

THE RESPONSE REGULATOR NPUN_F1278 IS ESSENTIAL FOR SCYTONEMIN BIOSYNTHESIS IN THE CYANOBACTERIUM *NOSTOC PUNCTIFORME* ATCC 29133¹

Sejuti Naurin, Janine Bennett

Department of Biology, Indiana University-Purdue University Fort Wayne, Fort Wayne, Indiana 46805, USA

Patrick Videau, Benjamin Philmus

Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon 97331, USA

and Tanya Soule²

Department of Biology, Indiana University-Purdue University Fort Wayne, Fort Wayne, Indiana 46805, USA

Following exposure to long-wavelength ultraviolet radiation (UVA), some cyanobacteria produce the indole-alkaloid sunscreen scytonemin. The genomic region associated with scytonemin biosynthesis in the cyanobacterium *Nostoc punctiforme* includes 18 cotranscribed genes. A two-component regulatory system (Npun_F1277/Npun_F1278) directly upstream from the biosynthetic genes was identified through comparative genomics and is likely involved in scytonemin regulation. In this study, the response regulator (RR), Npun_F1278, was evaluated for its ability to regulate scytonemin biosynthesis using a mutant strain of *N. punctiforme* deficient in this gene, hereafter strain $\Delta 1278$. Following UVA radiation, the typical stimulus to initiate scytonemin biosynthesis, $\Delta 1278$ was incapable of producing scytonemin. A phenotypic characterization of $\Delta 1278$ suggests that aside from the ability to produce scytonemin, the deletion of the Npun_F1278 gene does not affect the cellular morphology, cellular differentiation capability, or lipid-soluble pigment complement of $\Delta 1278$ compared to the wildtype. The mutant, however, had a slower specific growth rate under white light and produced ~2.5-fold more phycocyanin per cell under UVA than the wildtype. Since $\Delta 1278$ does not produce scytonemin, this study demonstrates that the RR gene, Npun_F1278, is essential for scytonemin biosynthesis in *N. punctiforme*. While most of the evaluated effects of this gene appear to be specific for scytonemin, this regulator may also influence the overall health of the cell and phycobiliprotein synthesis, directly or indirectly. This is the first study to identify a regulatory gene involved in the biosynthesis of the sunscreen scytonemin and posits a link between cell growth, pigment synthesis, and sunscreen production.

Key index words: *Nostoc*; response regulator; scytonemin; two-component regulatory system; ultraviolet radiation; UVA

Abbreviations: AA, Allen and Arnon medium; *amp*^R, ampicillin-resistant; ANOVA, Analysis of Variance; *cm*^R, chloramphenicol-resistant; HK, histidine kinase; HPLC, high-pressure liquid chromatography; *kan*^R, kanamycin-resistant; ND, not detectable; PCR, polymerase chain reaction; RR, response regulator; *sacB*, levansucrase; TCRS, two-component regulatory system; UVA, long-wavelength ultraviolet radiation (320–400 nm); UVB, short-wavelength ultraviolet radiation (280–320 nm); UVR, ultraviolet radiation; $\Delta 1278$, *Nostoc punctiforme* ATCC 29133 containing a 552 bp in frame deletion of Npun_F1278

As oxygenic photosynthetic prokaryotes, cyanobacteria must live in environments open to solar ultraviolet radiation (UVR) in order to capture the visible energy required for photosynthesis (Castenholz and Garcia-Pichel 2012). Despite the various harmful effects of solar UVR on cyanobacteria, they have evolved several UVR defense strategies that allow survival in these environments. These strategies include the gliding movements of some filamentous cyanobacteria (Bebout and Garcia-Pichel 1995), development of efficient metabolic DNA repair systems (Van Baalen 1968, Levine and Thiel 1987), synthesis of UV shock proteins (Shibata et al. 1991, Shirkey et al. 2000), and production of sunscreens such as the mycosporine-like amino acids and the indole-alkaloid scytonemin (Garcia-Pichel and Castenholz 1991, 1993, Gao and Garcia-Pichel 2011). Scytonemin is a yellow to brown, lipid-soluble, non-fluorescent, stable pigment only produced by some strains of cyanobacteria, most of which live in near-surface habitats. It is synthesized upon exposure to UVA radiation (320–400 nm) and exported to the extracellular polysaccharide sheath where it absorbs incident UVA radiation with a maximum around

¹Received 14 December 2015. Accepted 15 February 2016.

²Author for correspondence: e-mail soulet@ipfw.edu.

Editorial Responsibility: J. Collier (Associate Editor)

370 nm in vivo (Garcia-Pichel and Castenholz 1991, Proteau et al. 1993).

The genomic region associated with scytonemin biosynthesis has been identified in the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133 (PCC 73102) and contains 18 adjacent genes (Npun_R1276 to Npun_R1259) transcribed in a single direction (Fig. 1; Soule et al. 2007). Most of the genes in the upstream region of the cluster encode unique proteins and have been shown to be involved directly in scytonemin biosynthesis. For example, *scyA* (Npun_R1276), *scyB* (Npun_R1275), and *scyC* (Npun_R1274) encode enzymes which form the precursors involved in the early stages of scytonemin biosynthesis (Balskus and Walsh 2008, 2009). This 18-gene genomic region was also found to be conserved in the genome of the closely related cyanobacterium *Anabaena* PCC 7120, as well as in the genomes of *Lyngbya* PCC 8106, *Nodularia spumigena* CCY 9414, and *Chlorogloeopsis* sp. Cgs-O-89 (Soule et al. 2009b). Furthermore, an updated comparative genomics analysis reveals that the conserved scytonemin gene cluster occurs in at least 15 sequenced strains of cyanobacteria (data not shown), although their ability to produce scytonemin has not yet been confirmed. Results from the comparative genomic analyses also described the conservation of a putative two-component regulatory system (TCRS) located upstream, adjacent, and divergently transcribed to the scytonemin gene cluster in *N. punctiforme* (Npun_F1277 and Npun_F1278). This TCRS is highly conserved in orientation and in its location upstream of the scytonemin gene cluster in at least 11 strains of cyanobacteria containing the putative scytonemin biosynthetic clusters, leading to the hypothesis that it is involved in scytonemin regulation.

In prokaryotes, TCRSs are a primary means by which cells recognize and respond to different environmental stimuli. TCRSs are typically characterized by a sensor kinase (often referred to as a histidine kinase; HK) and a response regulator (RR). The RR and subject of this study, Npun_F1278, is described

as having an AraC output DNA-binding domain (Ashby and Houmar 2006). In general, AraC output domains describe a family of bacterial transcriptional activators that are involved with stress responses that regulate diverse genetic systems (Martin and Rosner 2001). Analysis of the Npun_F1278 protein homologues associated with the scytonemin biosynthetic gene cluster in other cyanobacterial genomes shows that, like the Npun_F1278 protein in *N. punctiforme*, they are all AraC-type RRs, possibly indicating a conserved function (Soule et al. 2009b).

In this study, the putative RR Npun_F1278 gene was shown to be essential for the regulation of scytonemin biosynthesis in *N. punctiforme*. This is the first study to identify a gene that regulates scytonemin biosynthesis in cyanobacteria. This analysis not only provides a better understanding of scytonemin regulation but also provides further insight into the dynamic response of cyanobacteria to UVA radiation.

MATERIALS AND METHODS

Cultivation and media. *Nostoc punctiforme* ATCC 29133 batch cultures were grown in a 4-fold diluted formulation of Allen and Arnon medium (AA/4) (Allen and Arnon 1955) at 25°C. Nitrogen supplementation and buffering (AA/4 + N) were provided at a final concentration of 2.5 mM NH₄Cl and 5 mM MOPS, respectively, at pH 7.8 as required. All cultures were routinely grown under visible white light, which was provided by cool-white fluorescent bulbs at an intensity of 30–50- $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. *Escherichia coli* strains were cultured in Lysogeny Broth medium in liquid and on plates solidified with 1% Bacto agar (Becton Dickinson, Franklin Lakes, NJ, USA). Plasmids were selected in *N. punctiforme* by supplementing the media with neomycin (25 $\mu\text{g} \cdot \text{mL}^{-1}$) and chloramphenicol (10 $\mu\text{g} \cdot \text{mL}^{-1}$) and in *E. coli* with the addition of kanamycin (50 $\mu\text{g} \cdot \text{mL}^{-1}$) and ampicillin (100 $\mu\text{g} \cdot \text{mL}^{-1}$; Risser and Meeks 2013).

Plasmid and strain construction. Plasmid construct inserts were prepared according to established protocols (Risser and Meeks 2013), and all plasmid inserts were sequenced to ensure accuracy. Table 1 lists the strains and plasmids used

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i> UC585	Contains pRK24 (mobilizes shuttle vectors) and pRL528 (methylated to protect against nucleases); <i>cm^R</i> , <i>amp^R</i>	Liang et al. (1993)
<i>Nostoc punctiforme</i> ATCC 29133	Wildtype (scytonemin-producer)	Rippka et al. (1979)
Δ 1278	Npun_F1278 mutant	This study
Plasmids		
pRL278	<i>kan^R</i> , <i>cm^R</i> , <i>sacB</i>	Elhai and Wolk (1988)
pSN1501	<i>kan^R</i> , <i>cm^R</i> , <i>sacB</i>	This study
<i>kan^R</i> , kanamycin-resistant; <i>cm^R</i> , chloramphenicol-resistant; <i>amp^R</i> , ampicillin-resistant; <i>sacB</i> , levansucrase.		

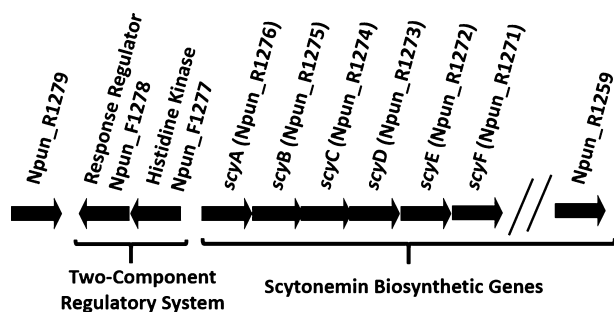


FIG. 1. Scytonemin biosynthetic gene cluster and associated two-component regulatory system in *Nostoc punctiforme*. The break represents genes Npun_R1270 through Npun_R1260 and arrows show the direction of transcription. Image is not drawn to scale.

in this study, Table 2 lists the primers used in the construction of the Δ Npun_F1278 gene fusion product and in the gene expression analyses described below; Figure S1 in the Supporting Information details the procedure utilized to create the in frame deletion mutants. To create a 552 bp in frame deletion within the coding region of Npun_F1278, the regions up- and downstream of the desired deletion were amplified by PCR from chromosomal DNA using the primer pairs 1278.1/1278.2 and 1278.3/1278.4. The products were joined through fusion PCR (Frohmann 1994) and included 78 amino acids of the N-terminus and 22 amino acids of the C-terminus of the Npun_F1278 protein sequence along with the adjacent flanking regions, effectively deleting 184 amino acids from within the central region of the protein. The Δ Npun_F1278 gene fusion product was digested with *Xho*I-*Bam*HI at sites added onto the primers and cloned into the same sites in the conjugal vector pRL278 (Black et al. 1993) to generate plasmid pSN1501.

Bi-parental conjugation was done as previously described (Cohen et al. 1998, Risser and Meeks 2013) to introduce plasmid pSN1501 into *N. punctiforme* to cleanly delete a portion of the Npun_F1278 protein-coding region. This deletion was designed such that it would not disrupt expression of the downstream convergently transcribed Npun_R1279 gene or inactivate the upstream cotranscribed Npun_F1277 gene (Janssen and Soule 2016). Double-recombinant colonies were preliminarily screened by their ability to grow on 5% sucrose and their sensitivity to neomycin, the resulting phenotype from the loss of pSN1501. The genomic DNA from double-recombinant colonies was also screened for deletion of the central region of the Npun_F1278 gene according to established protocols (Risser and Meeks 2013, Risser et al. 2014, Campbell et al. 2015, Hudek et al. 2015). For this, the genomic regions outside and within the Npun_F1278 gene were amplified through colony PCR using the following primer pair combinations: 1278.5/1278.8, 1278.7/1278.6, 1278.5/1278.6, 1278.7/1278.8 (Fig. S2 in the Supporting Information). The PCR products from positive colonies were then sequenced to confirm the mutant genotype.

Pigment and growth analysis of *N. punctiforme* strain Δ 1278. To assess the ability of the *N. punctiforme* Δ 1278 mutant strain to produce scytonemin, both Δ 1278 and the wildtype were exposed to white light only ($33 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and white light supplemented with UVA ($6 \text{ W} \cdot \text{m}^{-2}$) in UVA-transparent tissue culture flasks for 1 week. Cells were then homogenized and the pigments were extracted in 100% acetone overnight at 4°C in the dark. Scytonemin was then measured spectrophotometrically by absorbance at 384 nm (Garcia-Pichel et al. 1992).

HPLC analyses of the lipid-soluble pigment profiles of Δ 1278 and wildtype cells with and without UVA exposure were accomplished using an Agilent 1100 HPLC instrument, which consisted of a quaternary pump, vacuum degasser, autosampler, column thermostat (set to 30°C), and diode array detector. Separation was achieved using a Luna C8 column ($4.6 \times 150 \text{ mm}$, $5 \mu\text{m}$; Phenomenex, Torrance, CA, USA) with a flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$ where line A was water and line B was methanol with the following program. The column was pre-equilibrated in 85% A/15% B, and upon injection this composition was held for 2 min. The composition of the mobile phase was then changed to 0% A/100% B over 20 min utilizing a linear gradient. This composition was held for 15 min followed by changing to 85% A/15% B over 2 min. The column was equilibrated in 85% A/15% B for 6 min prior to the next injection. Under these chromatographic conditions scytonemin, myxoxanthophyll, echinenone, chl *a*, and β -carotene eluted at 22, 23.5, 24, 26.5, and 30.2 min, respectively. The HPLC was operated with and data was viewed using ChemStation software (version B.04.03;

TABLE 2. Oligonucleotide primers used in this study.

Primer name	Primer sequence (5'-3')	Melting temperature ($^\circ\text{C}$)	Characteristics
1278.1	TACATGCTCGAGAACAGCCCTGCATAGACAAAA	57.6	5' <i>Xho</i> I
1278.2	AAGGTGCTGATGGAAAACCTTTGCCCGTAAAGATCTTGG	60.2	5' Complementary sequences for fusion PCR
1278.3	AGTTTCCCAGTCACGACGTTCCGCCAACATCAGGGTTACCT	59.9	5' Complementary sequences for fusion PCR
1278.4	GGAATAGGATCCGACTGGCAGTATGCTGA	60.0	5' <i>Bam</i> HI
1278.5	ATTCTTCTAAGGCAGAAAGCAGA	55.7	Genomic region upstream of Npun_F1278, outside of cloned region
1278.6	ATAGATTGTTAGCTGGGAACGC	55.3	Genomic region downstream of Npun_F1278, outside of cloned region
1278.7	CTGACAACGGTTTTGCTGGT	56.1	Genomic region upstream of Npun_F1278, within cloned region
1278.8	TTACGTAAGGGCAGGGTGGA	58.1	Genomic region downstream of Npun_F1278, within cloned region
1276F	CCAGTGGGGCCAGAAAGTATTGAT	59.5	Q-PCR of <i>soyA</i> (Npun_R1276)
1276R	CCTGTAACGGGGGTGACCCATT	59.9	Q-PCR of <i>soyA</i> (Npun_R1276)

Agilent, Santa Clara, CA, USA). Relative quantitation of compounds was accomplished through the analysis of three replicate samples. Data were processed with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

The water-soluble phycobilisome pigments of $\Delta 1278$ and wildtype cells were measured as previously described (Tandeu de Marsac and Houmard 1988, Soule et al. 2007) with the following modifications. Following exposure to white light with or without UVA for 1 week in shallow UVA-transparent glass Petri dishes, cells were pelleted for 10 min at 1,500 *g* and resuspended in a 10 mM Tris and 25 mM EDTA, pH 8.0 buffer. Lysozyme was then added to a final concentration of 10 mg · mL⁻¹ and cells were incubated for 30 min at room temperature. Cells were then sonicated with a sonic dismembrator using a 1/8" microtip (model 120; Fisher-Scientific, Pittsburgh, PA, USA) at 30% amplitude with 10 cycles of 10 s on/off pulsing. The extracts were allowed to precipitate in 1% streptomycin sulfate overnight at 4°C prior to spectroscopic measurements as described previously (Tandeu de Marsac and Houmard 1988, Soule et al. 2007).

Dry weights were determined gravimetrically for $\Delta 1278$ and wildtype batch cultures exposed to white light daily for five continuous days (Soule et al. 2007). Growth rates were then calculated from the exponential phase (Ingraham et al. 1983).

Microscopy. Following exposure of $\Delta 1278$ and wildtype cells to white light and white light supplemented with UVA for 1 week, cultures were viewed on an Olympus CX22LED brightfield microscope (Olympus Scientific Solutions, Waltham, MA, USA). Scytonemin as a yellow-brown pigment, cell size and morphology, and the presence of heterocysts and akinetes were examined.

Transcript abundance of *scyA*. Wildtype and $\Delta 1278$ batch cultures were filtered onto membrane filters (EMD Millipore, Billerica, MA, USA) placed on top of AA/4 + N liquid media in glass Petri dishes to minimize self-shading and maximize light exposure. Cells were exposed for 48 h to each condition, white light only or white light supplemented with UVA, to achieve maximal expression of the scytonemin biosynthetic genes as previously determined (Soule et al. 2009a). Cells were then harvested through centrifugation and the total RNA was extracted with LiCl precipitation as previously described (Schmidt-Goff and Federspiel 1993, Summers et al. 1995). The total RNA was treated with TURBO DNase (Life Technologies Corporation, Grand Island, NY, USA) and cDNA was synthesized using the Sensi-Fast cDNA Synthesis Kit (Bioline, Taunton, MA, USA) according to the manufacturer's instructions. This cDNA was then measured using quantitative-PCR on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each 20- μ L reaction was done in triplicate and contained 10 μ L 2 \times iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μ M of each forward and reverse primer (1276F/1276R; Integrated DNA Technologies, Coralville, IA, USA), and 1 μ L of cDNA with the following conditions: 95°C for 3 min and 39 cycles of 95°C for 10 s and 55°C for 30 s followed by a melt curve analysis (CFX Manager Software; Bio-Rad Laboratories). The 182 bp product targeted Npun_R1276 (*scyA*), the first gene in the scytonemin biosynthetic gene cluster (Fig. 1). The reference gene was DNA gyrase (Npun_F0025) (Sorrels et al. 2009, Janssen and Soule 2016), and all values obtained in parallel for the reference gene were used to normalize the gene expression data for *scyA*.

Statistical analysis. All quantitative analyses were conducted from cultures of each strain and treatment carried out in triplicate. All pigment data were normalized to

biomass and presented as the average along with the standard deviation of the triplicate samples. The pigment data were analyzed with a one-way ANOVA followed by post hoc analysis using Tukey's HSD to identify differences between strains and treatments using a *P*-value of 0.05 for significance. The specific growth rate and gene expression data were analyzed with a Student's *t*-test using a *P*-value of 0.05 for significance.

RESULTS

Phenotypic analysis of *Nostoc punctiforme* strain $\Delta 1278$. The highly conserved nature of the TCRS encoded by the Npun_F1277 and Npun_F1278 genes upstream of the scytonemin gene cluster indicates that it may be involved in the regulation of the scytonemin gene cluster. To determine whether Npun_F1278 regulates the production of scytonemin, a clean in frame deletion mutant was created that removed 552 bp of the Npun_F1278 open reading frame, generating strain $\Delta 1278$. Scytonemin synthesis was then assessed following UVA exposure of the *N. punctiforme* wildtype and $\Delta 1278$ mutant strains for 1 week. After UVA exposure, scytonemin was not detected spectroscopically in the $\Delta 1278$ mutant (Fig. 2). This was in contrast to the wildtype exposed to UVA, which displayed a strong absorbance peak at 384 nm indicative of scytonemin. Microscopic images of the wildtype compared to strain $\Delta 1278$ with and without exposure to UVA confirmed the presence of scytonemin as a yellow-brown pigment only in the wildtype subjected to UVA radiation (data not shown). The wildtype and $\Delta 1278$ mutant strains were both similar in colony morphology and in the size and shape of vegetative cells. Both heterocysts and akinetes produced by $\Delta 1278$ appeared to be similar to the wildtype, with

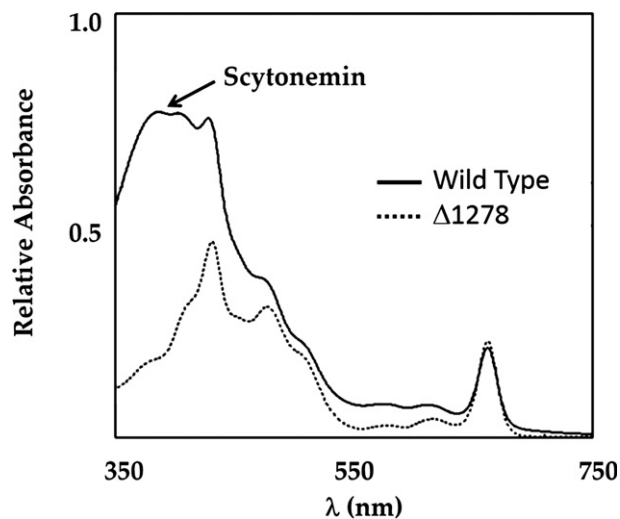


FIG. 2. Absorption spectra of lipid-soluble pigments extracted from the wildtype and the $\Delta 1278$ mutant strain after exposure to UVA for 1 week. The strong absorption at 384 nm is indicative of scytonemin in the wildtype.

no observable differences (data not shown). The ability to differentiate into hormogonia was not assessed.

The phenotype of the $\Delta 1278$ strain was further characterized to determine the extent of difference from the wildtype. This was accomplished by comparing the lipid-soluble pigment profiles, water-soluble phycobilin pigment concentrations, and growth rates of $\Delta 1278$ and the wildtype. The lipid-soluble pigments scytonemin, chl *a*, and the carotenoids myxoxanthophyll, echinone, and β -carotene were detected using HPLC. Comparative analysis of the pigments produced by each strain revealed that scytonemin was the only difference under either white light alone or white light supplemented with UVA (Fig. 3 and Fig. S3 in the Supporting Information). In particular, the mutant $\Delta 1278$ had no detectable level of scytonemin while the wildtype produced significantly more scytonemin under UVA rather than under white light alone ($n = 3$, $P < 0.05$).

The water-soluble phycobilin pigment analysis did show a difference in phycocyanin; the mutant strain $\Delta 1278$ produced about 2.5-fold more phycocyanin under UVA compared to white light or the wildtype in either condition (Table 3). There was no significant difference in phycoerythrin production for either strain or treatment and concentrations of allophycocyanin were below detectable limits in all samples and conditions tested ($n = 3$, $P < 0.05$).

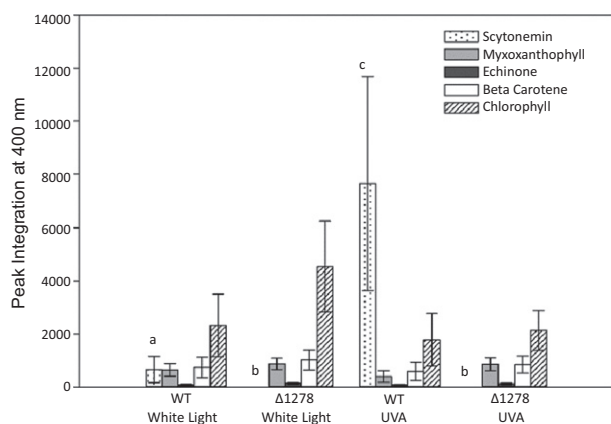


Fig. 3. Lipid-soluble pigments analyzed by HPLC and detected by absorbance at 400 nm. Error bars represent the standard deviation of triplicate samples with similar letters denoting values that are statistically similar ($P < 0.05$) for scytonemin regardless of the strain (note that significant differences were not observed for the other pigments). Samples were normalized by gravimetrically measured biomass.

There was a significant difference in the growth rate (*g*), expressed as doublings per day (Ingraham et al. 1983), between the wildtype and $\Delta 1278$. During logarithmic growth under white light, *g* was 0.335 ± 0.10 for the wildtype and 0.139 ± 0.08 for $\Delta 1278$ ($n = 3$, $P < 0.05$).

Transcript abundance of scyA. Expression of the first biosynthetic gene in the scytonemin gene cluster, *scyA*, was measured using quantitative-PCR. Following exposure to white light supplemented with UVA for 48 h, *scyA* from the wildtype was significantly up-regulated, ~40-fold, over the white light only controls (Table 4). This was in contrast to the mutant strain, $\Delta 1278$, in which *scyA* did not differ significantly when exposed to UVA as compared to the white light controls ($n = 3$, $P > 0.05$).

DISCUSSION

In this study, we sought to determine the relationship between the putative RR gene Npun_F1278 and scytonemin biosynthesis in the cyanobacterium *N. punctiforme*. The approach was to construct an in frame deletion of the Npun_F1278 gene to avoid disrupting the upstream cognate HK, Npun_F1277, and to ensure localization of the mutation to the Npun_F1278 gene. Using the mutant strain, *N. punctiforme* $\Delta 1278$, we have shown that the RR encoded by Npun_F1278 is indeed essential for scytonemin biosynthesis. This was demonstrated visually through microscopy, spectroscopically through pigment extraction and analysis, and molecularly through *scyA* transcript quantification.

It is worth noting that the basal level of scytonemin produced by the wildtype under white light was absent in strain $\Delta 1278$. While UVA is the dominant environmental signal to induce scytonemin biosynthesis, high light, ranging from 33 to 183 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, has been found to induce production of this pigment in other strains of cyanobacteria (Garcia-Pichel and Castenholz 1991). The complete lack of scytonemin production by the mutant strain under white light suggests that the absence of the Npun_F1278 gene in strain $\Delta 1278$ may not only affect the ability of the cell to respond to UVA but also impair any scytonemin production due to basal expression of Npun_F1278. This effect on lipid-soluble pigment production, however, appears to be limited to scytonemin since the amounts of chl *a* and the

Strain	Treatment	Phycoerythrin	Phycocyanin	Allophycocyanin
Wildtype	White light	36.05 ± 36.92	92.19 ± 24.24	ND
	UVA	24.11 ± 4.59	109.41 ± 5.60	ND
$\Delta 1278$	White light	74.21 ± 11.36	115.79 ± 11.45	ND
	UVA	82.15 ± 27.85	249.39 ± 69.03^a	ND

TABLE 3. Water-soluble phycobilin pigment profile averages and standard deviations as mg pigment per g cells.

^aStatistically different from the other treatments, $n = 3$, $P < 0.05$; ND = not detectable.

TABLE 4. Transcript abundance of *scyA* in the wildtype and Δ 1278 mutant strain following 48 h of UVA irradiation.

Strain	<i>scyA</i> fold change in transcript abundance (UVA vs. white light) ^a	$t_{(2),2}$	<i>P</i> -value ^b
Wildtype	40.36 ± 13.50	6.37	0.024
Δ 1278	1.58 ± 0.58	1.27	0.333

^aAverage and standard deviation of three replicates.

^b $P < 0.05$.

carotenoids myxoxanthophyll, echinone, and β -carotene were not significantly different between Δ 1278 and the wildtype under white light or white light supplemented with UVA. Phycocyanin levels, however, were affected in the mutant strain, but only when exposed to UVA, otherwise the mutant produced similar levels as the wildtype. The change in phycocyanin, which absorbs maximally ~615–640 nm (Sidler 1994), as well as the absence of a basal level of scytonemin during growth in white light, may suggest that the Npun_F1278 protein responds to light quality beyond UVA, perhaps even into the visible range. Another possibility, as a more indirect effect, would be that multiple RRs are involved in the regulation of phycocyanin and that the deletion of the Npun_F1278 gene interferes with this network. It may also be that phycocyanin production was over-expressed in strain Δ 1278 to compensate for the lack of scytonemin in the presence of UVA, which may have made the cells vulnerable to phycobilisome bleaching (Rastogi et al. 2015).

It is interesting that there are differences between the two strains and treatments with respect to phycocyanin, while the lipid-soluble pigments and phycoerythrin did not appear to be affected (see Table 3 and Fig. 3). Perhaps the differences in the response of the pigments measured in this study are not due to how the light is sensed, but rather due to the stability of the pigments. The protein-nature of phycobilin proteins may make them more susceptible to degradation by UVR stress. Indeed, several studies have found that phycobilin proteins are typically degraded under combined UVA and UVB radiation (Sinha et al. 1995, Six et al. 2007). Recently, the distinct effects of UVA and UVB on phycobilisomes were evaluated in a *Lyngbya* strain, where UVA alone was shown to degrade phycobiliproteins, although not as dramatically as UVB (Rastogi et al. 2015). Furthermore, both UVA and UVB have been shown to reduce the expression of genes involved in phycobilisome biosynthesis (i.e., linker proteins) while increasing the expression of genes involved in phycobilisome degradation in cyanobacteria (Huang et al. 2002, Soule et al. 2013). Interestingly, deletion of the Npun_F1278 gene resulted in an increase in phycocyanin production, but only after exposure to UVA. Perhaps the phycocyanin

degradation proteins are down-regulated or the phycocyanin rod-core linker proteins are up-regulated in this mutant, which is the opposite of what was observed in a microarray study of *N. punctiforme* under UVA stress (Soule et al. 2013), although further study will be necessary to better understand this response. Another point worth noting is that the pigments in this study were assessed after 1 week of exposure since it takes days for scytonemin to accumulate. Unfortunately, the duration of this experiment makes it challenging to find a suitable comparative study since most of the aforementioned studies were done within 24 h. In future studies, the specific effect of UVA and the Npun_F1278 protein on phycobilisome proteins will be evaluated. Perhaps the relationship between Npun_F1278 and allophycocyanin will also be revealed with an alternative approach, since concentrations were below the detection limit of the method used in this study.

Interestingly, the scytonemin biosynthetic mutant of *N. punctiforme*, SCY59, which harbors a transposon insertion in the third gene of the cluster, Npun_R1274 (*scyC*), did not exhibit any appreciable differences in the production of any of the pigments measured in this work, regardless of whether or not the cells were stressed with UVA (Soule et al. 2007). One explanation for the difference in phenotype between the *scyC* biosynthetic mutant and Δ 1278 regulatory mutant could be that the Npun_F1278 protein regulates more than just scytonemin biosynthesis. Indeed, the Npun_F1278 gene responds to UVB and high light, as indicated by increased transcript levels within 20 min postexposure (Janssen and Soule 2016). Both of these are conditions that have not been shown to stimulate scytonemin production in *N. punctiforme*, suggesting that the Npun_F1278 protein may act on other cellular processes, such as phycocyanin production.

In addition to pigment production, the Npun_F1278 protein may be involved in regulating other cellular processes as evidenced by the decreased growth rate of the Δ 1278 mutant strain under white light. Interestingly, this is in contrast to the biosynthetic mutant SCY59, which did not differ in *g* as compared to the wildtype during growth under white light or UVA (Soule et al. 2007). The SCY59 growth curve, however, was done using chl *a* levels as a measure of biomass as opposed to the dry weight analysis done in this study. Perhaps the difference in approach explains why the biosynthetic mutant grew comparably well as opposed to the regulatory mutant of this study. It is also possible that the result of the mutation in *scyC*, a biosynthesis gene, in SCY59 was limited to a deficiency in scytonemin biosynthesis while Npun_F1278 is a regulatory gene. The impaired growth of the Δ 1278 mutant may also be the result of decreased phycobilin production or stability, which could suggest a

broader dependency on Npun_F1278 to maintain homeostasis during light stress.

The level of UVA used in these experiments is not lethal to *N. punctiforme*, even without the protection afforded by scytonemin. As with the biosynthetic mutant SCY59, other photo-protective mechanisms likely continue to function in the absence of scytonemin. In a laboratory environment, where growth is favored and nutrients are plentiful to support central metabolism, *N. punctiforme* is able to invest the energy necessary to synthesize efficient UV shock proteins (Shibata et al. 1991, Shirkey et al. 2000), support DNA repair systems (Van Baalen 1968, Levine and Thiel 1987), and synthesize antioxidant enzymes and carotenoids (Canfield et al. 1992) in the presence of UVA stress. In the natural environment or in a desiccated state, however, mutant strains Δ 1278 and SCY59 may not be able to survive as well as the wildtype since the nutrient and metabolic support would be more limited and/or pulsed. Thus, this is likely the reason scytonemin is only found in cyanobacteria from desiccated or nutrient-limited environments, as opposed to more nutrient-rich environments (Garcia-Pichel and Castenholz 1991). With an impaired regulatory gene, it is unclear as to what extent the UVA/light-associated stress response is affected in strain Δ 1278, and future studies will continue to evaluate how Δ 1278 responds to a variety of light-associated conditions at both the physiological and molecular levels.

We wish to thank Dr. Jaiyanth Daniel for technical assistance and Dr. Jordan Marshall for support with the statistical analyses. This project was partially supported by the Office of Research, Engagement, and Sponsored Projects at Indiana University-Purdue University Fort Wayne and by the Indiana Academy of Sciences Senior Research Grant. The authors declare that they have no conflicts of interest associated with this research.

- Allen, M. & Arnon, D. I. 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen-fixation by *Anabaena cylindrica*. *Plant Physiol.* 30:366–72.
- Ashby, M. & Houmard, J. 2006. Cyanobacterial two-component proteins: structure, diversity, distribution, and evolution. *Microbiol. Mol. Biol. Rev.* 70:472–509.
- Balskus, E. P. & Walsh, C. T. 2008. Investigating the initial steps in the biosynthesis of cyanobacterial sunscreen scytonemin. *J. Am. Chem. Soc.* 130:15260–1.
- Balskus, E. P. & Walsh, C. T. 2009. An enzymatic cyclopentyl[b]indole formation involved in scytonemin biosynthesis. *J. Am. Chem. Soc.* 131:14648–9.
- Bebout, B. M. & Garcia-Pichel, F. 1995. UV-B-induced vertical migrations of cyanobacteria in a microbial mat. *Appl. Environ. Microbiol.* 61:4215–22.
- Black, T. A., Cai, Y. & Wolk, C. P. 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol. Microbiol.* 9:77–84.
- Campbell, E., Hagen, K., Chen, R., Risser, D., Ferreira, D. & Meeks, J. 2015. Genetic analysis reveals the identity of the photoreceptor for phototaxis in hormogonium filaments of *Nostoc punctiforme*. *J. Bacteriol.* 197:782–91.
- Canfield, L. M., Forage, J. W. & Valenzuela, J. G. 1992. Carotenoids as cellular antioxidants. *Proc. Soc. Exp. Biol. Med.* 200:260–5.
- Castenholz, R. W. & Garcia-Pichel, F. 2012. Cyanobacterial responses to UV radiation. In Whitton, B. A. [Ed.] *Ecology of Cyanobacteria II*. Springer, Dordrecht, pp. 481–502.
- Cohen, M. F., Meeks, J. C., Cai, Y. A. & Wolk, C. P. 1998. Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. *Methods Enzymol.* 297:3–17.
- Elhai, J. & Wolk, C. P. 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. *Gene* 68:119–38.
- Frohman, M. A. 1994. Cloning PCR products. In Mullis, K. B., Ferré, F., & Gibbs, R. A. [Eds.] *The Polymerase Chain Reaction*. Birkhäuser, Boston, MA, pp. 14–37.
- Gao, Q. & Garcia-Pichel, F. 2011. Microbial ultraviolet sunscreens. *Nat. Rev. Microbiol.* 9:1–12.
- Garcia-Pichel, F. & Castenholz, R. W. 1991. Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. *J. Phycol.* 27:395–409.
- Garcia-Pichel, F. & Castenholz, R. W. 1993. Occurrence of UV-absorbing, mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity. *Appl. Environ. Microbiol.* 59:163–9.
- Garcia-Pichel, F., Sherry, N. D. & Castenholz, R. W. 1992. Evidence for an ultraviolet sunscreen role of the extracellular pigment scytonemin in the terrestrial cyanobacterium *Chlorogloeopsis* sp. *Photochem. Photobiol.* 56:17–23.
- Huang, L., McCluskey, M. P., Ni, H. & LaRossa, R. A. 2002. Global gene expression profiles of the cyanobacterium *Synechocystis* sp. strain PCC 6803 in response to irradiation with UV-B and white light. *J. Bacteriol.* 184:6845–58.
- Hudek, L., Bräu, L., Michalczyk, A., Neilan, B., Meeks, J. & Ackland, M. 2015. The ZntA-like NpunR4017 plays a key role in maintaining homeostatic levels of zinc in *Nostoc punctiforme*. *Appl. Microbiol. Biotechnol.* 99:10559–74.
- Ingraham, J. L., Maaloe, O. & Neidhardt, F. C. 1983. *Growth of the Bacterial Cell*. Sinauer Associates, Sunderland, Massachusetts.
- Janssen, J. & Soule, T. 2016. Gene expression of a two-component regulatory system associated with sunscreen biosynthesis in the cyanobacterium *Nostoc punctiforme* ATCC 29133. *FEMS Microbiol. Lett.* 363:fnv235.
- Levine, E. & Thiel, T. 1987. UV-inducible DNA repair in the cyanobacterium *Anabaena* spp. *J. Bacteriol.* 169:3988–93.
- Liang, J., Scappino, L. & Haselkorn, R. 1993. The *patB* gene product, required for growth of the cyanobacterium *Anabaena* sp. strain PCC 7120 under nitrogen-limiting conditions, contains ferredoxin and helix-turn-helix domains. *J. Bacteriol.* 175:1697–704.
- Martin, R. G. & Rosner, J. L. 2001. The AraC transcriptional activators. *Curr. Opin. Microbiol.* 4:132–7.
- Proteau, P. J., Gerwick, W. H., Garcia-Pichel, F. & Castenholz, R. W. 1993. The structure of scytonemin, an ultraviolet sunscreen pigment from the sheaths of cyanobacteria. *Experientia* 49:825–9.
- Rastogi, R. P., Sonani, R. R. & Madamwar, D. 2015. Effects of PAR and UV radiation on the structural and functional integrity of phycocyanin, phycoerythrin and allophycocyanin isolated from the marine cyanobacterium *Lyngbya* sp. A09DM. *Photochem. Photobiol.* 91:837–44.
- Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. 1979. Generic assignments, strain histories, and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111:1–61.
- Risser, D. D., Chew, W. G. & Meeks, J. C. 2014. Genetic characterization of the hmp locus, a chemotaxis-like gene cluster that regulates hormogonium development and motility in *Nostoc punctiforme*. *Mol. Microbiol.* 92:222–33.
- Risser, D. D. & Meeks, J. C. 2013. Comparative transcriptomics with a motility-deficient mutant leads to identification of a

- novel polysaccharide secretion system in *Nostoc punctiforme*. *Mol. Microbiol.* 87:884–93.
- Schmidt-Goff, C. M. & Federspiel, N. A. 1993. In vivo and in vitro footprinting of a light-regulated promoter in the cyanobacterium *Fremyella diplosiphon*. *J. Bacteriol.* 175:1806–13.
- Shibata, H., Baba, K. & Ochiai, H. 1991. Near-UV induces shock proteins in *Anacystis nidulans* R-2, possible role of active oxygen. *Plant Cell Physiol.* 32:771–6.
- Shirkey, B., Kovarcik, D. P., Wright, D. J., Wilmoth, G., Prickett, T. F., Helm, R. F., Gregory, E. M. & Potts, M. 2000. Active Fe-containing superoxide dismutase and abundant *sodF* mRNA in *Nostoc commune* (cyanobacteria) after years of desiccation. *J. Bacteriol.* 182:189–97.
- Sidler, W. A. 1994. Phycobilisome and phycobiliprotein structure. In Bryant, D. A. [Ed.] *The Molecular Biology of Cyanobacteria*. Kluwer Academic Publishers, Dordrecht, pp. 139–216.
- Sinha, R., Lebert, M., Kumar, A., Kumar, H. & Häder, D. P. 1995. Spectroscopic and biochemical analyses of UV effects on phycobiliproteins of *Anabaena* sp. and *Nostoc carmium*. *Botanica Acta* 108:87–92.
- Six, C., Joubin, L., Partensky, F., Holtzendorff, J. & Garczarek, L. 2007. UV-induced phycobilisome dismantling in the marine picocyanobacterium *Synechococcus* sp. WH8102. *Photosyn. Res.* 92:75–86.
- Sorrels, C. M., Proteau, P. J. & Gerwick, W. H. 2009. Organization, evolution, and expression analysis of the biosynthetic gene cluster for scytonemin, a cyanobacterial UV-absorbing pigment. *Appl. Environ. Microbiol.* 75:4861–9.
- Soule, T., Gao, Q., Stout, V. & Garcia-Pichel, F. 2013. The global response of *Nostoc punctiforme* ATCC 29133 to UVA stress, assessed in a temporal DNA microarray study. *Photochem. Photobiol.* 89:415.
- Soule, T., Garcia-Pichel, F. & Stout, V. 2009a. Gene expression patterns associated with the biosynthesis of the sunscreen scytonemin in *Nostoc punctiforme* ATCC 29133 in response to UVA radiation. *J. Bacteriol.* 191:4639–46.
- Soule, T., Palmer, K., Gao, Q., Potrafka, R., Stout, V. & Garcia-Pichel, F. 2009b. A comparative genomics approach to understanding the biosynthesis of the sunscreen scytonemin in cyanobacteria. *BMC Gen.* 10:336–46.
- Soule, T., Stout, V., Swingley, W. D., Meeks, J. C. & Garcia-Pichel, F. 2007. Molecular genetics and genomic analysis of scytonemin biosynthesis in *Nostoc punctiforme* ATCC 29133. *J. Bacteriol.* 189:4465–72.
- Summers, M. L., Wallis, J. G., Campbell, E. L. & Meeks, J. C. 1995. Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in nitrogen fixation and dark growth of the cyanobacterium *Nostoc* sp. strain ATCC 29133. *J. Bacteriol.* 177:6184–94.
- Tandeu de Marsac, N. & Houmard, J. 1988. Complementary chromatic adaptation: physiological conditions and action spectra. *Methods Enzymol.* 167:318–28.
- Van Baalen, C. 1968. The effects of ultraviolet radiation on a coccoid blue-green alga: survival, photosynthesis, and photoreactivation. *Plant Physiol.* 43:1689–95.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Deletion of the Npun_F1278 gene using fusion PCR. The blue line represents the Npun_F1278 gene and black lines represent the adjacent flanking genomic sequence in the wildtype. The 5' primer overhang modifications on the primers are labeled with either the restriction enzyme site (green) or with the complementary region on the 1278.2 and 1278.3 primers that anneal to form the fused product (red). Shown are the wildtype gene and flanking region (a), priming sites on the wildtype chromosomal template (b), products from the initial PCR (c), and the fusion product of Npun_F1278 with the central region replaced by the fused complementary sequences engineered onto primers 1278.2 and 1278.3 (d).

Figure S2. Priming sites on the *Nostoc punctiforme* genome used to confirm the Δ 1278 genotype prior to sequencing. The Npun_F1278 gene with the deleted region of 552 bp is represented by the large blue arrow and flanked by the genome shown as dotted lines. The expected product amplified by each primer set is represented by the colored arrows, with the size for the wildtype (WT) and Δ 1278 mutant strains provided. Vertical alignment of the image is set to show the priming positions relative to the Npun_F1278 gene and the flanking genomic regions.

Figure S3. HPLC chromatograms for the wildtype (red) and mutant Δ 1278 strain (blue) lipid-soluble pigment extracts under (a) white light and (b) white light supplemented with UVA irradiation detected by absorbance at 400 nm. Pigments are labeled as follows: scy, scytonemin; myx, myxoxanthophyll; ech, echinenone; chl, chlorophyll *a*; and β -c, β -carotene.