transcription of *hrmK*

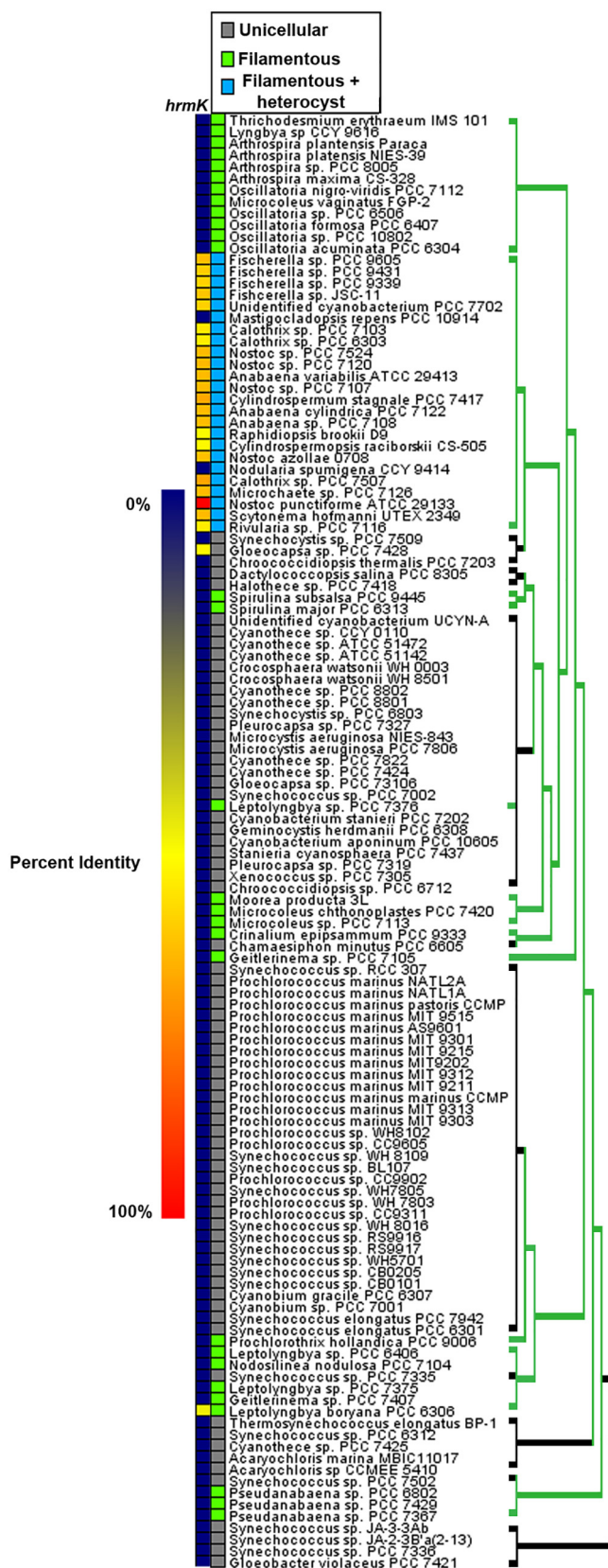


FIG 4 Evolutionary conservation of HrmK in cyanobacteria. Shown is a heat map depicting the percent identity for orthologs of *N. punctiforme* HrmK in cyanobacteria, derived from data reported by Cho et al. (26). The species organization and phylogenetic tree are based on the phylogeny reported by Shih et al.

(Continued on next page)

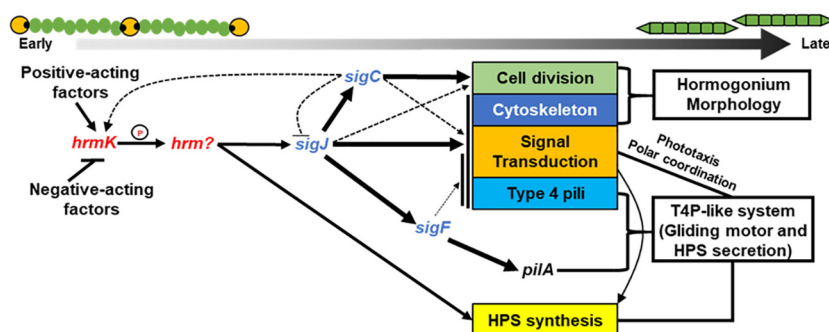


FIG 5 A model depicting the major findings of this report on the role of *hrmK* in the hormogonium GRN. Arrows indicate positive regulation, and lines with bars indicate negative regulation. The thickness of the arrows represents a combination of the number of genes regulated and the stringency of the regulation.

is reduced in the absence of both *sigJ* and *sigC* indicates the presence of an indirect positive-feedback loop between these three genes, where *sigJ* promotes expression of *sigC*, which in turn enhances the transcription of *hrmK*. This model is also supported by previous experiments showing that enhanced expression of *sigJ* in hormogonia is *sigC* dependent (7). Given the presence of this positive-feedback loop, care must be taken in establishing the order of each component in the GRN; thus, while the available evidence is most consistent with *hrmK* as the earliest acting factor in the network, additional experimentation may be needed to definitively resolve the order.

Notably, despite the static expression of *sigJ* in the absence of *sigC*, the *sigJ*-dependent regulon is still rapidly upregulated upon hormogonium induction (7), an observation that could be accounted for by posttranscriptional regulation of *sigJ*. In contrast to the deletion of *sigC*, the deletion of *hrmK* drastically reduced *sigJ* expression to levels well below that of wild-type vegetative filaments and also abolished the upregulation of *sigJ*-dependent genes, indicating that *hrmK* regulates *sigJ* at the transcriptional level and is essential for its expression. These findings demonstrate that the hormogonium GRN is similar to those that control other developmental processes in bacteria, perhaps most notably sporulation in the *Bacillus* genus, where a phosphorelay system controls the initiation of a sigma factor cascade (21). It is also notable that the Δ *hrmK* mutant strain displayed increased sensitivity to sucralose with respect to the formation of aseriate colonies. Currently, little is known about the genetic regulation of this morphology, but it appears to be negatively influenced by the activity of *hrmK*. Thus, it seems likely that *hrmK* may function as a switch that simultaneously promotes the hormogonium GRN while suppressing the mutually exclusive aseriate morphology.

Presumably, HrmK is not directly affecting transcription but is modulating the phosphorylation state of a downstream response regulator, possibly a transcriptional activator or repressor, to control its activity. Typically, HHKs participate in phosphorelays where the phosphate is passed from the REC domain of the HHK to a standalone Hpt protein and subsequently to a response regulator (19). However, HrmK is an orphan HHK with no obvious partner Hpt or response regulator proteins encoded at the same genetic locus. This makes identifying the additional components of the putative phosphorelay more challenging. Moreover, the genomes of *N. punctiforme* and several other cyanobacteria investigated in a previous study are largely devoid of obvious Hpt proteins that would serve as intermediaries between HrmK and its cognate response regulator (27). Perhaps, cyanobacteria possess cryptic Hpt proteins that are not easily identified based on sequence similarity. It is also possible that HrmK participates in a less common type of phosphorelay similar to that which controls type III chromatic

FIG 4 Legend (Continued)

(36) but depicting the finding, as reported by Schirmeister et al. (37), that most extant cyanobacteria are derived from a filamentous ancestor. For the phylogenetic tree, green indicates filamentous and black indicates unicellular.

acclimation in the cyanobacterium *Fremyella diplosiphon* (28). In this system, the histidine kinase RcaE passes phosphate to a standalone REC domain protein, RcaF, which in turn phosphorylates an Hpt domain on RcaC, a transcriptional regulator that contains both an Hpt and multiple REC domains (29). Notably, there are several RcaC homologs encoded in the *N. punctiforme* genome (24). Alternatively, HrmK may directly phosphorylate a response regulator. Identifying the additional components of the HrmK signal transduction system is a critical area for future work.

Another aspect of HrmK that requires further investigation is the role of the N-terminal sensory domain. This domain is found exclusively in heterocyst-forming cyanobacteria and has not been previously characterized, making the prediction of its sensory capacity difficult. Given the absence of any detectable transmembrane domains within HrmK, it is likely to sense an intracellular, rather than extracellular, signal. It is clear, based on its conservation, that HrmK probably plays a critical role in promoting hormogonium development in all heterocyst-forming cyanobacteria. Further studies will be required to answer these outstanding questions.

MATERIALS AND METHODS

Strains and culture conditions. For a detailed description of the strains used in this study, refer to Table S1 in the supplemental material. *N. punctiforme* ATCC 29133 and its derivatives were cultured in Allen and Arnon medium diluted 4-fold (AA/4), without supplementation of fixed nitrogen, as previously described (30), with the exception that liquid cultures were supplemented with 4 mM (for the wild-type strain) or 2 mM (for the $\Delta hrmK$ mutant strain) sucralose, and solid medium was supplemented with 10 mM sucralose, to inhibit hormogonium formation (25). For small-scale hormogonium induction for phenotypic analysis, the equivalent of 30 $\mu\text{g ml}^{-1}$ chlorophyll *a* (Chl *a*) of cell material from cultures at a Chl *a* concentration of 10 to 20 $\mu\text{g ml}^{-1}$ was harvested at $2,000 \times g$ for 3 min, washed two times with AA/4, and resuspended in 2 ml of fresh AA/4 without sucralose. For large-scale hormogonium induction for RNA extraction and subsequent RT-qPCR analysis, this process was repeated but starting with the equivalent of 300 $\mu\text{g ml}^{-1}$ Chl *a* of cell material and resuspension in 50 ml of fresh AA/4. For selective growth, the medium was supplemented with 50 $\mu\text{g ml}^{-1}$ neomycin. *Escherichia coli* cultures were grown in lysogeny broth (LB) for liquid cultures or LB supplemented with 1.5% (wt/vol) agar for plates. Selective growth medium was supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin, 50 $\mu\text{g ml}^{-1}$ ampicillin, and 15 $\mu\text{g ml}^{-1}$ chloramphenicol.

Plasmid and strain construction. For a detailed description of the plasmids, strains, and oligonucleotides used in this study, refer to Tables S1 and S2. All constructs were sequenced to ensure fidelity.

To construct a plasmid for in-frame deletion of *hrmK*, approximately 900 bp of flanking DNA on either side of the gene and several codons at the beginning and end of the gene were amplified via overlap extension PCR (see Tables S1 and S2 for details) and cloned into pRL278 (31) as BamHI-SacI fragments using restriction sites introduced on the primers.

To construct a mobilizable shuttle vector containing *hrmK* and its respective promoter region, the coding region and 5' intergenic region were amplified via PCR (see Tables S1 and S2 for details) and subsequently cloned into pAM504 (32) as a BamHI-SacI fragment using restriction sites introduced on the primers.

The generation of transposon mutants and identification of transposon insertion sites were performed as previously described (22) using plasmid pRL1063a (33). Gene deletions were performed as previously described (16) with *N. punctiforme* cultures supplemented with 4 mM sucralose to inhibit hormogonium development and enhance conjugation efficiency (22, 25). To construct the $\Delta hrmK$ mutant strain, pDDR420 was introduced into wild-type *N. punctiforme*, creating strain UOP139.

Motility assays. Both plate and time-lapse motility assays were performed as previously described (15).

RT-qPCR. Total RNA was extracted from the equivalent of 300 $\mu\text{g ml}^{-1}$ Chl *a* of cell material for each of 3 biological replicates from each strain at 0 and 18 h following hormogonium induction, using previously published methods (30). Five hundred nanograms of total RNA was used to synthesize cDNA with the ProtoScript first-strand cDNA synthesis kit and random hexamer primers (New England BioLabs, Inc.), following the specifications of the manufacturer, after which 2 μl of cDNA was used as the template for quantitative PCR (qPCR). Transcripts were amplified with the primer sets indicated in Table S2, using a StepOnePlus real-time PCR system (Applied Biosystems) and SensiFAST SYBR No-ROX kit (Bioline), following the manufacturer's specifications. Quantification of transcript abundance was calculated from the average of two technical replicates from each of the three biological replicates using the $2^{-\Delta\Delta CT}$ method (34), with expression normalized relative to *mpbB*. The primer efficiencies for each primer pair were all greater than 90%.

Immunological and lectin-based analyses. Preparation of cell material, protein extraction, and detection of PilA, RbcL, and HmpD by immunoblot analysis were performed as previously described (26). Detection of HPS by fluorescent lectin staining was performed as previously described (26).

Microscopy. Light microscopy of filament morphology was performed using a Leica DM E light microscope with a 40 \times lens objective and equipped with a Leica DFC290 digital camera controlled by

micromanager imaging software (35). Quantification of cell length and the percentage of filaments with attached heterocysts were determined as previously described (22).

Fluorescence microscopy was performed with an Evos FL fluorescence microscope (Life Technologies) equipped with a 10 \times lens objective. Excitation and emission were as follows: EVOS light cube, green fluorescent protein (GFP; AMEP4651), excitation at 470 \pm 22 nm and emission at 525 \pm 50 nm for UEA-fluorescein labeled HPS, and Evos light cube, red fluorescent protein (RFP; AMEP4652), excitation at 531 \pm 40 nm and emission at 593 \pm 40 nm for cellular autofluorescence.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

SUPPLEMENTAL FILE 2, AVI file, 2.1 MB.

ACKNOWLEDGMENTS

We thank Carrie Kozina and the Microbiology (BIOL 145) laboratory course teaching assistants and students for purification of chromosomal DNA from nonmotile transposon mutants of *N. punctiforme*.

This work was supported by NSF award number 1753690 to D.D.R.

D.D.R. and E.G.Z. designed the experiments, D.D.R., E.G.Z., N.M.F., A.G., and A.P.P. performed the experiments, and D.D.R. and E.G.Z. prepared the manuscript.

REFERENCES

- Meeks JC, Campbell EL, Summers ML, Wong FC. 2002. Cellular differentiation in the cyanobacterium *Nostoc punctiforme*. Arch Microbiol 178: 395–403. <https://doi.org/10.1007/s00203-002-0476-5>.
- Kumar K, Mella-Herrera RA, Golden JW. 2010. Cyanobacterial heterocysts. Cold Spring Harb Perspect Biol 2:a000315. <https://doi.org/10.1101/cshperspect.a000315>.
- Perez R, Forchhammer K, Salerno G, Maldener I. 2016. Clear differences in metabolic and morphological adaptations of akinetes of two Nostocales living in different habitats. Microbiology 162:214–223. <https://doi.org/10.1099/mic.0.000230>.
- Meeks JC. 2006. Molecular mechanisms in the nitrogen-fixing Nostoc-bryophyte symbiosis. Prog Mol Subcell Biol 41:165–196. https://doi.org/10.1007/3-540-28221-1_9.
- Wong FC, Meeks JC. 2002. Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation. Microbiology 148:315–323. <https://doi.org/10.1099/00221287-148-1-315>.
- Kluge M, Mollenhauer D, Wolf E. 2003. The *Nostoc-Geosiphon* endocytobiosis, p 1–30. In Rai AR, Bergman B, Rasmussen U (ed), Cyanobacteria in symbiosis. Springer, Dordrecht, the Netherlands.
- Gonzalez A, Riley KW, Harwood TV, Zuniga EG, Risser DD. 2019. A tripartite, hierarchical sigma factor cascade promotes hormogonium development in the filamentous cyanobacterium *Nostoc punctiforme*. mSphere 4:e00231-19. <https://doi.org/10.1128/mSphere.00231-19>.
- Campbell EL, Meeks JC. 1989. Characteristics of hormogonia formation by symbiotic *Nostoc* spp. in response to the presence of *Anthoceros punctatus* or its extracellular products. Appl Environ Microbiol 55:125–131.
- Campbell EL, Wong FC, Meeks JC. 2003. DNA binding properties of the Hrm protein of *Nostoc punctiforme* responsible for transcriptional regulation of genes involved in the differentiation of hormogonia. Mol Microbiol 47:573–582. <https://doi.org/10.1046/j.1365-2958.2003.03320.x>.
- Cohen MF, Meeks JC. 1997. A hormogonium regulating locus, *hrmUA*, of the cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its response to an extract of a symbiotic plant partner *Anthoceros punctatus*. Mol Plant Microbe Interact 10:280–289. <https://doi.org/10.1094/MPMI.1997.10.2.280>.
- Khamar HJ, Breathwaite EK, Prasse CE, Fraley ER, Secor CR, Chibane FL, Elhai J, Chiu WL. 2010. Multiple roles of soluble sugars in the establishment of Gunnera-Nostoc endosymbiosis. Plant Physiol 154:1381–1389. <https://doi.org/10.1104/pp.110.162529>.
- Liaimer A, Helfrich EJN, Hinrichs K, Guljamow A, Ishida K, Hertweck C, Dittmann E. 2015. Nostopeptolide plays a governing role during cellular differentiation of the symbiotic cyanobacterium *Nostoc punctiforme*. Proc Natl Acad Sci U S A 112:1862–1867. <https://doi.org/10.1073/pnas.1419543112>.
- Meeks JC, Elhai J. 2002. Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol Mol Biol Rev 66:9–121.
- Hashidoko Y, Nishizuka H, Tanaka M, Murata K, Murai Y, Hashimoto M. 2019. Isolation and characterization of 1-palmitoyl-2-linoleoyl-sn-glycerol as a hormogonium-inducing factor (HIF) from the coralloid roots of *Cycas revoluta* (Cycadaceae). Sci Rep 9:475–478. <https://doi.org/10.1038/s41598-019-39647-8>.
- Khayatan B, Meeks JC, Risser DD. 2015. Evidence that a modified type IV pilus-like system powers gliding motility and polysaccharide secretion in filamentous cyanobacteria. Mol Microbiol 98:1021–1036. <https://doi.org/10.1111/mmi.13205>.
- Risser DD, Meeks JC. 2013. Comparative transcriptomics with a motility-deficient mutant leads to identification of a novel polysaccharide secretion system in *Nostoc punctiforme*. Mol Microbiol 87:884–893. <https://doi.org/10.1111/mmi.12138>.
- Risser DD, Chew WG, Meeks JC. 2014. Genetic characterization of the hmp locus, a chemotaxis-like gene cluster that regulates hormogonium development and motility in *Nostoc punctiforme*. Mol Microbiol 92: 222–233. <https://doi.org/10.1111/mmi.12552>.
- Riley KW, Gonzalez A, Risser DD. 2018. A partner-switching regulatory system controls hormogonium development in the filamentous cyanobacterium *Nostoc punctiforme*. Mol Microbiol 109:555–569. <https://doi.org/10.1111/mmi.14061>.
- Jung K, Fried L, Behr S, Heermann R. 2012. Histidine kinases and response regulators in networks. Curr Opin Microbiol 15:118–124. <https://doi.org/10.1016/j.mib.2011.11.009>.
- Sun H, Shi W. 2001. Genetic studies of *mrp*, a locus essential for cellular aggregation and sporulation of *Myxococcus xanthus*. J Bacteriol 183: 4786–4795. <https://doi.org/10.1128/JB.183.16.4786-4795.2001>.
- Hoch JA. 1993. Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. Annu Rev Microbiol 47:441–465. <https://doi.org/10.1146/annurev.mi.47.100193.002301>.
- Khayatan B, Bains DK, Cheng MH, Cho YW, Huynh J, Kim R, Omoruyi OH, Pantoja AP, Park JS, Peng JK, Splitt SD, Tian MY, Risser DD. 2017. A putative O-linked β -N-acetylglucosamine transferase is essential for hormogonium development and motility in the filamentous cyanobacterium *Nostoc punctiforme*. J Bacteriol 199:e00075-17. <https://doi.org/10.1128/JB.00075-17>.
- Möller S, Croning MD, Apweiler R. 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics 17:646–653. <https://doi.org/10.1093/bioinformatics/17.7.646>.
- Meeks JC, Elhai J, Thiel T, Potts M, Larimer F, Lamerdin J, Predki P, Atlas R. 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth Res 70:85–106. <https://doi.org/10.1023/A:1013840025518>.

25. Splitt S, Risser D. 2015. The non-metabolizable sucrose analog sucralose is a potent inhibitor of hormogonium differentiation in the filamentous cyanobacterium *Nostoc punctiforme*. *Arch Microbiol* <https://doi.org/10.1007/s00203-015-1171-7>.
26. Cho YW, Gonzales A, Harwood TV, Huynh J, Hwang Y, Park JS, Trieu AQ, Italia P, Pallipuram VK, Risser DD. 2017. Dynamic localization of HmpF regulates type IV pilus activity and directional motility in the filamentous cyanobacterium *Nostoc punctiforme*. *Mol Microbiol* 106:252–265. <https://doi.org/10.1111/mmi.13761>.
27. Ashby MK, Houmard J. 2006. Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. *Microbiol Mol Biol Rev* 70:472–509. <https://doi.org/10.1128/MMBR.00046-05>.
28. Montgomery BL. 2017. Seeing new light: recent insights into the occurrence and regulation of chromatic acclimation in cyanobacteria. *Curr Opin Plant Biol* 37:18–23. <https://doi.org/10.1016/j.pbi.2017.03.009>.
29. Kehoe DM, Grossman AR. 1997. New classes of mutants in complementary chromatic adaptation provide evidence for a novel four-step phosphorelay system. *J Bacteriol* 179:3914–3921. <https://doi.org/10.1128/jb.179.12.3914-3921.1997>.
30. Campbell EL, Summers ML, Christman H, Martin ME, Meeks JC. 2007. Global gene expression patterns of *Nostoc punctiforme* in steady-state dinitrogen-grown heterocyst-containing cultures and at single time points during the differentiation of akinetes and hormogonia. *J Bacteriol* 189:5247–5256. <https://doi.org/10.1128/JB.00360-07>.
31. Cai YP, Wolk CP. 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J Bacteriol* 172:3138–3145. <https://doi.org/10.1128/jb.172.6.3138-3145.1990>.
32. Wei TF, Ramasubramanian TS, Golden JW. 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. *J Bacteriol* 176:4473–4482. <https://doi.org/10.1128/jb.176.15.4473-4482.1994>.
33. Wolk CP, Cai Y, Panoff JM. 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. *Proc Natl Acad Sci U S A* 88:5355–5359. <https://doi.org/10.1073/pnas.88.12.5355>.
34. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>.
35. Edelstein AD, Tsuchida MA, Amodaj N, Pinkard H, Vale RD, Stuurman N. 2014. Advanced methods of microscope control using μ Manager software. *J Biol Methods* 1:e10. <https://doi.org/10.14440/jbm.2014.36>.
36. Shih PM, Wu D, Latifi A, Axen SD, Fewer DP, Talla E, Calteau A, Cai F, Tandeau de Marsac N, Rippka R, Herdman M, Sivonen K, Coursin T, Laurent T, Goodwin L, Nolan M, Davenport KW, Han CS, Rubin EM, Eisen JA, Woyke T, Gugger M, Kerfeld CA. 2013. Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. *Proc Natl Acad Sci U S A* 110:1053–1058. <https://doi.org/10.1073/pnas.1217107110>.
37. Schirmermeister BE, de Vos JM, Antonelli A, Bagheri HC. 2013. Evolution of multicellularity coincided with increased diversification of cyanobacteria and the Great Oxidation Event. *Proc Natl Acad Sci U S A* 110:1791–1796. <https://doi.org/10.1073/pnas.1209927110>.