A New Circadian Class 2 Gene, *opcA*, Whose Product Is Important for Reductant Production at Night in *Synechococcus elongatus* PCC 7942

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Gene expression in the cyanobacterium *Synechococcus elongatus* PCC 7942 is under the control of a circadian oscillator, such that peaks and troughs of expression recur with a periodicity of about 24 h in the absence of environmental cues. This can be monitored easily as light production from luciferase gene fusions to *S. elongatus* promoters. All promoters seem to exhibit circadian oscillation of expression, but the phasing of peak and trough times differs among different genes. The majority of genes are designated class 1, with expression peaks near dusk or subjective dusk (the time corresponding to dusk in the absence of a diurnal cycle). A minority, of which *purF* is an example, have expression peaks approximately 12 h out of phase with class 1 genes. A screen of Tn5 mutants for those in which *purF* phasing is altered revealed a mutant that carries an insertion in the *opcA* gene, previously identified as essential for glucose-6-phosphate dehydrogenase function. However, a different enzymatic reporter and in vitro luciferase assays revealed that the expression pattern of the *purF* promoter is not altered by *opcA* inactivation, but rather the reduced flavin mononucleotide substrate of luciferase is limiting at the time of the natural circadian peak. The results suggest that OpcA is involved in temporally separated reductant-generating pathways in *S. elongatus* and that it has a role outside of its function in activating glucose-6-phosphate dehydrogenase. The *opcA* gene, expected to be cotranscribed with *fbp* and *zwf*, was shown to have its own class 2 promoter, whereas the *fbp* promoter was determined to be in class 1. Thus, *opcA* expression is likely to be constitutive by virtue of the activity of two promoters in nearly opposite circadian phases.

Cyanobacteria, like diverse eukaryotes, possess an internal timekeeping mechanism termed a circadian clock that controls oscillations of biological functions with an endogenous period of about 24 h (2, 6, 12, 14, 34). A circadian system is conventionally divided into three parts: a central oscillator that determines the circadian period, input pathways that synchronize the phasing of the oscillator with daily environmental cycles, and output pathways that confer circadian rhythmicity on the processes that are controlled by the clock. A locus of three genes, *kaiA*, *kaiB*, and *kaiC*, has been identified in the cyanobacterium *Synechococcus elongatus* PCC 7942 that is fundamentally important for circadian rhythmicity of gene expression (16). Mutations in any of these genes can alter the circadian period or cause arhythmicity of all genes that are examined.

Most or all of the genes in the *S. elongatus* genome are transcribed with a circadian rhythm. One demonstration of this was the insertion of promoterless *Vibrio harveyi* luciferase genes (*luxAB*) randomly throughout the genome. All bioluminescent colonies, indicating fusion of the reporter genes with a promoter, showed a circadian rhythm of light production (19). These and other data suggest that transcription is globally rhythmic in the organism. However, there is specialization in the phasing (timing of peaks and troughs), amplitude, and duration of the day with which individual genes are expressed. Most *S. elongatus* promoters drive *luxAB* with a peak of bioluminescence at dusk (in a light-dark cycle) or subjective dusk (in continuous light [LL]) and a trough at dawn or subjective

dawn. By convention, subjective dusk is around 12 h, 36 h, and so on, and subjective dawn is around 24 h, 48 h, and so on, in LL after a 12-h pulse of darkness to entrain the clock. These dusk-peaking genes, including *psbAI*, *psbAII*, *psbAIII*, *kaiA*, *kaiBC*, and *rrnA*, are categorized as having class 1 expression patterns (16, 19). Even P_{conII}, a synthetic promoter with a consensus *Escherichia coli* σ^{70} binding site, drives *luxAB* with a class 1 phase in *S. elongatus* (10, 35).

A minority of genes, among which only *purF* has been characterized (18), show the opposite phase of expression. Expression of these genes, grouped as class 2, peaks at dawn or subjective dawn and troughs at dusk or subjective dusk. *purF* encodes a key enzyme in the de novo purine biosynthesis pathway, glutamine phospho-ribosyl-pyrophosphate (PRPP) amidotransferase (amidophosphoribosyltransferase, EC 2.4.2.14). However, the *purL* gene, which is immediately upstream of *purF* and encodes a 5′-phospho-ribosyl *N*-formyl-glycinamidine (FGAM) synthetase (EC 6.3.5.3), another enzyme in the same pathway, belongs to class 1 (18). A potential advantage for this phasing of expression is that glutamine PRPP amidotransferase is oxygen sensitive, and the O_2 concentration is low at dawn after a night of respiration, which consumes O_2, the by-product of photosynthesis (18).

Mutagenesis has revealed that there are specific output pathways that affect subsets of the class 1 genes (17, 35). However, none of these affects circadian expression of the *purF* gene. To understand what decides the peaking of class 2 gene expression at dawn and how the temporal separation between class 1 and 2 gene expression is achieved, we attempted to identify mutations that would specifically eliminate the specialized phasing pattern of class 2 genes. We performed transposon mutagenesis on a bioluminescent reporter strain in which the *purF* promoter drives expression of the *V. harveyi luxAB* genes with a class 2 expression pattern and screened for mu-

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics or relevant genotype	Reference
E. coli		
DH10B	Host for plasmids	
AM1037	HB101 containing plasmid pAM1037, which carries a Tn*5* derivative	17
AM1460	HB101 containing plasmid pRK2013 to provide conjugal transfer functions	9
S. elongatus strains		
PCC 7942	Wild-type *S. elongatus* strain	
AMC393	*psbAI*::*luxAB* fusion in NSII (Cm^r) and *psbAI*::*luxCDE* fusion in NSI (Sp^r)	1
AMC395	*psbAI*::*luxCDE* fusion in NSI (Sp^r)	
AMC408	*purF*::*luxAB* fusion in NSII (Cm^r) and *psbAI*::*luxCDE* fusion in NSI (Sp^r)	
AMC517	Tn*5* no. 91, uncharacterized class 2 reporter (Km^r and Sp^r)	
AMC530	Tn*5* no. 61, uncharacterized class 2 reporter (Km^r and Sp^r)	
AMC534	AMC395 with *opcA* inactivated by Tn*5* insertion	
AMC537	*psbAIII*::*luxAB* fusion in NSI (Sp^r) and *psbAI*::*luxCDE* fusion in NSII (Cm^r)	
AMC601	*purF*::*luc* in NSII (Km^r)	
AMC617	*opcA*::*luxAB* fusion (intact *opcA* gene) in NSII (Cm^r) and *psbAI*::*luxCDE* fusion in NSI (Sp^r)	
AMC636	*opcA*::*luxAB* fusion (N-terminal coding region only of *opcA*) in NSII (Cm^r) and *psbAI*::*luxCDE* fusion in NSI (Sp^r)	
AMC661	Derivative of AMC408 in which *opcA* has been inactivated by Tn*5* insertion (reconstruction of the OP strain)	
AMC662	Derivative of AMC636 in which *opcA* has been inactivated by Tn*5* insertion	
AMC663	*purF*::*luxAB* reporter strain, different from AMC408, in which *zwf* has been inactivated by Tn*5* insertion	
AMC866	*fbp*::*luxAB* fusion in NSII (Cm^r) and *psbAI*::*luxCDE* fusion in NSI (Sp^r)	
Plasmids		
pBGS18	pUC18 with Ap^r gene replaced by Km^r gene	31
PIC20H and pIC20R	Cloning vectors	21
pAM1037	Tn*5* derivative pRL1058, further modified by insertion of a 0.7-kb fragment into the *Xba*I site to add outward-reading promoters from the *Anabaena* sp. strain PCC 7120 *glnA* and *rbcL* genes	1
pAM1406	Cm^r cassette inserted in *Eco*RV site of pIC20H	
pAM1580	NSII vector with promoterless *luxAB*; Cm^r selection for transformation of *Synechococcus*	
pAM2076	Plasmid recovered by digestion of DNA from the OP mutant; Tn*5* inserted in *opcA*	
pAM2079	Intact *opcA* gene upstream of *luxAB* in pAM1580	
pAM2080	N-terminal coding region of *opcA* upstream of *luxAB* in pAM1580	
pAM2084	Cm^r cassette inserted in *opcA* with the promoter of Cm^r cassette convergent with the *opcA* promoter	
pAM2085	Cm^r cassette inserted in *opcA* with the promoter of Cm^r cassette reading in the same direction as the *opcA* promoter	
pAM2092	*opcA Sal*I-*Cla*I fragment in pIC20R	
pAM2093	*Eco*RI *opcA* fragment from pAM2092 inserted in the *Eco*RI site of pBGS18	
pAM2095	Km^r cassette in pIC20R with the promoter of Km^r cassette reading in the same direction as the *lacZ* promoter	
pAM2258	P$_{rbcLglnA}$ removed from pAM2076 by digestion with *Xba*I followed by intramolecular ligation	
pAM2259	Plasmid recovered by digestion of a low-bioluminescence mutant; Tn*5* inserted in *zwf*	
pAM2260	P$_{rbcLglnA}$ removed from pAM2259 by digestion with *Xba*I followed by intramolecular ligation	
pAM2510	*Hinc*II-*Hin*dIII fragment of *fbp* upstream of *luxAB* in pAM1580	

tants in which the phasing of the bioluminescence rhythm had reverted to class 1. The screen revealed a mutant that carries an insertion in the *opcA* gene, whose function was previously known only as being essential for function of glucose-6-phosphate dehydrogenase (G6PD), encoded by the upstream *zwf* gene (32, 33). G6PD is the first enzyme in the oxidative pentose phosphate pathway, the primary catabolic source of reductant in cyanobacteria (28).

Additional analysis revealed that the actual expression pattern of the *purF* promoter was unchanged in the *opcA* mutant, but that the reduced flavin mononucleotide (FMNH$_2$) substrate of the luciferase was limiting at the time of the natural circadian peak. The *opcA* gene overlaps the upstream *zwf* gene by 1 bp, suggesting cotranscription (22). However, the phenotype of an *opcA* mutant is more severe than that of a *zwf* mutant, which cannot synthesize G6PD, indicating a broader function for OpcA than previously identified and lack of polarity of the *zwf* insertion on *opcA*. The *opcA* gene, whose product is important for reductant production at night in *S. elongatus*, has a class 2 promoter, independent of class 1 expression from the upstream *fbp* promoter.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. A list of all strains of cyanobacteria, *Escherichia coli*, and plasmids used in this work is presented in Table 1. Endpoints for restriction fragments used in reporter constructs are shown in Fig. 2. All reporter strains were derived from *S. elongatus* PCC 7942. This strain has previously been reported without a specific name as *Synechococcus* sp. strain PCC 7942. However, a pending update to *Bergey's Manual of Determinative Bacteriology* will support designation of the closely related strain PCC 6301 (13, 36) as the living neotype of *S. elongatus* (23, 24); PCC 7942 is also appropriately assigned to this species (R. Rippka, personal communication). *S. elongatus* strains were grown in liquid culture or on agar plates of modified BG-11 medium (BG-11M) (7) with appropriate antibiotics under continuous light at 30°C. *E. coli* strains were grown in liquid or on solid Luria-Bertani (LB) or Terrific broth (TB) medium (25) with appropriate antibiotics. Antibiotic concentrations in BG-11M were as follows: chloramphenicol (Cm), 2 μg/ml; spectinomycin (Sp) and streptomycin (Sm), 2 μg/ml each for selection of the omega cassette; and kanamycin (Km), 5 μg/ml. In LB or TB, concentrations were as follows: ampicillin (Ap), 100 μg/ml; Cm, 17 μg/ml; and Km, 50 μg/ml.

Transposon mutagenesis. A modified Tn*5* element that transposes in *S. elongatus* and confers Km resistance (Km^r) was introduced into *S. elongatus* on pAM1037, a derivative of pRL1058 (37), by triparental conjugation. Details of the procedure have been described elsewhere (1, 17).

Assay for bioluminescence rhythms. Reporter strains were assayed for bioluminescence rhythms on a Packard TopCount luminometer (1, 17) in either LL or 12-h light–12-h dark cycles (LD). Luciferase reporter strains also carried a P$_{psbAI}$::*luxCDE* cassette to provide the long-chain aldehyde substrate for luciferase in vivo. Strains that carried *purF* fusions to the firefly luciferase gene (*luc*)

were measured in the presence of firefly D-luciferin (Biosynth International, Naperville, Ill.) as described previously (17). Entrainment of circadian rhythms by temperature cycles in LL was performed with the following regimen while samples were in stackers on the TopCount: 12 h at 38°C, 12 h at 28°C, 12 h at 38°C, and then 28°C continuously. The cells perceive 12 h at 38°C as day and 12 h at 28°C as night (C. Inoue and T. Kondo, unpublished results). Except where indicated, figures show representative traces; in all cases in which different strains are compared, the wells chosen for comparison are comparable with respect to cell age, density, and position on the microtiter plate, so that quantitative values are meaningful.

Recovery of Tn5, sequencing of the flanking region, and removal of P$_{rbcLglnA}$. Restriction enzymes and most modifying enzymes were purchased from Promega and used as directed by the manufacturer. Genomic DNA from the *S. elongatus* mutant was isolated as described previously (8), digested with *Kpn*I (*Kpn*I does not cut within the Tn5), and religated by T4 DNA ligase. The ligation mixture was introduced into *E. coli* DH10B cells by electroporation, and Km^r *E. coli* colonies were selected on LB-agar plates. Plasmid DNA was prepared by standard methods (3). DNA flanking the transposon was sequenced on one strand by using a primer, AMO178, that is complementary to the N-terminal coding region of the transposase gene; this sequence should be adjacent to *S. elongatus* DNA after insertion of Tn5. The cycle sequencing method was used (dye terminator cycle sequencing ready reaction, ABI Prism; PE Applied Biosystems, Foster City, Calif.). P$_{rbcLglnA}$ was removed from the recovered plasmids from the *opcA* and *zwf* mutants (pAM2076 and pAM2259, respectively) by digestion with *Xba*I followed by intramolecular ligation to create pAM2258 and pAM2260, respectively.

Isolation and inactivation of *opcA*. The intact *opcA* gene was isolated as a *Sal*I-*Cla*I fragment (Fig. 2) from the plasmid that was recovered following Tn5 insertion into the *zwf* locus (pAM2259; U. Nair and S. Golden, unpublished results). The fragment was inserted into *Sal*I- and *Cla*I-digested pIC20R to create pAM2092. The Km^r cassette from pAM2095 and the Cm^r cassette from pAM1406 were isolated as *Xho*I-*Sal*I fragments and inserted individually into the *Xho*I site of *opcA* in both orientations. The resultant four constructs were used to transform appropriate cyanobacterial reporter strains to inactivate *opcA* (11).

Complementation of *opcA*-inactivated mutants. The *opcA* gene was excised from pAM2092 as an *Eco*RI fragment and inserted into *Eco*RI-digested pBGS18 to create pAM2093. *opcA* was then removed as an *Xba*I fragment and inserted at the *Nhe*I site of pAM1580 (1), a promoterless *luxAB* reporter vector for insertion at neutral site II (NSII) of the *S. elongatus* genome, creating pAM2079. In this construct, the entire *opcA* open reading frame (ORF) precedes *luxA* and *luxB* as a tricistronic operon. pAM2079 was digested with *Xho*I and religated to remove the carboxy-terminal coding region of *opcA*, resulting in pAM2080. In this construct, *opcA* upstream sequences drive expression of *luxAB*, but only a fragment of the OpcA polypeptide is encoded. pAM2079 and pAM2080 were used to transform AMC395, an *S. elongatus* strain that carries *luxCDE* driven by the *psbAI* promoter. These genes encode the synthetase responsible for biosynthesis of the luciferase long-chain aldehyde substrate.

In vitro luciferase assay and in vivo bioluminescence measurements. Reporter strains were grown to an optical density at 750 nm of ca. 0.8 in flasks before they were split into two sets. One set was given a 12-h dark pulse and then 12-h light for the ZT12 group (*Zeitgeber* time is time relative to an entraining environmental signal). The other set was given 10-h light, 12-h darkness, and then 2-h light for the ZT2 group. The two groups were assayed at the same time. For in vivo assays, bioluminescence was measured from 200-μl samples of cultures after addition of 4 μl of decanal (*n*-decyl aldehyde; Sigma, St. Louis, Mo.) by a scintillation counter with coincidence disabled for photon counting (Beckman LS3801 liquid scintillation system). Strains with strongly expressed reporters were diluted before the assay. For in vitro assays, cells were broken with a French press, and luciferase activities were measured by the flavin injection assay (30, 38). Total protein concentrations were determined by the Lowry assay (20).

RESULTS

An opposite-phase mutant. The *purF*::*luxAB* reporter strain AMC408 was mutagenized by the introduction of pAM1307, which carries a Tn5 transposon derivative. Exconjugants were transferred to agar pads in microtiter plates for screening of circadian phenotypes on a TopCount (Packard) cycling luminometer. Among approximately 3,000 screened colonies, a putative mutant was found, named OP, with low bioluminescence and an opposite phasing relative to the wild-type *purF*::*luxAB* bioluminescence rhythm (Fig. 1). The mutant phenotype was confirmed by several independent TopCount runs and also observed when the running conditions were 12-h light–12-h dark cycles (LD) (data not shown). Circadian rhythms of gene expression in *S. elongatus* can also be entrained by temperature cycles. The apparent phase change phenotype was also ob-
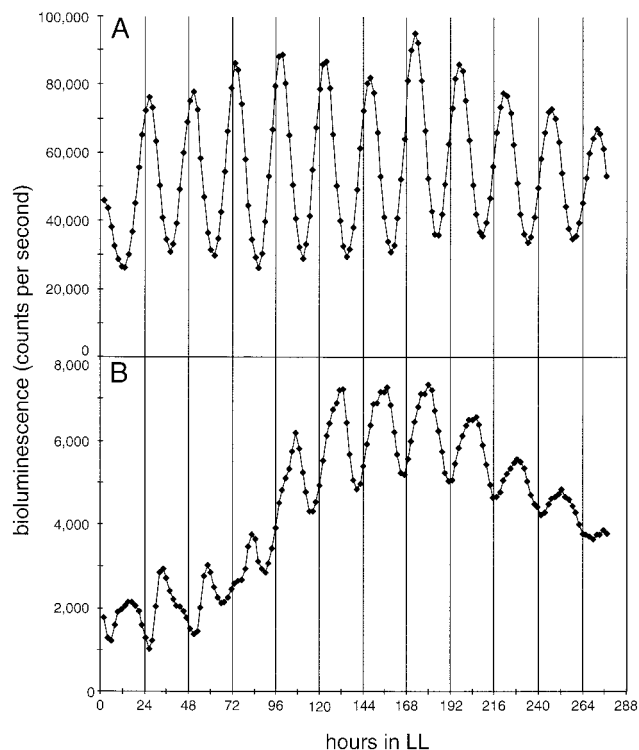

FIG. 1. Circadian rhythms of bioluminescence from the *purF*::*luxAB* reporter in AMC408 (wild-type genetic background) (A) and the OP mutant (B). These representative traces demonstrate the altered phasing of peaks and troughs in OP as well as the characteristic decrease in overall bioluminescence level (peaks approximately 10% of the levels of those in a wild-type background). The *x* axis in these and all subsequent bioluminescence traces is hours in LL after a phase-synchronizing 12-h incubation in darkness. Bioluminescence values are counts per second measured from microtiter wells that contain reporter strains supported on agar cushions.

served for the mutant when the circadian clock was entrained by a 38°C-28°C temperature cycle (data not shown).

The Tn5 element and its flanking regions were recovered as a plasmid, pAM2076. This plasmid and a derivative, in which the outward-reading P$_{rbcLglnA}$ had been removed from the Tn5 element, were linearized and used to transform AMC408 by homologous recombination at the original insertion locus (1). All transformants showed the original OP phenotype (data not shown). This demonstrated that the mutant phenotype results from the insertion of Tn5 at the locus in OP rather than from a secondary mutation and that it did not depend on activity of P$_{rbcLglnA}$. The *S. elongatus* DNA flanking Tn5 in pAM2076 was sequenced, revealing identity with the *opcA* gene previously sequenced from *S. elongatus* (GenBank accession numbers U33285 and X64768) (27). The transposon inserted at nucleotide 3124 in the GenBank DNA sequence corresponds to amino acid (aa) 133 of the 455-aa OpcA coding region (Fig. 2).

The biochemical activity of OpcA is unknown. However, it is required for the oxidative pentose phosphate (OPP) pathway (32, 33), specifically for the function of G6PD (525 aa) encoded by *zwf*. A Tn5 insertion into *zwf*, which is immediately upstream of *opcA*, was isolated independently in another screen (U. Nair and S. Golden, unpublished results) (Fig. 2). The insertion site corresponds to the codon for aa 197 of G6PD (GenBank accession numbers U33285 and X64768) (27). In the *zwf* mutant, P$_{rbcLglnA}$ reads upstream toward *fbp* (Fig. 2). The *zwf* insertion mutant was found initially in a *psbAIII* reporter background, AMC537. Tn5 and its flanking
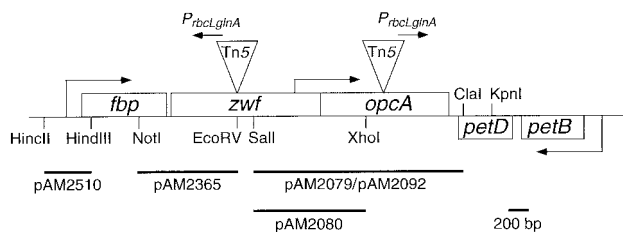
FIG. 2. Physical and genetic map of the *Synechococcus opcA* locus. Genes are represented by labeled boxes. Triangles indicate the positions of insertions of Tn*5* in mutants described in the text. Arrows show direction and approximate originations of transcription. Restriction sites are indicated for enzymes used in constructing relevant clones, and the inserts of wild-type DNA in specific plasmids are indicated by bars underneath the map, labeled with the plasmid names.

regions were recovered and used to transform both AMC537 (*psbAIII*::*luxAB*) and AMC408 (*purF*::*luxAB*). Bioluminescence from both reporter strains decreased, as previously observed for the original *zwf* insertion mutant in the AMC537 background (Fig. 3A). However, unlike the *opcA* insertion, *zwf* inactivation did not cause a phase change in the AMC408 background (data not shown). *zwf* and *opcA* overlap by one nucleotide and are assumed to be cotranscribed (26, 27). If OpcA were involved only in the OPP pathway and could only be cotranscribed with *zwf*, we would expect the OP phenotype when *zwf* is inactivated.
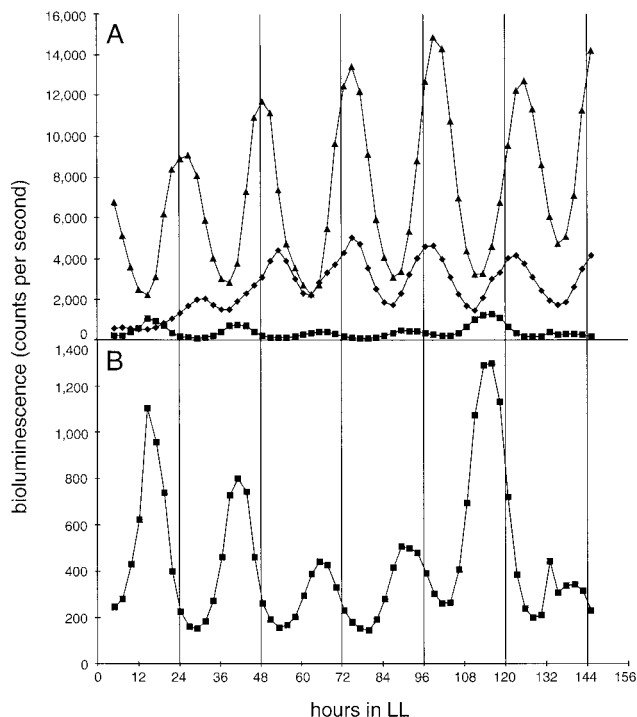

FIG. 3. Bioluminescence traces from AMC408 and derivatives that carry Tn*5* insertions in *opcA* or *zwf* that lack the outward-reading P*rbcLglnA* promoter. The fragment that carries P*rbcLglnA* was removed from the Tn*5*-bearing plasmids recovered from OP and AMC537(Tn*5zwf*). The resulting plasmids were then used to transform AMC408 to recreate Tn*5* insertions in the *zwf* and *opcA* genes [AMC408(Tn*5zwf*) and AMC408(Tn*5opcA*), respectively]. (A) Bioluminescence traces from AMC408 (triangles), AMC408(Tn*5zwf*) (diamonds), and AMC408 (Tn*5opcA*) (squares). (B) Expansion of the scale for the trace from AMC408 (Tn*5opcA*) to demonstrate that the circadian rhythm persists with a phasing like that of OP in Fig. 1.
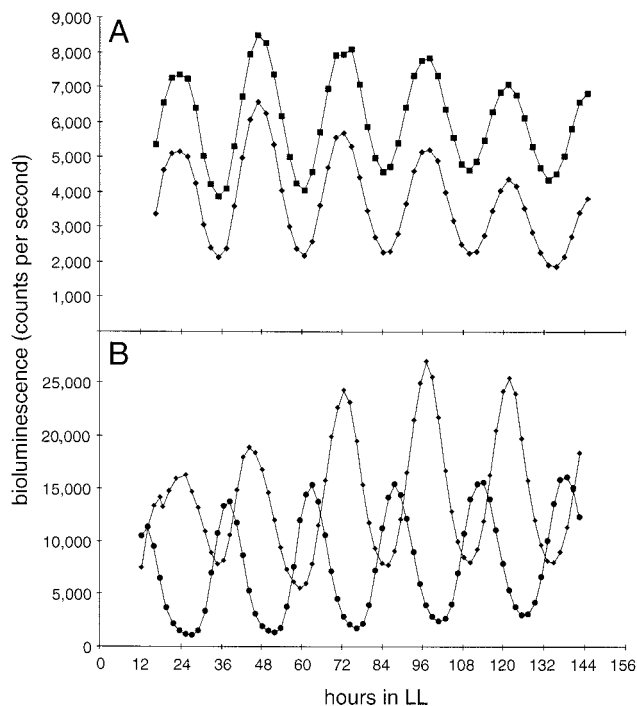

FIG. 4. Rhythmic class 2 expression from *luxAB* fusions driven by the *opcA* upstream region. (A) In AMC617 (diamonds), *luxAB* is fused downstream of a complete *opcA* ORF and potential terminator sequences (see pAM2079 insert in Fig. 2). In AMC636 (squares), the reporter is fused after the first 439 bp of *opcA* (see pAM2080 insert in Fig. 2). The left endpoint of the regulatory region, within the *zwf* gene, is the same for both constructs (Fig. 2). The traces are mean averages from 12 samples of each strain. The mean peak levels (± SD) for the five complete oscillations are 5,403 ± 811 and 7,738 ± 548 for AMC617 and AMC636, respectively; trough values are 2,139 ± 155 and 4,290 ± 329, respectively. (B) Class 1 expression from the *fbp* upstream region in AMC866 (circles) compared with class 2 expression from *opcA* (diamonds). Average expression levels from the *opcA* reporter were approximately twice that from the *fbp* reporter.

***opcA* is transcribed independently as a circadian class 2 gene.** To determine whether *opcA* is transcribed independently of *zwf*, two constructs were made in which all or part of the *opcA* gene, including upstream sequences internal to *zwf*, were used to drive promoterless *luxAB* genes. Two *opcA*::*luxAB* plasmids, pAM2079 and pAM2080, were used to transform AMC395, an *S. elongatus* strain that expresses the *luxCDE* genes to instruct in vivo synthesis of the long-chain aldehyde substrate of the luciferase (Table 1). In pAM2079, the entire *opcA* ORF is present upstream of *luxAB*, as are potential downstream terminator sequences from between *opcA* and *petD* (Fig. 2). The reporter fusion in pAM2080 carries only the first 439 bp of *opcA* (of 1,334 bp) upstream of *luxAB* (Fig. 2). Both plasmids target the reporters to a defined cloning site in the *S. elongatus* genome (NSII) (1). The resulting transformants were screened for circadian rhythms of bioluminescence, and all showed robust bioluminescence with a class 2 phase (Fig. 4A). This demonstrated that *opcA* has its own transcription signals in addition to expected cotranscription with *zwf* and that this independent transcription is in a class 2 phase. Assay of a fragment including *zwf* upstream sequences (Fig. 2) did not show promoter activity in this region (data not shown). The upstream region of *fbp* drives *luxAB* expression with a high-amplitude oscillation in class 1 (Fig. 4B). Thus, the independent transcription of *opcA* allows it to be expressed at a time when the remainder of the operon is quiescent.
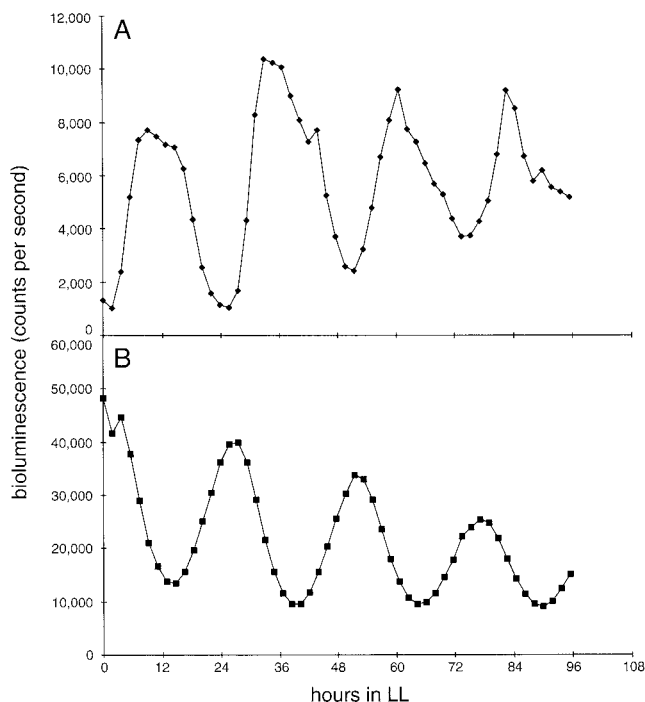
FIG. 5. Phasing of bioluminescence from the *opcA* promoter is itself sensitive to *opcA* inactivation. A strain in which *opcA* is inactivated by Tn*5* insertion was transformed with reporter plasmids that carry either an incomplete (A) or complete (B) copy of *opcA* fused to *luxAB*. When no intact *opcA* is present, the *opcA*::*luxAB* fusion shows the lower bioluminescence and reversed phasing of expression characteristic of the OP mutant (*purF*::*luxAB* reporter).

Mutant phenotype caused by inactivation of *opcA*. Mutants were constructed in which Km^r or Cm^r cassettes were used to inactivate *opcA* in various reporter backgrounds to determine whether bioluminescence rhythms from other genes are affected. Each inactivating resistance cassette was inserted in both orientations into the *Xho*I site of *opcA* (Fig. 2). The two Km^r constructs were used to transform AMC408 (*purF*::*luxAB*, class 2). All transformants showed low bioluminescence and a class 1 phase, as first observed for the Tn*5* mutant OP (data not shown). The two orientations of the Cm^r cassette in *opcA* (pAM2084 and pAM2085) were used to transform two uncharacterized class 2 reporter strains, AMC517 and AMC530, obtained by random transposition of Tn*5*::*luxAB* throughout the *S. elongatus* genome (1; J. Shelton and S. Golden, unpublished results). All transformants showed low bioluminescence and class 1 phase (data not shown). These results indicate that the interruption of *opcA* can cause the mutant phenotype in other class 2 reporters.

We then tested whether *opcA* inactivation affects the pattern of *opcA*::*luxAB* expression. The *opcA*::*luxAB* reporter plasmids pAM2079 and pAM2080 were individually used to transform AMC534, a strain that carries the aldehyde biosynthesis genes and Tn*5*-inactivated *opcA*. Of the two sets of transformants, those from pAM2080 showed lower bioluminescence and a class 1 phase (Fig. 5A), indicating that the *opcA* bioluminescence rhythm, like that of *purF*, is affected by inactivation of *opcA*. However, the transformants from pAM2079 showed higher bioluminescence and a class 2 phase (Fig. 5B). This indicated that the *opcA*::*luxAB* construct in pAM2079, which carries the entire *opcA* ORF fused to *luxAB*, not only reported *opcA* expression but also complemented the mutant phenotype. In a wild-type (*opcA*$^+$) background, pAM2080 transformants also exhibit class 2 phasing and the higher level of

bioluminescence (Fig. 4A). These results demonstrate that the OP mutant phenotype—low bioluminescence and change of phase from class 2 to class 1—was caused by inactivation of *opcA*.

Inactivation of *opcA* affects reductant production at night and causes luciferase substrate limitation. Several pieces of evidence suggested that the role of *opcA* in the OPP, an important pathway for generating reductant (NADPH) in the absence of photosynthesis, might be an important aspect of the phenotype. The Tn*5* interruption of *opcA* was transferred to some class 1 reporters, including the *psbAIII*::*luxAB* reporter AMC537 (Fig. 6A). In general, the bioluminescence was lowered, more so at subjective dawn than at dusk; this caused an increase in the amplitude of the bioluminescence rhythm but did not change the phase (Fig. 6B). When *opcA* was inactivated in the *psbAI*::*luxAB* reporter AMC393 by transformation with pAM2076, bioluminescence from the transformants dropped dramatically during the first 3 days of monitoring (approximately 10 days after appearance of transformants), and the strains could not survive beyond that time. This early-death phenotype is not apparent in other reporter backgrounds or in an *opcA*$^+$ *psbAI*::*luxAB* reporter background. An explanation for toxicity of the strong *psbAI*::*luxAB* expression in an *opcA*-null background might be consumption of limiting reductant by the luciferase enzyme. The promoter of the *psbAI* gene is the strongest we have measured in *S. elongatus* (among more than 20), and luciferase expression from this fusion is very high. The involvement of OpcA in the OPP pathway, which is the main catabolic source of reductant, made us suspect that inactivation of *opcA* caused limitation of FMNH$_2$ substrate for the luciferase (4), especially at night.

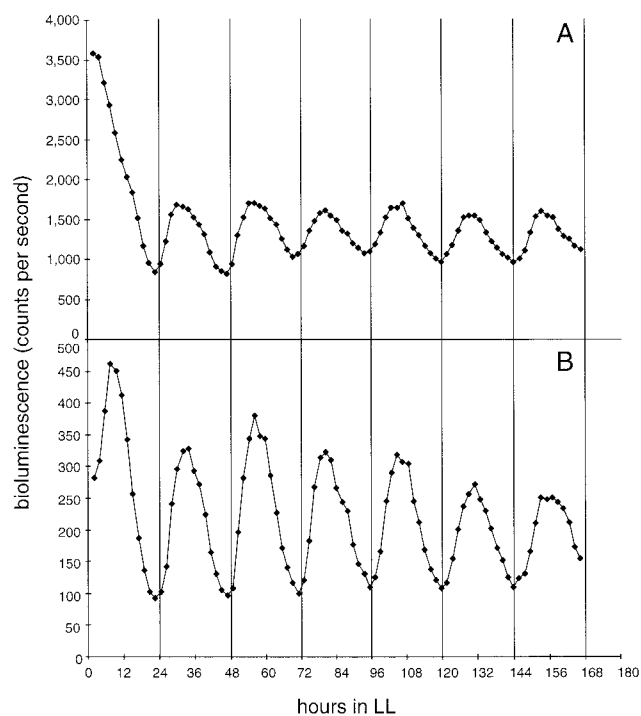If the mutant phenotype resulted from substrate limitation,

FIG. 6. Inactivation of *opcA* does not change the phasing of bioluminescence from a class 1 phase *luxAB* reporter. The Tn*5* insertion in *opcA* was transferred to a *psbAIII*::*luxAB* reporter strain. Bioluminescence was monitored from the reporter in the wild-type background (AMC537) (A) and the *opcA*-inactivated background [AMC537(Tn*5opcA*)] (B).
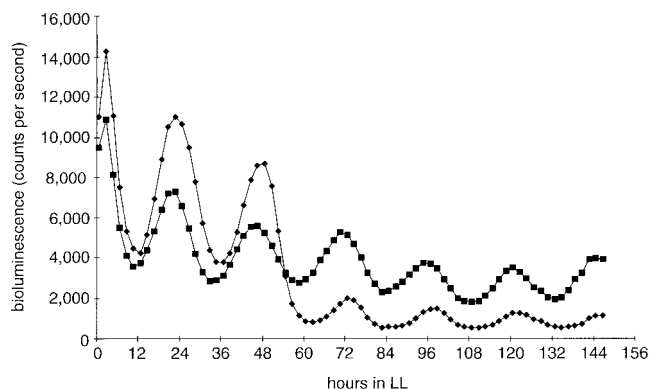
FIG. 7. Inactivation of *opcA* does not affect either the phasing or magnitude of *purF* expression when measured by a different reporter. A reporter was constructed in which the firefly luciferase gene, *luc*, is driven by the *purF* promoter. Phasing and overall magnitude of bioluminescence were similar from this gene fusion whether *opcA* was intact (AMC601, diamonds) or inactivated by insertion of a Cmʳ cassette [AMC601(Cm*opcA*), squares].

we expected that the phasing of class 2 genes would be unaltered in an *opcA* mutant when measured by a reporter that does not depend on FMNH$_2$. Strain AMC601 carries a translational fusion between *purF* and the firefly luciferase gene, *luc*. The luciferase encoded by *luc*, although it is an oxygenase like the *luxAB* product, does not use the reductant FMNH$_2$ as a substrate, but rather requires ATP and a luciferin in addition to O$_2$. We transformed AMC601 with pAM2084 and pAM2085 individually to inactivate *opcA*. All transformants tested showed the same bioluminescence phenotype as AMC601 (Fig. 7). This indicates that *purF* expression itself is not altered by loss of *opcA* and supports the likelihood that the OP phenotype is reporter specific, perhaps as a result of FMNH$_2$ limitation.

To test directly for substrate limitation in the *opcA* background, we performed in vitro assays for luciferase activity from extracts of *purF*::*luxAB* and *opcA*::*luxAB* strains. Luciferase activity showed the wild-type pattern of higher activity at dawn and lower at dusk in both *opcA* and *zwf* mutants (AMC661 and AMC663, respectively) when exogenous FMNH$_2$ and decanal were provided (Table 2). In vivo assays of *opcA*-inactivated strains still showed opposite phasing of bioluminescence from *purF*::*luxAB* (AMC661) and *opcA*::*luxAB* (AMC662) reporters relative to the wild-type controls even when decanal was added exogenously. The only untested substrate is O$_2$, which is also a substrate for firefly luciferase. We did not see a mutant phe-

notype from the *purF*::*luc* reporter when *opcA* was inactivated. We conclude that the limitation of FMNH$_2$ is solely responsible for the low bioluminescence and the phase change in the *purF*::*luxAB* reporter when *opcA* is absent. The FMNH$_2$ level was especially low at dawn, such that the bioluminescence at dawn became lower than that at dusk, resulting in the apparent reversed phase.

DISCUSSION

When the OP mutant data are reexamined with the knowledge that the *opcA* mutation causes a limitation in the reductant supply, the nature of the apparent phasing change of the *purF*::*luxAB* bioluminescence rhythm is evident. The absolute bioluminescence level at subjective dusk (when class 1 genes peak and *purF* troughs) is much less affected than the level at subjective dawn (when class 1 genes trough and class 2 genes peak). The same phenomenon is responsible for the increased amplitude of class 1 reporters when *opcA* is inactivated, as a result of lower bioluminescence during the trough at subjective dawn and a lesser effect on the subjective dusk peak. This asymmetry of the effect of decreased luciferase substrate suggests that the metabolic pathways for generating FMNH$_2$ are not constitutive in *S. elongatus* but oscillate in a circadian fashion. A simplistic hypothesis is that photosynthetic activity is largely responsible for generating reductant during the day and subjective day, whereas respiratory pathways such as the OPP take over this role during subjective night. Surprisingly, firefly luciferase activity was not affected by inactivation of *opcA*, indicating that the cell has means of maintaining ATP concentrations when the OPP is disrupted.

A central question that remains regarding these data is why inactivation of *opcA* has a more severe phenotype than inactivation of *zwf*. The known function of OpcA is that it is required for G6PD activity. G6PD monomers are encoded by *zwf*. OpcA is involved in the oligomerization and activation of G6PD, as the subunits of the enzyme are monomeric, and there is little activity in an *opcA* mutant (33). Both *zwf* and *opcA* mutants appear to have defects in FMNH$_2$ production, but only the inactivation of *opcA* lowered the substrate level enough at dawn to reverse the phase of bioluminescence. Our data suggest that OpcA has a role in a reductant-generating step beyond its involvement in activating G6PD. This could be another target within the OPP or in another metabolic pathway, but it is likely to be more active in the night or subjective night and under circadian control.

TABLE 2. Luciferase activities

Strain	ZT time	In vitro		In vivo	
		Activity[a]	Class[b]	Activity[c]	Class
AMC408 (*purF*::*luxAB*) *opcA*⁺	ZT2	6.03 ± 1.00	2	7.90 ± 0.54	2
	ZT12	3.15 ± 1.00		3.44 ± 0.19	
AMC661 (*purF*::*luxAB*) *opcA* null	ZT2	5.60 ± 0.84	2	2.80 ± 0.86	1
	ZT12	2.54 ± 1.01		6.35 ± 1.84	
AMC636 (*opcA*::*luxAB*) *opcA*⁺	ZT2	4.67 ± 1.02	2	13.02 ± 2.37	2
	ZT12	2.02 ± 0.95		4.58 ± 0.22	
AMC662 (*opcA*::*luxAB*) *opcA* null	ZT2	6.41 ± 0.60	2	19.58 ± 0.27	1
	ZT12	3.48 ± 0.13		44.33 ± 3.03	
AMC663 (*purF*::*luxAB*) *zwf* null	ZT2	5.87 ± 0.03	2	NT[d]	
	ZT12	2.88 ± 0.219		NT	

[a] Luminometer values (arbitrary units) per microgram of total protein ± standard deviation.
[b] Class of expression; higher value at subjective dusk ZT12 (class 1) or dawn ZT2 (class 2).
[c] Excess *n*-decyl aldehyde provided exogenously; values are 10⁵ cpm ± standard deviation.
[d] NT, not tested.

The opposite phasing of *opcA* and *fbp* expression is consistent with a role outside of OPP for OpcA; its independent transcription allows expression when the *fbp* and *zwf* genes are silent. Cotranscription of *opcA* with *zwf* may coordinate OpcA function in the OPP pathway, in which G6PD encoded by *zwf* is a crucial enzyme. Our data demonstrate that *opcA* can be transcribed independently and that its independent transcription is in class 2 phase, i.e., peaks at dawn. This, and the fact that the *opcA* mutant has such a low bioluminescence specifically at dawn, such that the original peak at dawn has become a trough, suggests that OpcA made at dawn is involved in a reductant production pathway at that time of day.

We have partially analyzed the *purF* promoter region (unpublished data). The identification of *opcA* as another class 2 gene affords the opportunity to compare potential regulatory elements. Functional analysis of these elements is in progress to determine the sequence information that is required for class 2 phasing of circadian expression.

The goal of the project was to identify mutants affected in generating class 2 expression, but these were not identified in our screen of approximately 5,000 Tn*5* insertion mutants. There are several potential explanations, both technical and biological. One is that Tn*5*, thus far the only useful transposon for the organism, is simply a limited mutagen, which shows bias of insertion sites (5). Indeed, we have identified the *zwf/opcA* locus four more times in various screens with Tn*5* mutagenesis and have yet to isolate an insertion in the known *kaiABC* locus that is required for circadian rhythmicity, even though loss of these genes is not deleterious under lab conditions.

Another more intriguing possibility is that there is a general mechanism underlying all class 2 gene expression and the classes of mutations we seek may be lethal. Temporal separation may be the original drive for the evolution of circadian clocks. Like *purF*, nitrogenase genes are expressed at night in unicellular diazatrophic cyanobacteria (12, 15, 29). The products of these genes are sensitive to O_2, which is produced by photosynthesis during the (subjective) day. Phasing the expression of these genes at night may be advantageous, and changing all class 2 gene expression to class 1 may be disastrous. Support for this idea is provided by the *cpmA* mutant, in which some class 1 genes show dramatically altered phasing and the cells have linked growth and pigment defects (17). However, it is not clear whether the change in phasing itself is responsible for the poor fitness of the strain. Screens for conditional mutants may allow us to identify loci that are necessary for the generation of class 2 gene expression.

Prokaryotic circadian rhythms were first recognized in diazotrophic cyanobacteria because of temporal separation of the incompatible processes of oxygen-sensitive nitrogen fixation and oxygen-evolving photosynthesis (12). However, only gene expression has been examined as a circadian phenomenon in *S. elongatus*, the strain in which genetic analysis has identified components of the circadian clock. The current work provides an entry point to explore the extent and consequence of temporal regulation of metabolism in this genetically tractable strain.

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