THE HUNT FOR CikR: RESPONSE REGULATOR FOR CikA IN *Synechococcus elongatus* PCC 7942

A Senior Scholars Thesis

by

HANNAH R. MALCOLM

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2006

Major: Chemistry

THE HUNT FOR CikR: RESPONSE REGULATOR FOR CikA IN *Synechococcus elongatus* PCC 7942

A Senior Scholars Thesis

by

HANNAH R. MALCOLM

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor: Susan S. Golden
Associate Dean for Undergraduate Research: Robert C. Webb

April 2006

Major: Chemistry

ABSTRACT

The hunt for CikR: response regulator for CikA in *Synechococcus elongatus* PCC 7942
(April 2006)

Hannah R. Malcolm
Departments of Chemistry and Biology
Texas A&M University

Research Advisor: Dr. Susan S. Golden
Department of Biology

Circadian rhythms are daily cycles of activity that have been demonstrated in many organisms including bacteria, fungi, insects, plants, and mammals. A clock system is composed of three parts: the input pathway, the central oscillator, and the output pathway. The input pathway takes temporal and environmental signals and transfers that information to the central oscillator, which contains the core components of the clock, to synchronize the endogenous clock with the environment. Temporal cues are then sent through output pathways to control certain cell processes. The CikA (**c**ircadian **i**nput **k**inase) protein is an integral part of the input pathway because a *cikA* mutant cannot reset its circadian rhythm in response to dark pulses. CikA contains a histidine protein kinase (HPK) domain, which suggests that CikA is part of a bacterial two-component signal transduction system in which CikA autophosphorylates in response to a signal and transfers that phosphate to a putative partner response regulator (RR), named CikR. The purpose of this project is to identify CikR. Bioinformatic analysis of the *Synechococcus elongatus* PCC 7942 genome identified nineteen potential *cikR* genes. Thirteen of the *cikR* candidates were eliminated due to their proximity to another HPK, or because their

function was known and unrelated to the clock. Five of the remaining six genes were cloned and their protein products were overexpressed in *Escherichia coli.* Each protein was purified by affinity chromatography and used in *trans*-phosphorylation experiments to determine if a phosphoryl group can be transferred from radio-labeled, phosphorylated CikA to any CikR candidate. To date, no phosphoryl transfer has been detected; however, the sixth gene is currently being cloned for future tests. *In vivo* analysis of null alleles and overexpression constructs in the cyanobacterium has shown that at least two of the *cikR* candidate genes is necessary for normal circadian clock function as measured by luciferase reporters.

DEDICATION

To those that have encouraged my love of science over the years: my wonderful mother, Susan Malcolm and my patient mentor, Shannon Mackey.

ACKNOWLEDGMENTS

I would like to give a special thanks to Shannon Mackey for her constant tutelage and encouragement throughout this research project. Also, thanks to Dr. Eugenia Clerico for her assistance in protein purification and isolation methods. I would like to thank Run-Zhi Lai from Dr. Michael Manson's lab for the purified CheY protein, and Carl Carruthers in Dr. Andy LiWang's lab for the 134RR2 plasmid. I would like to thank Dr. Susan S. Golden for her constant guidance and giving me the opportunity to work in her lab for several years.

The funding for this research was generously provided as a grant from National Institutes of Health to Dr. Golden. Funding was also provided from the American Society for Microbiology as an Undergraduate Research Fellowship to Hannah R. Malcolm.

NOMENCLATURE

Ap	Ampicillin
CikA	Circadian input kinase
Cm	Chloramphenicol
HPK	Histidine protein kinase
IPTG	Isopropyl-beta-D- thiogalactopyranoside
kD	Kilodalton
Km	Kanamycin
LD	12 h light/12 h dark
LL	Constant light
Ni-NTA	Nickel-nitrilotriacetic acid
NS	Neutral site
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PsR	*Pseudo*-receiver
RR	Response regulator
SasA	*Synechococcus* adaptive sensor
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Sm	Streptomycin
Sp	Spectinomycin
6xHis	Six adjacent histidine residues

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION ◊

Circadian rhythms are cycles of daily behavioral activity that have been reported in many different organisms such as cyanobacteria, fungi, mice, and even humans (2, 34). These circadian rhythms are approximately 24 h, and closely match the light/dark cycle of a day on Earth. The biological rhythm controls many of the processes of a cell or an organism. Plants regulate their leaf movement to optimize their time in the sun for energy production, but not lose too much moisture at night; mice exhibit nocturnal wheel-running behavior as dictated by their biological rhythm.

In all organisms there are many rhythmic biological processes with periodicities that can be on the order of minutes to hours; some are driven by changes in the environment while others are anticipatory to those changes to provide a competitive fitness advantage. In order for a biological rhythm to be considered a circadian rhythm it must satisfy three requirements (5, 24). (1) The period of the oscillation must remain near 24 h in the absence of environmental cues, such as light/dark cycles. (2) In order for the rhythm to remain in synchrony with its environment, the phase of the rhythm must be reset by changes in the environment, *e.g.* the change from dark to light. (3) Circadian rhythms are also temperature-compensated such that the rhythmicity of the clock is not significantly altered by changes in temperature within the organism's physiological range. The components that make up the circadian clocks are different in each organism, but the function of the system remains the same.

◊ This thesis follows the format of the *Journal of Bacteriology*.

An oversimplified model of a circadian clock system consists of a central oscillator that maintains a near 24-h oscillation, input pathways that transmit environmental information to the oscillator, and output pathways that transduce temporal information to clock-controlled processes. To identify components of the clock, a monitoring system is needed that allows for thousands of mutants to be easily screened in order to discover the proteins required for internal timekeeping. The unicellular photoautotrophic cyanobacterium *Synechococcus elongatus* PCC 7942 possesses a circadian clock system that maintains approximate 24 h time. Because there is no overt circadian behavior that can be measured by non-invasive methods in *S. elongatus*, one was designed for this bacterium. Transcription of the genome is regulated by the circadian clock in *S. elongatus* (18), which allows for the use of a reporter construct to monitor the rhythm of gene expression *in vivo* in high-throughput assays. The luciferase genes (*luxAB*) from *Vibrio harveyi* can be expressed from any cyanobacterial promoter and the emission of light fluctuates over the course of a day to reflect the endogenous biological rhythm of the cell (15, 17). The light emitted from the cyanobacterial reporter strains is measured by an automated luminometer on 96-well plates, which allows for the rapid screening of the clones (3).

In *S. elongatus* the central oscillator is composed of at least three known proteins: KaiA, KaiB, and KaiC. The Kai proteins were identified through the chemical mutagenesis of a luciferase reporter strain (16). After the mutagenesis, circadian periods ranging from 16

h to 60 h, as well as arrhythmic mutants, were detected. The mutants could be complemented by a single locus that contains three consecutive genes to restore wild-type periods of rhythmicity (8). The three genes were named *kaiA, kaiB,* and *kaiC* from the Japanese word *kaiten,* which means 'cycle.'

Once the *kai* genes were identified as core clock components, the function of each individual gene was investigated. Individual deletion of any *kai* gene causes an arrhythmic phenotype, but the growth of the cell is not affected (8). The *kaiA* gene is expressed from a single promoter (P*kaiA*) while *kaiB* and *kaiC* are expressed together by a single promoter (P*kaiBC*) (8). KaiA is a positive regulator of *kaiB* and *kaiC* expression, while KaiC is a negative regulator of P*kaiBC* (8). All three Kai proteins have been shown to interact with one another (10). The interaction between KaiA and KaiB is weaker than either protein's interaction with KaiC, and the presence of KaiC enhances the KaiA/KaiB interaction. A primary motif search of the *kaiC* coding sequence shows two ATP-binding sites, which suggest the ability of KaiC to autophosphorylate (8). The autokinase activity of KaiC was demonstrated both *in vitro* and *in vivo* (23, 32). The phosphorylation state of KaiC cycles throughout the day in wild-type *S. elongatus* (9). The amino acid sequence of KaiA and KaiB do not provide any clues to their function (8); however, they are able to influence the rate of KaiC autophosphoylation *in vitro* (9, 14, 31). In the presence of KaiA, the rate of KaiC autophosphorylation increases. The addition of KaiB alone does not change the rate of KaiC phosphorylation; however, if KaiB is added to KaiC in the presence of KaiA, KaiB reduces the positive effect that

KaiA has on KaiC kinase activity. These changes in the rates of phosphorylation of KaiC by the other Kai proteins indicate that this reaction is likely an important factor in the timing mechanism in *S. elongatus*.

Crystallography and electron microscopy were used to determine the structure of KaiC (20, 32). Phosphorylated KaiC can form a higher order complex comprised of six KaiC monomers. This KaiC hexamer forms even higher molecular weight complexes with at least KaiA and KaiB, with the largest complexes forming during the middle of the night (12). The current model shows that the rate of phosphorylation of KaiC increases in the presence of KaiA, and it is thought that KaiA interacts with KaiC at the beginning of the day to induce the formation of the hexameric structure. In the early evening, KaiB interacts with the KaiA/KaiC complex to diminish the positive effect of KaiA on KaiC, which decreases the rate of KaiC phosphorylation (and increases the rate of dephosphorylation) and the central oscillator proteins begin to disassemble. The formation and degradation of the Kai complex occurs on an approximate 24-h timescale and is believed to be a crucial part of maintaining rhythms in the cell.

Temporal information is sent from the central oscillator to the remainder of the cell through different output pathways at the appropriate time of day. In *S. elongatus* little information is known about the output pathways that confer timing cues to cellular processes. One protein known to be involved in these output pathways is SasA (***S****ynechococcus* **a**daptive **s**ensor). SasA was identified through a yeast two-hybrid screen

that used KaiC as 'bait' to search for KaiC-interacting proteins (11). SasA is involved in the control of global gene expression in the cell because a strain that lacks *sasA* maintains a functional clock, but the ability of the clock to control downstream processes is severely disrupted (11). SasA is part of the family of histidine protein kinase (HPK) proteins involved in bacterial two-component signal transduction pathways and it is expected that SasA has a partner response regulator (RR) protein that is needed for the temporal signal to be propagated to downstream processes. Other output pathways proteins, such as *cpmA* (13) and the family of group 2 sigma factors (22, 30), affect the phase, period, or amplitude of gene expression of a subset of transcriptional reporters, which demonstrate the presence of multiple clock-controlled outputs.

To maintain synchrony with the environment, circadian clocks must be able to reset their rhythms by environmental cues and it is the input pathway that receives and transmits that information to the central oscillator. In *S. elongatus*, one major component of the input pathway is CikA (**c**ircadian **i**nput **k**inase). A *cikA* mutant has a shorter period (~22 h) than the wild-type cells and is not able to reset its phase of peak expression by a 5-h dark pulse at times that would reset the wild-type strain by up to 8 h (26). The inability to reset indicates that CikA is the one of the proteins needed to interpret external cues.

The primary amino acid sequence of CikA shows that the protein contains an HPK domain (26). The HPK of CikA is capable of autophosphorylation at a conserved

histidine residue (21). HPKs are usually thought to be part of bacterial two-component signal transduction systems, which are composed of the HPK and at least one corresponding RR protein. In response to a specific signal, the HPK autophosphorylates and then transfers its phosphoryl to its partner RR, which will move through the cell to perform its duty to elicit a response to the original signal (29). CikA contains a *pseudo*-receiver (PsR) domain that was initially thought to be the corresponding RR for its own HPK. The PsR was eliminated as the true RR through experiments involving two different mutants. A *cikA* mutant lacking the PsR domain (ΔPsR) was allowed to autophosphorylate for an hour and was then combined with a *cikA* mutant that cannot autophosphorylate (H393A) but contains a functional PsR (21). A 2-h time course attempted to measure the phosphotransfer from ΔPsR to H393A but no transfer could be detected (21). It is thus assumed that CikA instead transfers its phosphoryl to a true RR that is involved in the input pathway.

Because we hypothesize that there is an RR for CikA, our goal is to identify that protein (or those proteins), which we have named CikR, and determine its role in the transfer of environmental cues from CikA to the Kai complex. After the sequencing and nearly-finished annotation of the full *S. elongatus* genome, we used bioinformatics to search for gene sequences that encode proteins that share sequence similarity to the receiver domains of known RRs. The search identified nineteen potential RRs. Several of the

potential genes were eliminated based on additional bioinformatics criteria. Five putative RRs remained and were named 124RR1, 132RR2, 134RR2, 135RR1, and 135RR4.

We used both *in vitro* and *in vivo* methods to identify CikR. The *in vitro* techniques ultimately tested for an interaction between CikA and the potential CikR proteins that would permit phosphotransfer of radio-labeled ATP. *In vivo* analysis of inactivated alleles of *cikR* candidates in bioluminescent reporter strains was conducted to determine the circadian phenotype that resulted from the absence of these genes.

METHODS

Maintenance of cultures

The *Escherichia coli* cultures were maintained on LB agar plates with antibiotics in the following concentrations: chloramphenicol (Cm), 17 μg/mL; ampicillin (Ap), 100 μg/mL; and kanamycin (Km), 50 μg/mL. All *S. elongatus* cultures were maintained in modified BG-11 (BG-11M) medium either as a liquid culture or on an agar plate. Antibiotics were added at the following concentrations: spectinomycin/streptomycin (Sp/Sm), 2 μg/mL each; Cm, 7.5 μg/mL; and Km, 5 μg/mL.

Cloning techniques

The potential *cikR* genes were amplified using the polymerase chain reaction (PCR). The forward primer for each gene contained a 5' CACC sequence for directional cloning into the pENTR™/D-TOPO® vector (Invitrogen); the resulting plasmid was then used to transform DH10B *E. coli* cells. The plasmid DNA was sequenced using the BigDye™ terminator mix (Applied Biosystems) as per manufacturer's instructions. From the pENTR™/D-TOPO® plasmid, each *cikR* gene was then cloned into the pDEST™17 (Invitrogen) vector using an LR recombinase (Invitrogen) reaction and used to transform DH10B cells. The resulting plasmids were isolated from *E. coli* using a QIAprep® kit (Qiagen) and the genes were sequenced to assure no mutations occurred during the recombinase reaction. The pDEST™17 derivatives were used to transform BL21(DE3)pLysE (Cm^R) *E. coli* cells (Novagen), which harbor an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible T7 polymerase because the pDEST™17 vector

uses a T7 promoter to drive expression of the *cikR* candidate gene. The coding sequence for six histidine (6xHis) residues lies just upstream of the potential CikR open reading frame (ORF) and serves as an affinity tag for purification of the protein of interest.

The 134RR2 gene was also cloned into the pET15b vector (Novagen) by Carl Carruthers in Dr. Andy LiWang's lab. This vector includes a T7 promoter and codons that encode an N-terminal 6xHis tag. This plasmid was also used to transform BL21(DE3)pLysE cells for overexpression and purification of 134RR2.

Protein overexpression

The pDEST17-*cikR* and pET15b/134RR2 plasmids in the BL21(DE3)pLysE *E. coli* strain were used to overexpress each CikR candidate protein. CikA was overexpressed from pAM2478 (Ap^R), which is a pQE80 (Qiagen) derivative that drives expression of *cikA* from a T7 promoter and produces an N-terminal 6xHis-tagged CikA protein. A 5 mL overnight culture was used to inoculate 500 mL LB with appropriate antibiotics, and the culture was grown at 30ºC shaking at 250 rpm until an OD_{600} ~0.5. The culture was then induced with IPTG (1 mM final concentration). 124RR1, 132RR2, and 134RR2 were induced for approximately 4 h before the entire culture was collected by centrifugation at 5000 rpm (JA-10 rotor) for 10 minutes and frozen at –80ºC until ready for use. CikA, 135RR1, and 135RR4 were induced overnight at room temperature before being collected by centrifugation at 5000 rpm (JA-10 rotor) for 10 minutes and frozen at –80ºC until ready for use.

Purification of CikR candidates and CikA

The frozen *E. coli* pellets were thawed on ice for 15 minutes before being resuspended in 3 mL lysis buffer per gram pellet (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). Once the cells were resuspended, lysosyme was added (1 mg/ml final concentration) and the cells were incubated on ice for 30 minutes. The cells were broken open by sonication for 5 minutes (30 seconds on, 30 seconds off). After the cells were broken open, RNase (10 μg/mL) and DNase (10 μg/mL) were added and the cells were incubated on ice for 15 minutes. The cells were then collected by centrifugation at 10,000 rpm (JA-20 rotor) for 30 minutes at 4ºC to separate soluble and insoluble protein. Soluble protein was then transferred to a 15 mL conical tube.

2 mL nickel-nitrilotriacetic acid (Ni-NTA) slurry (Qiagen) were added to a flow column and allowed to settle before draining the buffer. The column was washed with 5 mL lysis buffer. The soluble protein containing each overexpressed CikR candidate protein was added to the equilibrated column, allowed to flow through the column, and collected. The collected soluble protein was then added to the column again to assure that all 6xHis-tagged CikA or CikR protein bound to the column. The column was washed with 1 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole) 8 times. The protein was eluted using 3 volumes of 1 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole) each. Each eluate was collected and stored at 4ºC until ready

to use. Purified CheY protein was obtained by Run-Zhi Lai in the laboratory of Dr. Michael Manson in the Department of Biology at Texas A&M University.

Immunoblot analysis

Whole-cell extracts of *E. coli* cells that carry *cikR* plasmids were prepared as follows. 0.5 mL of an overnight culture was used to inoculate 8 mL LB+ApCm and cells were grown at 37^{o}C until OD_{600} of 0.4, when a 1 mL uninduced sample was collected. 1 mM IPTG (final concentration) was added to the cells to induce expression of the potential *cikR* genes for 3 h and a 1 mL induced sample was taken. The cells were resuspended in 1X sample buffer (50 mM Tris•HCl, pH 6.8, 10% glycerol, 2% SDS, 4% mercaptoethanol (added just before use), 0.1% bromophenol blue), boiled for 5 minutes and separated by 12.5% SDS-PAGE. Protein was transferred to 0.2 μm nitrocellulose membrane (Schleicher & Schuell) by semi-dry blot transfer according to the manufacturer's instructions (Bio-Rad). His-tagged proteins were detected using a Penta-His antibody (Qiagen) according to the manufacturer's directions and detected using a peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). The signal was visualized with the SuperSignal West Pico chemiluminescent substrate detection system (Pierce) and exposed to X-ray film.

Phosphotransfer experiments

The purified CikR candidates and CikA were dialyzed against the storage buffer (50 mM Tris•HCl, pH 7.5, 4 C, 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT)

overnight, changing the buffer after the first 4 h. The dialyzed protein was then concentrated using an Amicon Ultra centrifugal filter device (Millipore). Purified CikA protein (10 μg/μL) was allowed to auto-phosphorylate (CikA~P) for 60 minutes in kinase buffer (50 mM Tris•HCl pH 8, 50 mM KCl, 10mM Mg_2Cl, 0.1 μCi [γ-^{32}P] ATP). The radio-labeled CikA~P was then added to purified CikR candidate protein [10 μg/μL] or CheY protein [13.5 μg/μL] and excess cold ATP (0.5 mM) for 10 seconds, 30 seconds, 1 minute, and 2 minutes. Reactions were stopped using equal volume 2X sample buffer (120 mM Tris•HCl, pH 6.8, 20% glycerol, 4% SDS, 10% mercaptoethanol (added just before use), 0.1% bromophenol blue). Samples were separated by electrophoresis on 16% polyacrylamide gels. The gels were dried and radiation was detected using a phosphoimager after either a 20-minute or overnight exposure. All experiments were completed at room temperature.

Construction of *cikR* null alleles

Independent null alleles of each *cikR* candidate gene were created by insertion of an antibiotic-resistance cassette in the coding region of each gene. A Cm-resistance *Mu* transposon was inserted in the 124RR1 gene to give rise to the 7G3-W6 clone, and into the 135RR4 gene to create 4G8-AAA4. The Sp/Sm-resistance Ω-cassette (Sp^R/Sm^R-Ω) from pHP45Ω-Sp (6) was used to inactivate the 134RR2 gene. The full-length sequence of 134RR2 was cloned into pBluescript (Stratagene) at *Eco*RI and *Xho*I sites. The Sp^R/Sm^R-Ω cassette was digested with *Eco*RI and ligated at the *Mfe*I site of the subcloned 134RR2 gene to create pAM3337. Plasmids that contain genes interrupted by

antibiotic-resistance cassettes were used to transform cyanobacterial reporter strains(4, 7). Following homologous recombination, mutant allele replacement at the native locus was verified by PCR as compared to the WT allele.

Measurement of *in vivo* bioluminescence

Reporter strain AMC1300 (P*kaiBC::luxAB*) and its derivatives integrate the luciferase reporter at neutral site I (NS1) in the *S. elongatus* genome and the proteins required for synthesis of the long-chain aldehyde substrate, driven by the *psbAI* promoter (P*psbAI::luxCDE*), are integrated at neutral site II (NS2). AMC1004 (P*kaiBC::luxAB*) carries the luciferase reporter at NS2.1 and P*psbAI::luxCDE* at the adjacent NS2.2. AMC669 (P*psbAI::luxAB;* P*psbAI::luxCDE*) and its derivatives encode both the luciferase reporter and necessary enzymes at NS2.1. These neutral sites are regions of the *S. elongatus* chromosome that can be interrupted without any noticeable growth or circadian phenotype (1, 3). Automated measurement of bioluminescence from the various *S. elongatus* reporter strains was performed by inoculating each strain to an agar pad in a 96-well plate. The cultures were allowed to grow for 24 h in constant light (LL). Each plate was then placed on the Packard TopCount™ 96-well microplate scintillation and bioluminescence counter, exposed to two 12 h light/12 h dark (LD) cycles for synchronization, and placed in LL. All data acquired by the TopCount were graphed and analyzed by the Import and Analysis (I&A) Excel interface (S. A. Kay Laboratory, Scripps Institute; (25).

RESULTS AND DISCUSSION

Identification and cloning of *cikR* candidate genes

The CikA protein has been shown to autophosphorylate at its HPK domain and it is predicted that CikA transfers that phosphoryl group to a yet-to-be-identified partner RR protein, CikR. The entire *S. elongatus* genome has been sequenced, which facilitates using consensus sequences to find genes that encode proteins of interest. To identify candidate *cikR* genes, the consensus sequence for genes that encode receiver domains for RR proteins was used to search for similar sequences in the *S. elongatus* genome. Initially, nineteen ORF had sequence similarity that provided an E-value of 1e-05 or less. The sequence encoding the receiver domains for each of those nineteen candidates was then used to search the genome sequence database until no new sequences were found.

Of the nineteen sequences predicted to encode receiver domains, thirteen were eliminated. Elimination occurred because the function of the encoded protein was already known to be unrelated to the clock, or the gene was located just downstream of the ORF for an HPK other than CikA; it is predicted that those HPK and RR proteins act as partners. Other genes were removed from the list because their encoded receiver domain does not contain the conserved aspartic acid residue that has been shown to receive the phosphoryl group from HPK proteins. Because CikR is predicted to be a single domain protein, gene sequences that encode multi-domain proteins were not considered as possible *cikR* candidates. At the end, five putative *cikR* genes remained

and were named 124RR1, 132RR2, 134RR2, 135RR1, and 135RR4. The gene and predicted protein sizes are shown in Figure 1A.

Once these five ORFs were identified, the goal of this project was to test their role in the input pathway of the circadian clock system by showing information transfer from CikA to one of these CikR candidates by way of phosphotransfer. The five genes to be investigated were cloned using Gateway® Technology (Invitrogen) because it allows for the rapid insertion of genes into vectors without the need for restriction enzyme digests and ligations. This project was the first in Dr. Golden's laboratory to use Gateway® Technology for protein production in *E. coli* and served to determine the success of the system. The Gateway® cloning system is composed of two main steps: the entry vector and the destination vector.

To insert the gene of interest into the entry vector, each gene sequence was amplified from *S. elongatus* genomic DNA using PCR (Figure 1B) with primers that allowed for directional TOPO® cloning into the entry vector pENTR™/D-TOPO®. This entry vector was used because it allows for rapid introduction of a PCR product into the vector at room temperature. The commercial vector has been linearized by the topoisomerase I enzyme from the *Vaccinia* virus, which binds to double-stranded DNA, cuts the backbone, and forms a covalent bond with the DNA (28). The 5' hydroxyl group of the PCR product can reverse this reaction to allow the PCR product to anneal to the linearized pDEST™17 vector (27). The production of a circular plasmid can then be

A

	Base pairs	Amino acids	Predicted kD
124RR1	375	125	16.9
132RR2	360	120	16.4
134RR2	624	208	26.8
135RR1	399	133	17.7
135RR4	363	121	16.7

B

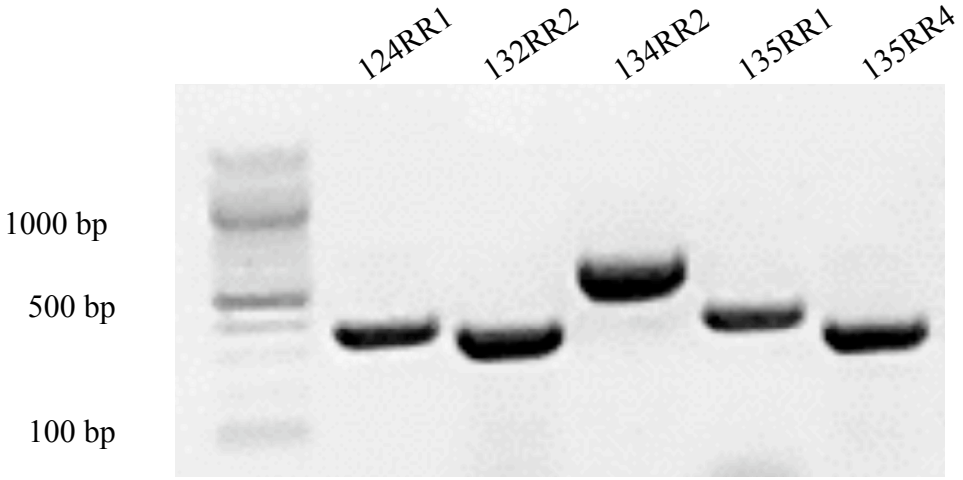

FIG. 1. A, Predicted sizes of *cikR* candidate genes and their protein products.
B, Amplification of *cikR* candidate genes. The entire ORF of each potential *cikR* was amplified by PCR using a high-fidelity DNA polymerase. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide fluorescence.

propagated during cell division; the vector contains a Km^R cassette for easy identification of positive clones. Initially, we attempted to verify the presence of the insert using restriction enzyme analysis, but the expected DNA fragment is small and difficult to visualize on the agarose gel; insertion was instead verified using PCR amplification of the insert (data not shown). After the insert was verified the pENTR™ vector was used to transfer the gene insert into the destination vector, pDEST™17, through an LR recombination reaction. We used the pDEST™17 vector because it contains a T7 promoter upstream of the cloning site, codons that encode an N-terminal 6xHis tag for protein purification, and an Ap^R cassette for clone selection. The resulting pDEST™17/*cikR* plasmids were used to transform either the BL21(DE3)pLacI or BL21(DE3)pLysE *E. coli* cells, both of which harbor an IPTG-inducible T7 RNA polymerase that recognizes the T7 promoter of the pDEST™17 vector. Thus, the addition of IPTG to the cell cultures will ultimately result in the overexpression of each CikR candidate protein.

Overexpression and purification of CikR proteins

In order to test the phosphotransfer from CikA to the potential CikR proteins, each protein needed to be overexpressed and purified from the *E. coli* cells. The BL21(DE3)pLacI cells were the first strain of *E. coli* used for overexpression because they contain an IPTG-inducible T7 RNA polymerase. However, in the absence of inducer the protein was expressed at high levels and, when IPTG was added, there was no noticeable increase in production (Figure 2). Because this project was the first to use

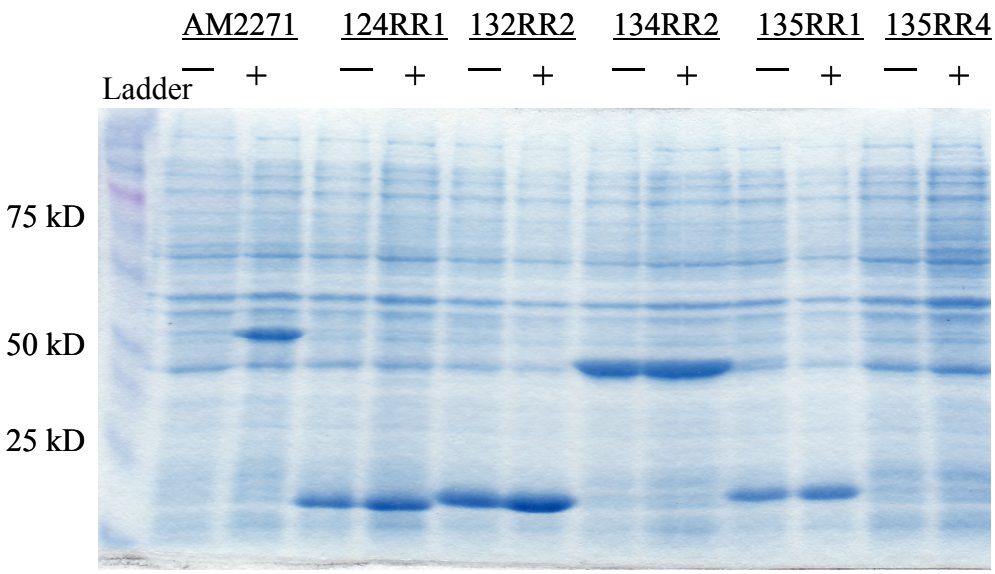

FIG. 2. Overexpression of *cikR* candidates and AM2271 in BL21(DE3)pLacI. The BL21(DE3)pLacI host cells were transformed with CikR candidate-containing plasmids. The cells were grown in LB+Ap until OD_{600}= ~0.5 at 37°C before being induced with 1 mM IPTG for 3 h. An uninduced sample was taken (indicated with the -) and an induced sample was taken (indicated with the +). The cells were resuspended in sample buffer and boiled for 5 min before being separated on a 12.5 % SDS-PAGE. The gel was stained with Coomassie Brilliant Blue.

the pDEST™17 vector in our lab, it was not known if the high levels of expression were due to a leaky promoter on the vector, or a high basal level of T7 RNA polymerase in the cells before induction. To test the latter possibility, another plasmid that uses a T7 promoter to transcribe the core clock component *kaiB* was used to transform the BL21(DE3)pLacI cells. Almost undetectable amounts of KaiB protein can be visualized on an SDS-polyacrylamide gel before induction, but a large band at the expected size of KaiB is seen after the addition of IPTG (Figure 2, lanes 2 and 3). These results suggested that the cells were not producing high basal levels of T7 RNA polymerase, but instead that the T7 promoter on the pDEST™17 vector was allowing transcription to occur in the absence of high polymerase levels. In order to attempt to repress the leaky expression, the *cikR* plasmids were introduced to the BL21(DE3)pLysE strain of *E. coli*. Other labs in the department had used this strain to aid in the reduction of leaky expression from T7 promoters. The new BL21(DE3)pLysE cells still allowed some uninduced expression from the T7 promoter of the pDEST™17 vector, but the basal levels are lower than was seen in the BL21(DE3)pLacI cells, and an increase of expression after induction with IPTG can be seen (compare Figures 2 and 3). Once the gene was able to be overexpressed in the presence of inducer, the problem of insolubility needed to be addressed.

When protein is made too quickly, the protein often folds incorrectly which leads to the insolubility of the protein. Many factors contribute to the speed of protein production including temperature, concentration of inducer, and length of induction. To determine

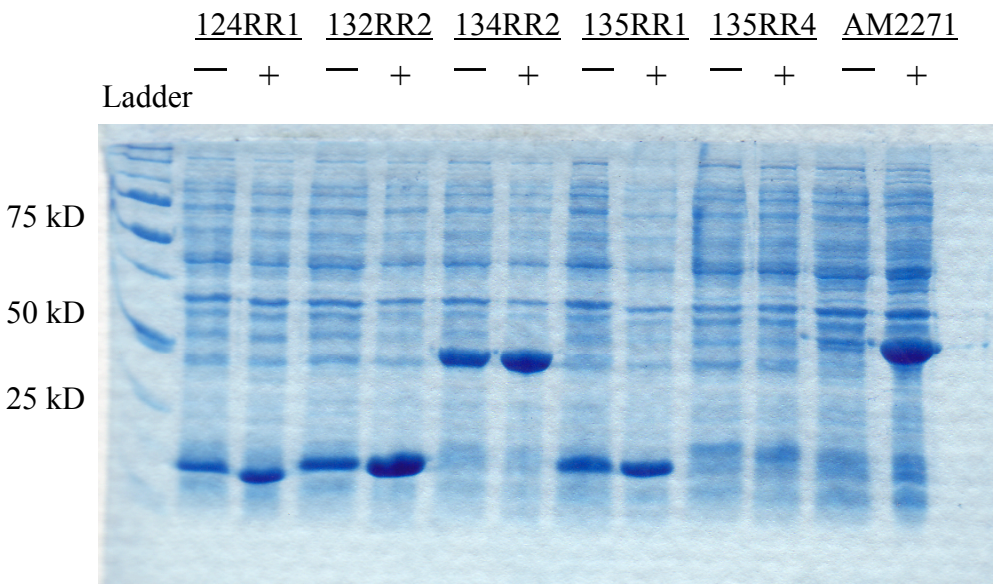

FIG. 3. Overexpression of *cikR* candidates and AM2271 in BL21(DE3)pLysE. The BL21(DE3)pLysE samples were treated as described as in Figure 2.

the correct conditions needed to recover maximal levels of soluble protein, many different trials were executed. The amount of IPTG used was a final concentration of 1 mM. This one consistency allowed for the other conditions to be tested. Four different temperatures were tested that ranged between 18°C and 37°C, with the optimal temperatures being 30°C for 3 h induction and 25°C for overnight induction. At the same time that the potential temperature range was investigated, the optimal time of induction was also tested in the range of 3 h to overnight induction. Each protein is different and the optimal conditions for each overexpression protocol varied slightly (see Methods).

Once the conditions for the optimal levels of soluble protein were determined, the proteins needed to be purified for the phosphotransfer experiments. The *E. coli* pellets were broken open using sonication. The potential CikR proteins each have an N-terminal 6xHis tag, as was verified by immunoblot analysis using antibodies against the engineered tag (Figure 4). This tag also allows for purification using affinity chromatography with a Ni-NTA column. The 6xHis tag binds strongly to the Ni-NTA column and proteins that do not adhere to the column will flow through the matrix. Some proteins other than the protein of interest can bind to the column, but their bond is not as strong as the tagged protein and they will be washed off with low concentrations of the competitor imidazole. Despite many washes with low concentrations of imidazole, there were still some non-specific proteins bound to the column that were eluted along with the target protein (Figure 5). This small level of contaminating protein, although annoying, was permissible for the phosphotransfer experiments.

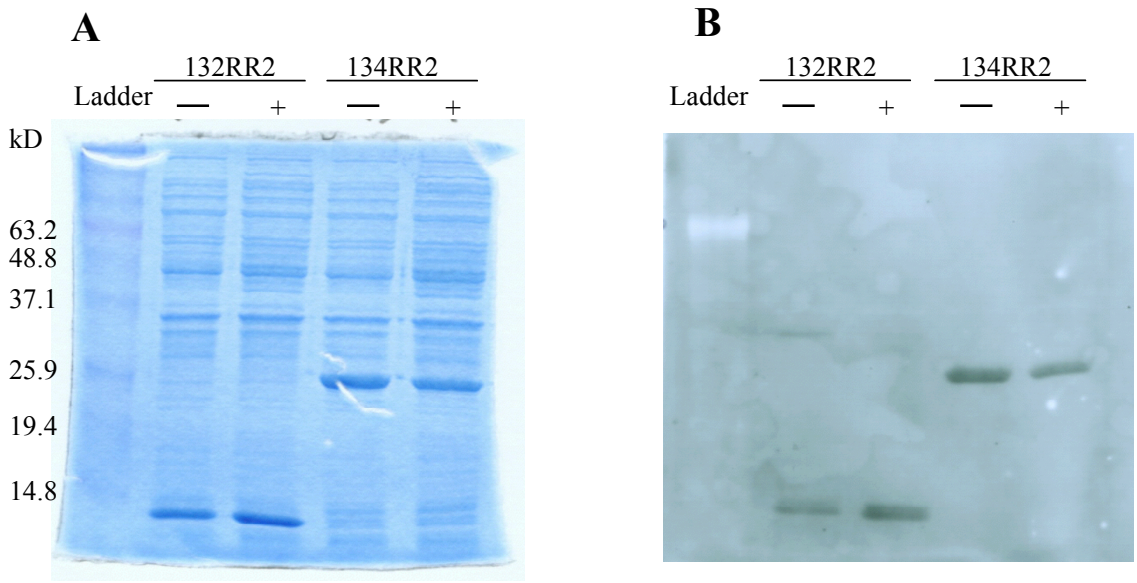

FIG. 4. His-tagged 132RR2 and 134RR2 are overexpressed in *E. coli.* pDEST™17 vectors that contain the full-length ORF for either 132RR2 or 134RR2 were used to transform BL21(DE3)pLysE *E. coli* cells, which harbor an IPTG-inducible T7 polymerase gene. The cloned RR genes are under control of a T7 promoter. A sample (0.5 ml) of an overnight culture was used to inoculate 8 ml LB+Ap and cells were grown at 37^{o}C until OD_{600}=~0.4, where an uniduced sample was collected (denoted by - symbol). 1 mM IPTG was added to the cells to induce expression of the potential *cikR* genes for 3 h, when the induced sample was taken (denoted by + symbol). The cells were resuspended in sample buffer, boiled for 5 min and separated by 12.5% SDS-PAGE. Samples were run in duplicate. One gel was stained with Coomassie Brilliant Blue (A) and the other was transferred to nitrocellulose membrane and immonublotted with Anti-His antibody to detect His-tagged proteins (B).

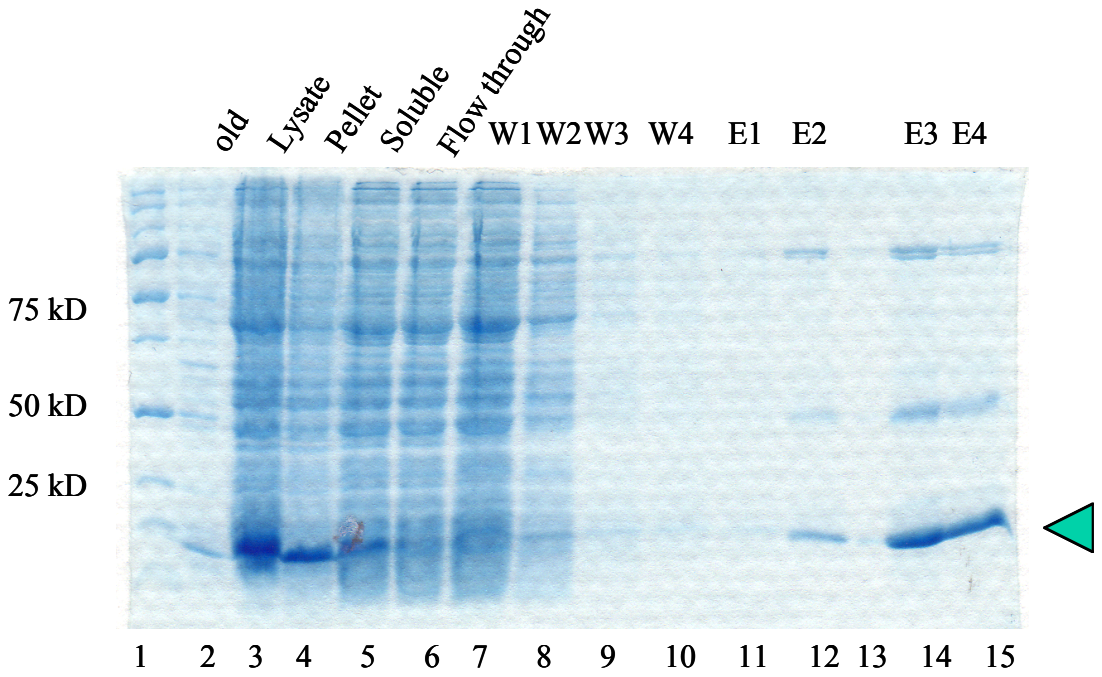

FIG. 5. Purification of 124RR1 in BL21(DE3)pLysE using an Ni-NTA column. 5 mL of an overnight culture was used to inoculate 500 mL LB+ApCm and cells were grown at 37°C until OD_{600}= 0.5. The cultures were then induced with 1 mM IPTG for 4 h. The cells were harvested and broken open using sonication. After sonication a lysate sample was taken (lane 3) and a pellet sample was taken (lane 4) the cell debris was separated from the soluble protein (lane 5) using a centrifuge. The soluble protein was added to a Ni-NTA column to purify the His-tagged 124RR1. After the soluble protein was filtered over the column a sample was taken (flow through, lane 6). The column was washed 4 x with 50 mM imidazole (lanes 7-10). The target protein was eluted using 3 1-mL volumes of 250 mM imidazole (lanes 11, 12, 14, 15). The samples were separated by 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue.

Phosphotransfer

Now that the soluble protein had been isolated, the conditions for the transfer of a phosphoryl group from CikA to CikR could be investigated *in vitro.* The first step of the phosphotransfer experiment was to establish conditions that allowed CikA to autophosphorylate. A recent publication in *The Journal of Biological Chemistry* had determined conditions that would allow for the autophosphorylation and phosphotransfer of almost all of the known HPK/RR systems from *E. coli* (33). The great success of these experiments encouraged the use of these protocols in the hunt for CikR. In the presence of [γ-^{32}P] ATP, CikA could autophosphorylate in a time-dependent manner (Figure 6A). For the remainder of the phosphotransfer experiments, CikA that had been incubated with radioactive ATP for either 40 or 60 minutes was used.

To limit the number of variables that existed when trying to detect transfer of the radio-labeled phosphoryl group from CikA to the CikR candidates, we first determined conditions that were favorable for CikA to transfer to the RR protein, CheY. CheY is involved in chemotaxis in *E. coli* and consists of a single receiver domain. CheY is a 'promiscuous' acceptor of information from HPK proteins *in vitro* as was shown by its ability to receive phosphoryl groups from a number of different HPK proteins when its cognate HPK protein is not present (33). The phosphorylation of CheY by its partner HPK was shown to occur within 10 seconds, with no detectable radioactive CheY present after only 2 minutes of incubation with a phosphorylated HPK protein (33). After CikA was allowed to autophosphorylate, purified CheY protein was added. Figure

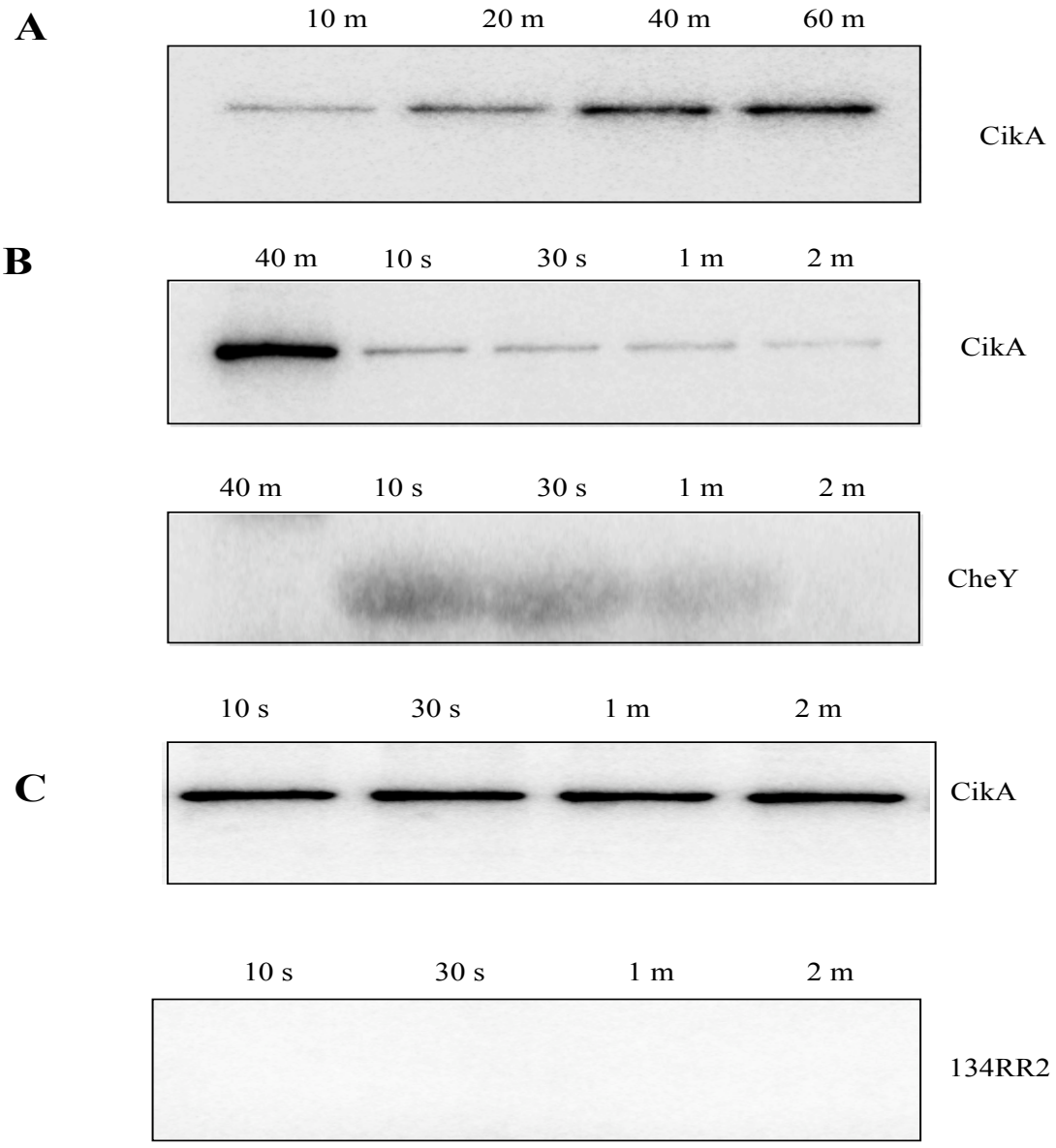

FIG. 6. Autophosphorylation of CikA and phosphotransfer experiments. A, Purified CikA (10 µg/ml) was combined with 0.1 µCi [γ-^{32}P] ATP for 10, 20, 40, or 60 min. Samples were stopped with 2X-stop buffer and separated on 16% SDS-PAGE. The gels were dried and radioactivity was detected using a phosphoimager. B, CheY is a single domain response regulator involved in chemotaxis in *E. coli* and was used to help determine conditions that favor phosphotransfer from CikA~P to an RR that is known to accept phosphate groups from HPK proteins. C, Using the conditions in B, phosphotransfer was attempted from CikA to CikR candidate 134RR2. No radio-labeled 134RR2 was detected. Phosphotransfer did not occur using any of the CikR proteins with these conditions (data not shown).

6B shows that the conditions used permitted the phosphotransfer from CikA to CheY within 10 seconds. The same conditions that allowed for the reactions to occur from CikA to CheY were used to measure the transfer to each of the five CikR candidate proteins. Despite numerous attempts, no phosphotransfer could be detected (Figure 6C; see Overall conclusions).

In vivo analysis of *cikR* inactivations

At the same time as the *in vitro* experiments were being conducted, three of the *cikR* genes were interrupted by antibiotic-resistance cassettes to study their effect on the circadian rhythm of *S. elongatus*. Mutant alleles of 124RR1, 134RR2, and 135RR4 were used to transform *S. elongatus* reporter strains to replace the wild-type copy of the gene with the disrupted gene. To verify that the wild-type copies of the gene were replaced by the inactivated allele, genomic DNA was extracted from wild-type and transformed cyanobacterial cultures. PCR was completed using primers that anneal just outside of the gene to differentiate wild-type and mutant alleles based on size. The wild-type 124RR1 and 135RR4 genes were completely replaced by the respective inactivated allele; however, both mutant and wild-type copies of 134RR2 were present in transformants (data not shown). *S. elongatus* carries 6 – 8 copies of its genome (19) and it is possible to replace enough copies of the gene to provide resistance to the selective antibiotic, but sufficient copies of the gene are present to serve the function of the gene product. These cells are called merodiploids, and any mutant phenotype that exists is a result of a partial loss of function.

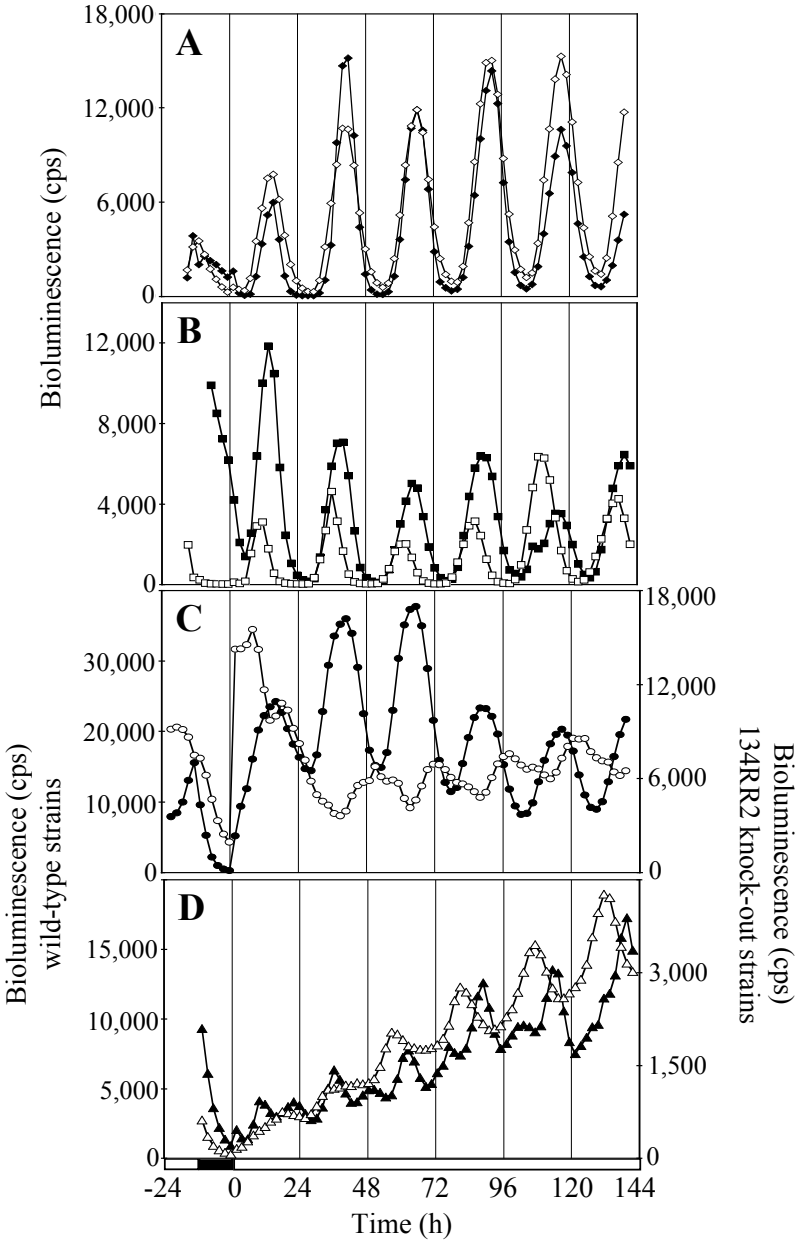

FIG. 7. Circadian phenotypes of *cikR* inactivation alleles in LL. A 135RR4 interrupted by a Cm^R *Mu* transposon insertion (open diamonds) does not display a difference in period or phase from the wild-type P*kaiBC::luxAB* reporter strain (closed diamonds). B A Cm^R *Mu* transposon insertion in 124RR1 (open squares) causes the peak of expression to occur 4 h before the wild-type P*kaiBC::luxAB* reporter (closed squares). 134RR2 was disrupted by a Sp^R/Sm^R-Ω cassette. The mutation causes altered periodicity and phasing in a (C) P*kaiBC::luxAB* reporter (134RR2, open circles; wild type, closed circles) or a (D) P*psbAI::luxAB* reporter (134RR2, open triangles; wild type, closed triangles).

The mutant cyanobacterial reporter strains were tested for rhythms in bioluminescence. The 135RR4 mutation did not produce any noticeable effect on the properties of the rhythm of gene expression in constant light conditions (LL; Figure 7A). Inactivation of 124RR1 produced rhythms with a period similar to the wild type, but the phase of peak expression occurred 4 h before the wild-type P*kaiBC::luxAB* reporter (Figure 7B). The merodiploid 134RR2 strain showed the most striking circadian phenotype. In a P*kaiBC::luxAB* reporter, the period and phase of the rhythm were different from the wild-type strain (Figure 7C). To verify that this phenotype was due to a disruption of the clock in general and not just a disruption of the regulation of the *kaiBC* promoter, a P*psbAI::luxAB* reporter strain was also transformed with the 134RR2 inactivation allele. The merodiploid state of the *psbAI* reporter was verified by PCR (data not shown) and also displayed altered phasing and a slightly shorter period than wild type (Figure 7D). Therefore, at least two of the CikR candidates have an effect on the biological timing system of the cyanobacterial cell.

OVERALL CONCLUSIONS

The importance of kinase proteins in the cyanobacterial clock system is becoming more evident with the identification of necessary clock components. A major protein of the central oscillator autophosphorylates and the levels of phosphorylation fluctuate throughout the circadian cycle to help maintain proper timing. Temporal information flows from the oscillator via a protein kinase to affect the global regulation of gene transcription. External information flows into the cell by way of a kinase to reset the clock to remain in synchrony with daily cycles.

The work presented in this thesis attempted to identify the cognate RR of CikA. The identification of CikR was explored using different cloning techniques than previously used in the lab. Gateway® Technology proved to be an easy way to manipulate DNA in order to produce the clones needed to overexpress proteins of interest. However, the lack of tight repression of gene expression required time to troubleshoot and determine methods that could minimize protein production in the absence of inducer. The use of Gateway® cloning could prove useful in other aspects of working on clock genes. In order for an expression construct to be introduced to the cyanobacterial genome, *S. elongatus* neutral site DNA must flank the construct to insert the exogenous DNA by homologous recombination. The techniques currently used require multiple cloning steps into vectors that are not easy to use. An ideal use for Gateway® Technology would be to create a neutral site vector that contained a recombination cassette. The construct of interest could then be amplified by PCR with primers containing necessary

recombination sequences at either end, and a quick reaction at room temperature would allow the construct to be incorporated in between the neutral site sequences.

No phosphotransfer could be detected from radio-labeled CikA to any of the predicted CikR proteins. There are many possibilities as to why no reaction occurred. One possibility is that *in vivo* a 'helper' protein is required to facilitate the transfer. It is known that when the PsR domain of CikA is removed, the level of autophosphorylation of CikA *in vitro* increases 10-fold (21). The PsR acts as a repressor of CikA kinase activity. It is also hypothesized that the PsR domain is involved in protein-protein interactions that tether CikA to the pole of the cell. Full-length CikA that is fused to a fluorescent protein is found only at the cell poles; a fluorescent CikAΔPsR variant has lost this polarity and is visible throughout the entire cell (35). The proposed model for CikA function is that the PsR domain binds to an unknown protein at the cell pole, and this binding removes the repression of the kinase activity to allow CikA to autophosphorylate at higher rates and then transfer that phosphoryl to its cognate RR protein. In the *in vitro* phosphotransfer experiments, it is possible that the correct conformation of CikA, or the correct interaction interfaces between CikA and CikR are not established because the helper protein is not present. Two candidate helper proteins have been identified through yeast two-hybrid experiments that used the PsR of CikA as bait (S.R. Mackey, J.-S. Choi, and S.S. Golden, unpublished results).

Another possibility for not detecting phosphotransfer could be that none of the five candidate proteins is CikR. It is predicted that CikR is a small, diffusable protein that likely consists of only a receiver domain. This prediction comes from preliminary experiments that showed cyanobacterial soluble protein extracts from a *cikA* null strain that have been radio-labeled with [γ-^{32}P]ATP in the presence or absence of recombinant full-length CikA protein show bands representing proteins of approximately 30 kDa and 45 kDa only when CikA is added (M. Mutsuda and S.S. Golden, unpublished results). Thus, the RR of CikA is predicted to be a protein of approximately 30 kDa. However, this prediction could be incorrect.

One candidate protein (133RR3) remains to be tested. This protein is very large – 929 amino acid residues – and contains seven predicted functional domains, one of which is a receiver domain with the conserved aspartic acid residue for accepting the phosphoryl group. None of the domains is predicted to be a kinase domain, and the ORF from 133RR3 does not lay immediately downstream of another HPK protein. The cloning of this gene is currently being conducted in order to conduct the same type of experiments with its protein product as were done with the first five candidates.

The *in vivo* analysis of inactivation alleles showed that 124RR1 and 134RR2 are needed to maintain proper phasing and periodicity of circadian rhythms. Interestingly, the 134RR2 protein appears to be essential not only for proper circadian timing, but also essential for cell viability because the wild-type copies of the gene could not be

completely replaced by the inactivated allele. The presence of a functional clock is not necessary for proper cellular processes, because *kai* deletions that render the clock arrhythmic do not display a discernable growth phenotype. The function of 134RR2 is likely not limited to the clock and may contribute to housekeeping cellular activities. The molecular mechanisms of these two receiver proteins can now be further studied. If either of these proteins is the true CikR, it would be expected that a null allele would also exhibit a difference in response to light/dark cues as is typical of input pathway components. Thus, these mutant strains will be tested for their ability to 'sense' differences in light intensities and to reset to dark pulses. Overall, two new players of the cyanobacterial clock system were discovered, and the tools used and developed during this project will allow for the roles of these components in the biological rhythm of *S. elongatus* to be assessed and fully appreciated.

REFERENCES

1. **Andersson, C. R., N. F. Tsinoremas, J. Shelton, N. V. Lebedeva, J. Yarrow, H. Min, and S. S. Golden.** 2000. Application of bioluminescence to the study of circadian rhythms in cyanobacteria. Methods Enzymol. **305:**527-42.

2. **Bell-Pedersen, D., V. M. Cassone, D. J. Earnest, S. S. Golden, P. E. Hardin, T. L. Thomas, and M. J. Zoran.** 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. **6:**544-56.

3. **Canales, S. R., J. L. Ditty, E. M. Clerico, and S. S. Golden.** 2006. Detection of rhythmic bioluminescence from luciferase reporters in cyanobacteria. *In* E. Rosato (ed.), Methods in Molecular Biology. Humana Press, Totowa, NJ., in press.

4. **Clerico, E. M., J. L. Ditty, and S. S. Golden.** 2006. Specialized techniques for site-directed mutagenesis in cyanobacteria. *In* E. Rosato (ed.), Methods in Molecular Biology. Humana Press, Totowa, NJ., in press.

5. **Dunlap, J. C., J. J. Loros, and P. J. DeCoursey (ed.).** 2004. Chronobiology: Biological Timekeeping. Sinauer Associates, Inc., Sunderland. MA.

6. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. Gene **52:**147-54.

7. **Golden, S. S.** 1988. Mutagenesis of cyanobacteria by classical and gene-transfer-based methods. Methods Enzymol. **167:**714-27.

8. **Ishiura, M., S. Kutsuna, S. Aoki, H. Iwasaki, C. R. Andersson, A. Tanabe, S. S. Golden, C. H. Johnson, and T. Kondo.** 1998. Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. Science **281:**1519-23.

9. **Iwasaki, H., T. Nishiwaki, Y. Kitayama, M. Nakajima, and T. Kondo.** 2002. KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. Proc. Natl. Acad. Sci. USA **99:**15788-93.

10. **Iwasaki, H., Y. Taniguchi, M. Ishiura, and T. Kondo.** 1999. Physical interactions among circadian clock proteins KaiA, KaiB and KaiC in cyanobacteria. EMBO J. **18:**1137-45.

11. **Iwasaki, H., S. B. Williams, Y. Kitayama, M. Ishiura, S. S. Golden, and T. Kondo.** 2000. A KaiC-interacting sensory histidine kinase, SasA, necessary to sustain robust circadian oscillation in cyanobacteria. Cell **101:**223-33.

12. **Kageyama, H., T. Kondo, and H. Iwasaki.** 2002. Circadian formation of clock protein complexes by KaiA, KaiB, KaiC and SasA in cyanobacteria. J. Biol. Chem. **278:**2388-95.

13. **Katayama, M., N. F. Tsinoremas, T. Kondo, and S. S. Golden.** 1999. *cpmA*, a gene involved in an output pathway of the cyanobacterial circadian system. J. Bacteriol. **181:**3516-24.

14. **Kitayama, Y., H. Iwasaki, T. Nishiwaki, and T. Kondo.** 2003. KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacteria circadian clock system. EMBO J. **22:**1-8.

15. **Kondo, T., C. A. Strayer, R. D. Kulkarni, W. Taylor, M. Ishiura, S. S. Golden, and C. H. Johnson.** 1993. Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc. Natl. Acad. Sci. USA **90:**5672-6.

16. **Kondo, T., N. F. Tsinoremas, S. S. Golden, C. H. Johnson, S. Kutsuna, and M. Ishiura.** 1994. Circadian clock mutants of cyanobacteria. Science **266:**1233-6.

17. **Liu, Y., S. S. Golden, T. Kondo, M. Ishiura, and C. H. Johnson.** 1995. Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria. J. Bacteriol. **177:**2080-6.

18. **Liu, Y., N. F. Tsinoremas, C. H. Johnson, N. V. Lebedeva, S. S. Golden, M. Ishiura, and T. Kondo.** 1995. Circadian orchestration of gene expression in cyanobacteria. Genes Dev. **9:**1469-78.

19. **Mori, T., B. Binder, and C. H. Johnson.** 1996. Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. Proc. Natl. Acad. Sci. USA **93:**10183-8.

20. **Mori, T., S. V. Saveliev, Y. Xu, W. F. Stafford, M. M. Cox, R. B. Inman, and C. H. Johnson.** 2002. Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNA. Proc. Natl. Acad. Sci. USA **99:**17203-8.

21. **Mutsuda, M., K. P. Michel, X. Zhang, B. L. Montgomery, and S. S. Golden.** 2003. Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in *Synechococcus elongatus* PCC 7942. J. Biol. Chem. **278:** 19102-10.

22. **Nair, U., J. L. Ditty, H. Min, and S. S. Golden.** 2002. Roles for sigma factors in global circadian regulation of the cyanobacterial genome. J. Bacteriol. **184:**3530-8.

23. **Nishiwaki, T., H. Iwasaki, M. Ishiura, and T. Kondo.** 2000. Nucleotide binding and autophosphorylation of the clock protein KaiC as a circadian timing process of cyanobacteria. Proc. Natl. Acad. Sci. USA **97:**495-9.

24. **Pittendrigh, C. S.** 1981. Circadian Systems: General Perspective and Entrainment, p. 57-80 and 95-124. *In* J. Aschoff (ed.), Handbook of Behavioral Neurobiology: Biological Rhythms. Plenum Press, New York.

25. **Plautz, J. D., M. Straume, R. Stanewsky, C. F. Jamison, C. Brandes, H. B. Dowse, J. C. Hall, and S. A. Kay.** 1997. Quantitative analysis of *Drosophila period* gene transcription in living animals. J. Biol. Rhythms **12:**204-17.

26. **Schmitz, O., M. Katayama, S. B. Williams, T. Kondo, and S. S. Golden.** 2000. CikA, a bacteriophytochrome that resets the cyanobacterial circadian clock. Science **289:**765-8.

27. **Shuman, S.** 1994. Novel approach to molecular cloning and polynucleotide synthesis using *Vaccinia* DNA topoisomerase. J. Biol. Chem. **269:**32678-84.

28. **Shuman, S.** 1991. Recombination mediated by *Vaccinia* virus DNA topoisomerase I in *Escherichia coli* is sequence specific. Proc. Natl. Acad. Sci. USA **88:**10104-8.

29. **Stock, A., V. Robinson, and P. Goudreau.** 2000. Two-component signal transduction. Annu. Rev. Biochem. **69:**183-215.

30. **Tsinoremas, N. F., M. Ishiura, T. Kondo, C. R. Andersson, K. Tanaka, H. Takahashi, C. H. Johnson, and S. S. Golden.** 1996. A sigma factor that modifies the circadian expression of a subset of genes in cyanobacteria. EMBO J. **15:**2488-95.

31. **Williams, S. B., I. Vakonakis, S. S. Golden, and A. C. LiWang.** 2002. Structure and function from the circadian clock protein KaiA of *Synechococcus elongatus*: A potential clock input mechanism. Proc. Natl. Acad. Sci. USA **99:**15357-62.

32. **Xu, Y., T. Mori, R. Pattanayek, S. Pattanayek, M. Egli, and C. H. Johnson.** 2004. Identification of key phosphorylation sites in the circadian clock protein KaiC by crystallographic and mutagenetic analyses. Proc. Natl. Acad. Sci. USA **101:**13933-8.

33. **Yamamoto, K., K. Hirao, T. Oshima, H. Aiba, R. Utsumi, and A. Ishihama.** 2005. Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli*. J. Biol. Chem. **280:**1448-56.

34. **Young, M. W., and S. A. Kay.** 2001. Time zones: a comparative genetics of circadian clocks. Nat. Rev. Genet. **2:**702-15.

35. **Zhang, X., G. Dong, and S. S. Golden.** 2006. The *pseudo*-receiver domain of CikA regulates the cyanobacterial circadian pathway. Mol. Microbiol., **60:** 658-68.

CONTACT INFORMATION

Hannah R. Malcolm received a Bachelors of Science in Chemistry from Texas A&M University in May of 2006. While an undergrad she worked under the guidance of Dr. Susan S. Golden for four years. She intends to enter graduate school at Washington University in St. Louis in the Fall of 2006 to earn the degree of Doctor of Philosophy with a specialization in Biological Chemistry.

Ms. Malcolm may be reached at hannah_malcolm@gmail.com. All professional correspondence can be sent to sgolden@tamu.edu.