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Molecular Genetics and Genomic Analysis of Scytonemin Biosynthesis in *Nostoc punctiforme* ATCC 29133▽

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The indole-alkaloid scytonemin is the most common and widespread sunscreen among cyanobacteria. Previous research has focused on its nature, distribution, ecology, physiology, and biochemistry, but its molecular genetics have not been explored. In this study, a scytonemin-deficient mutant of the cyanobacterium *Nostoc punctiforme* ATCC 29133 was obtained by random transposon insertion into open reading frame NpR1273. The absence of scytonemin under conditions of induction by UV irradiation was the single phenotypic difference detected in a comparative analysis of the wild type and the mutant. A cause-effect relationship between the phenotype and the mutation in NpR1273 was demonstrated by constructing a second scytonemin-less mutant through directed mutagenesis of that gene. The genomic region flanking the mutation revealed an 18-gene cluster (NpR1276 to NpR1259). Four putative genes in the cluster, NpR1274 to NpR1271, with no previously known functions, are likely to be involved in the assembly of scytonemin. Also in this cluster, there is a redundant set of genes coding for shikimic acid and aromatic amino acid biosynthesis enzymes, leading to the production of tryptophan and tyrosine, which are likely to be biosynthetic precursors of the sunscreen.

In order to get access to the solar radiation required for photosynthesis, cyanobacteria must live in environments exposed to some level of solar UV radiation (wavelengths shorter than 400 nm) (11), which is associated with biologically deleterious effects. UV-A irradiation (wavelengths of 315 to 400 nm) causes long-term damage through photosensitized oxidations, whereas UV-B irradiation (wavelengths of 280 to 315 nm) acts directly on DNA by forming pyrimidine dimers, cross-links, and double-strand breaks while altering disulfide bonds and aromatic amino acids in proteins (25). Despite the hazardous effects of solar UV radiation, cyanobacteria continue to thrive in habitats exposed to high doses, such as soil and rock surfaces (16), and have various known UV radiation defense mechanisms (10, 11). They possess specific UV radiation-sensing photoreceptors (35), are capable of efficient DNA repair processes such as excision repair and photoreactivation (29, 46), and express specific UV radiation shock proteins (41), including superoxide dismutase (42). Some of the motile, gliding cyanobacteria display behavioral UV radiation avoidance (4, 11), while others are able to synthesize and accumulate UV radiation-blocking sunscreens (14, 18, 19). Sunscreens serve as passive preventative mechanisms that allow the organism to intercept photons before they interact with cellular machinery or DNA or produce reactive oxygen species (11, 17). Because of this preventative potential, sunscreens seem to be particularly common among cyanobacteria that live under pulsed-growth regimes. Their physiological effect is most valuable in avoiding damage under conditions of quiescence, dormancy, or otherwise-restricted metabolism (17, 19).

The indole-alkaloid scytonemin is one such sunscreen, found exclusively among cyanobacteria. It is deposited as a yellow to brown, lipid-soluble pigment in the extracellular sheaths or other exopolysaccharide structures (17). Chemically, it consists of a dimeric carbon skeleton composed of fused heterocyclic units (see Fig. 1) (36). The conjugated double-bond distribution allows for the strong absorption of radiation in the UV-A range, with a maximum of approximately 384 nm, and thus the molecule effectively absorbs ambient UV-A irradiation before it can reach the cell (17). In model strains of cyanobacteria, UV-A irradiation has been shown to induce the synthesis of scytonemin, while UV-B, red, or green light does not (19). Scytonemin acts in a passive manner and has been shown to protect photosynthetic machinery from UV-A irradiation damage particularly when metabolic activity is not possible (19). In addition to its UV-A irradiation-blocking ability, scytonemin has potential for biomedical applications because of its strong antiproliferative and anti-inflammatory activities (43, 44). Although the biosynthetic pathway of scytonemin is not known, its chemical structure suggests that aromatic amino acids are precursors; this idea has found support in results from feedback inhibition and radiotracer experiments involving tryptophan and tyrosine (30).

The molecular genetics of sunscreen biosynthesis and regulation in cyanobacteria have not been explored. This lack of exploration is, in part, due to the lack of a suitable model for analysis; most cyanobacteria amenable to genetic manipulation do not produce sunscreens. Upon the completion of a large survey of cyanobacterial strains in pure culture (R. Apple, A. Mandalka, and F. Garcia-Pichel, unpublished), only one organism, the cyanobacterium *Nostoc punctiforme* ATCC 29133 (PCC 73102), was found to contain the necessary traits of producing sunscreens and being genetically tractable. *N. punctiforme* can be genetically modified using conjugation or electroporation and can be subjected to molecular mutagenesis

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
E. coli		
DH5α	*recA1 hsdR17*(r_K^- m_K^-) *endA1*	20
DH5α-MCR	DH5α *mcrA* Δ(*mrr hsdMRS mcrBC*)	20
HB101	*recA13 hsdS20*(r_B^- m_B^-)	7
N. punctiforme		
ATCC 29133 (PCC 73102)	Wild type (scytonemin producer)	38
SCY 59	*Nostoc* strain ATCC 29133 NpR1273::Tn*5*-1063a Scy$^-$	This study
SCY S7301	*Nostoc* strain ATCC 29133 carrying pTD59c integrated by a single recombination	This study
SCY M7301	Carries reconstruction of the SCY 59 mutation; Scy$^-$	This study
Anabaena strain PCC 7120	Scy$^-$	38
Plasmids		
pRK2013	RK2 derivative with an oriV; Km^r	15
pRL1063a	Tn*5*-1063 RK2 oriT Km^r Nm^r Sm^r	47
pRL1075	Used for reconstructing Tn*5*-1063a insertions; Cm^r Suc^s	6
pTD59a	Recovered 10-kb EcoRI fragment with NpR1273::Tn*5*-1063a from SCY 59; Km^r Nm^r Sm^r	This study
pTD59b	pTD59a with NotI site filled in; NpR1273::Tn*5*-1063a Km^r Nm^r Sm^r	This study
pTD59c	pTD59b with positive selection fragment from pRL1075; Cm^r Km^r Nm^r Sm^r Suc^s	This study

[a] Km^r, kanamycin resistant; Nm^r, neomycin resistant; Sm^r, streptomycin resistant; Cm^r, chloramphenicol resistant; Suc^s, sucrose sensitive.

involving random transposon insertions or targeted gene replacement (12). In addition, its genome is fully sequenced, and the sequence is available in the U.S. Department of Energy's Joint Genome Institute database (http://www.jgi.doe.gov). *N. punctiforme*'s genome is approximately 9.05 Mb (41.4% GC) and contains approximately 7,364 putative open reading frames (ORFs) (32).

N. punctiforme provides a unique model for the study of the molecular basis of cyanobacterial sunscreens. We report here the first of such studies based on the characterization and genomic analyses of a scytoneminless mutant.

MATERIALS AND METHODS

Strains, cultivation, and media. Inocula for all experiments were obtained from stocks of *N. punctiforme* ATCC 29133 (PCC 73102) that were kept on agar plates under axenic conditions at 25°C. The base growth medium was AA of Allen and Arnon (1). For liquid batch cultures, a fourfold-diluted formulation was used (AA/4), while full strength was used for plates solidified with 1.5% (wt/vol) Noble agar (Difco). The medium was supplemented as needed with 5 mM MOPS (morpholinepropanesulfonic acid) and 2.5 mM NH_4Cl at pH 7.8 (AA/4+N). Visible light was provided by cool-white fluorescent tubes at an intensity of 7 W m^{-2}; UV-A irradiation was provided by 20-W black-light fluorescent tubes (General Electric) at an approximate intensity of 10 W m^{-2}. The spectral output of these UV-A lamps has a maximum at 365 nm and has been previously described (19). *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth at 37°C (40), either in liquid or on plates solidified with 1.5% (wt/vol) Bacto agar (Difco). The growth of batch *E. coli* cultures was monitored by the optical density at 600 nm (OD_{600}). Media were supplemented with antibiotics, as needed, with final concentrations of 25 μg ml^{-1} each for kanamycin and neomycin, 1 μg ml^{-1} for streptomycin, and 60 μg ml^{-1} for chloramphenicol (47).

Transposon mutagenesis and screening of mutants. Strains and plasmids used in this study are listed in Table 1. Putative mutants incapable of synthesizing scytonemin were sought using transposon mutagenesis in a triparental conjugation system (12). Briefly, this system was composed of the cyanobacterial recipient strain, an *E. coli* HB101 (7) donor conjugal strain carrying the broad-host-range conjugal plasmid pRK2013 (15), and an *E. coli* DH5α (20) donor cargo strain carrying the transposon Tn*5*-1063a on plasmid pRL1063a (47). To prepare *N. punctiforme* for conjugation, filaments were sonicated using a microtip sonicator to produce short fragments of three to seven cells and allowed to recover on an orbital shaker at 25°C under white light in AA/4+N before mating. *E. coli* strains were grown to an OD_{600} of 0.6 in LB broth supplemented with kanamycin for the strain carrying pRK2013 and with both neomycin and streptomycin for the strain carrying pRL1063a. The cells were combined and washed twice in plain LB broth before mating. For conjugation, 0.5 ml of the *E. coli* mixture at an OD_{600} of 9 to 10 and 0.5 ml of *N. punctiforme* at a concentration corresponding to 75 μg of chlorophyll *a* ml^{-1} were combined and spread onto detergent-free membrane filters (Millipore HATF082), which were then placed onto AA/4+N plates supplemented with 0.5% (vol/vol) LB broth. The plates were incubated overnight under low light (2 to 3 W m^{-2}), after which the filters were transferred to AA/4+N plates and incubated for two additional days under low light to allow for gene expression. The filters were subsequently transferred to AA/4+N plates supplemented with streptomycin and neomycin to allow for the growth of only mutant colonies with resistance to streptomycin and neomycin as afforded by the transposon. Mutant colonies began to appear after approximately 10 days.

To identify colonies deficient in scytonemin production, mutant colonies were exposed to white light supplemented with UV-A irradiation (approximately 10 W m^{-2} each) to elicit the biosynthesis of the sunscreen (17). After 2 weeks, approximately 400 mutant colonies were examined microscopically for changes in extracellular pigmentation. For this examination, a piece of each colony was retrieved using watchmaker's forceps under a dissecting microscope, and a wet mount was prepared for bright-field microscopy. Putative scytonemin-deficient mutants were then examined spectroscopically to confirm the phenotype after extraction in acetone (17). One putative mutant, SCY 59, lacked the peak at 384 nm characteristic of scytonemin; this mutant was the subject of investigation.

Determination of the site of transposon insertion. In order to obtain information on the specific site of transposon insertion in the genome of SCY 59, total genomic DNA was extracted using a commercial kit (Ultra Clean plant DNA isolation kit; MoBio Laboratories Inc.) and digested with EcoRI; fragments were then ligated using T4 DNA ligase (5). The resulting plasmids, some of which contained the transposon (Tn*5*-1063a) and the genomic regions flanking the insertion, were used to chemically transform *E. coli* DH5α-MCR (20), and transformants were selected on the basis of streptomycin and neomycin resistance as afforded by the transposon. Three transformant colonies that contained plasmids with the transposon and SCY 59 genomic flanking regions were independently obtained. The plasmids were isolated using a commercial kit (standard mini plasmid prep kit; MoBio Laboratories Inc.) and sequenced using a primer (5′ TACTAGATTCAATGCTATCAATGAG 3′) complementary to the end of the transposon sequence. Only one plasmid (pTD59a) was chosen for use in further experiments. Unknown portions of pTD59a were then sequenced through the use of primer walking. All enzymes used in this study were from Fermentas Life Sciences, and all DNA products were confirmed by electrophoresis using 1% agarose gels.

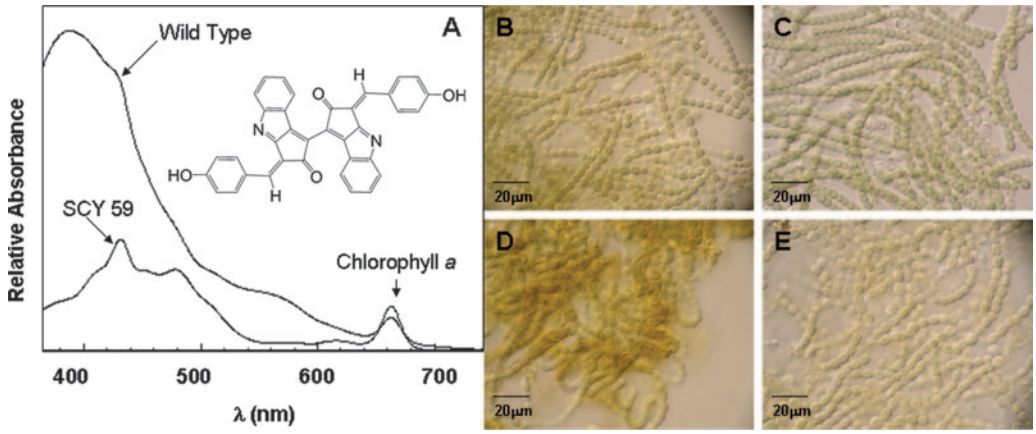

FIG. 1. (A) Chemical structure of scytonemin and the absorption spectra of the *N. punctiforme* wild-type and mutant SCY 59 lipid-soluble pigment extracts after incubation under white light supplemented with UV-A irradiation. The large characteristic peak at 384 nm for the wild type indicates the presence of scytonemin under UV-A irradiation. (B to E) The wild type (B and D) and mutant SCY 59 (C and E) under white light (B and C) and white light supplemented with UV-A irradiation (D and E). Extracellular scytonemin is clearly visible as dark areas only in the image of the wild type exposed to UV-A irradiation (D).

Verification of single-site transposition and reconstruction of the mutation. Southern analysis was performed to verify that a single transposition event had occurred in the genome of SCY 59. Southern blotting (40) was performed according to the directions of the manufacturer of the Gene Images AlkPhos direct labeling and detection system (Amersham Biosciences). Briefly, a total of 0.5 to 1.0 μg of whole genomic DNA was isolated as described above and digested with EcoRI. DNA was transferred onto a Zeta-Probe GT membrane (Bio-Rad) by using 0.4 M NaCl. The probe was designed to hybridize to a 373-bp region of the transposon and was labeled according to the manufacturer's instructions. The chemifluorescent signal generated was detected using Quantity One one-dimensional analysis software on a Fluor-S MultiImager (Bio-Rad).

To confirm the phenotype of SCY 59, the mutation was reconstructed through the direct insertion of a transposon into the wild-type strain at the same locus of insertion found in SCY 59. In preparation for this reconstruction, the transposase gene on pTD59a was inactivated through a frameshift mutation introduced by filling in a unique NotI site to yield plasmid pTD59b. To create a positive-selection vector, pTD59b was digested with EcoRV and subsequently ligated to the *sacB*-containing EcoRV fragment of pRL1075 (6) to obtain pTD59c. This final plasmid conferred sensitivity to 5% (wt/vol) sucrose and resistance to chloramphenicol, streptomycin, and neomycin (12). To reconstruct the mutation, pTD59c was inserted into wild-type *N. punctiforme* cells by the triparental conjugation method described above, except that pTD59c served as the cargo plasmid instead of pRL1063a. A single-recombinant strain, SCY S7301, was selected by its resistance to chloramphenicol and sensitivity to sucrose, while the double-recombinant strain, SCY M7301, was selected by its sensitivity to chloramphenicol and resistance to sucrose due to the loss of the EcoRV fragment of pRL1075 and its resistance to streptomycin and neomycin due to the insertion of the transposon (9). SCY M7301 was then assayed for scytonemin production upon UV-A irradiation by using the lipid-soluble-pigment extraction procedure described above (17).

Comparative mutant and wild-type characterizations. Growth rates of wild-type *N. punctiforme* and SCY 59 in batch cultures were determined using AA/4 liquid medium placed under white light and white light supplemented with UV-A irradiation with shaking at 25°C. The increase in biomass was monitored by measuring the chlorophyll *a* concentrations in 2-ml aliquots, from which chlorophyll *a* was extracted with 90% methanol (31), taken daily in triplicate through exponential phase and into stationary phase of growth.

For the evaluation of the lipid-soluble pigment complement, pigments were extracted from whole cells in triplicate with acetone by using previously published protocols (17) and assayed using high-pressure liquid chromatography (HPLC). Prefiltered, 50-μl concentrated extracts were used on a Waters Spherisorb S10 ODS2 (10-by-250-mm) semiprep analytical column filled with reverse-phase silica gel and run on a variable solvent gradient at a pressure of approximately 38×10^5 Pa. The gradient varied from 15% water in methanol to pure methanol in 6 min, then to pure acetone in 4 min, at which point the pure acetone was sustained for 5 min, and then back to 100% methanol for a total of 35 min at a flow rate of 1.8 ml min^{-1}. Carotenoids, chlorophyll *a*, and scytonemin were detected continuously using an online photodiode array detector in the 384-to-665-nm range. Pigments were identified by absorption spectra and quantified using previously published extinction coefficients for chlorophyll *a*, β-carotene, and myxoxanthophyll (27), echinenone (26), and scytonemin (19) with a chlorophyll *a* standard.

For the phycobilin complement, cultures were taken in triplicate and resuspended in a 20 mM sodium acetate buffer at pH 5.5. Cells were broken open using a French press at 1,000 lb/in^2, and the crude cell extracts were precipitated with 1% (wt/vol) streptomycin sulfate at 4°C to eliminate membrane fragments containing chlorophyll (45). The supernatant extracts were read from 350 to 750 nm on a spectrophotometer, and the absorbance values corresponding to the phycobiliproteins phycoerythrin (565 nm), phycocyanin (620 nm), and allophycocyanin (650 nm) were measured. Specific contents were established using a series of trichromatic equations (8, 45) and subsequent normalization of the absorbance values to biomasses (dry weights) determined gravimetrically.

For microscopic examination, all cultures were grown in AA/4 liquid medium as described above. All photographs were of wet-mount preparations on agar-covered slides, taken on a Zeiss AxioImager 8.1 Nomarski microscope using an Olympus C-7070 wide-zoom digital camera.

RESULTS

Comparative genetic and phenotypic characterization of *N. punctiforme* ATCC 29133 and SCY 59. We obtained approximately 400 mutant colonies after transposon mutagenesis. A microscopic observation of these colonies after the exposure of the plates to UV-A irradiation, the typical environmental cue that elicits the synthesis of scytonemin in cyanobacteria (17), revealed a single putative scytoneminless mutant, SCY 59, which had colorless sheaths (Fig. 1). The phenotype was then confirmed with spectroscopic analyses of lipid-soluble extracts from cells grown under UV-A light. SCY 59 was consistently unable to produce scytonemin, even under higher intensities of UV-A irradiation or longer exposure times than those sufficient for the induction of synthesis in the wild type.

The transposon used, Tn*5*-1063a, contains a plasmid origin of replication and antibiotic resistance markers. These properties allowed us to obtain plasmid clones of the transposon and regions flanking the insertion site from SCY 59 genomic DNA through the excision of these regions by restriction endonuclease cleavage (Materials and Methods). Three independent clones were thus obtained and sequenced using a primer

TABLE 2. Profiles of lipid-soluble and water-soluble pigments in the wild type and mutant SCY 59

Pigment	Amt[a] in strain under:			
	White light		White light and UV-A irradiation	
	Wild type	SCY 59	Wild type	SCY 59
Lipid-soluble pigments				
Scytonemin	ND[b]	ND	2.62 ± 2.18	ND
Chlorophyll *a*	1.12 ± 0.38	1.98 ± 0.46	1.08 ± 0.43	0.74 ± 0.25
Myxoxanthophyll[c]	0.28 ± 0.17	0.23 ± 0.12	ND	0.17 ± 0.08
Echinenone	0.03 ± 0.02	0.02 ± 0.01	0.05 ± 0.02	0.03 ± 0.01
β-Carotene	0.04 ± 0.02	0.05 ± 0.02	0.02 ± 0.00	0.03 ± 0.01
Phycobiliproteins				
Phycoerythrin	18.57 ± 4.48	15.43 ± 9.05	13.93 ± 5.08	9.51 ± 1.93
Phycocyanin	36.61 ± 14.08	31.63 ± 10.37	32.47 ± 13.14	27.89 ± 6.77
Allophycocyanin	19.51 ± 4.69	15.20 ± 8.53	16.50 ± 6.75	12.66 ± 3.05

[a] Pigment values are given as milligrams per gram (dry weight) and are means ± standard deviations.
[b] ND, not detectable.
[c] Values for myxoxanthophyll are the sums of results for three distinct forms of myxoxanthophyll differing in the sugar moiety.

complementary to the end of the Tn*5*-1063a sequence. Sequences obtained from the three clones were identical, indicating that the insertion site was common to all three and strongly suggesting, in fact, that SCY 59 contained a single insertion site. To confirm that a single-site transposition event had occurred in SCY 59, we also performed a Southern analysis of the mutant's genomic DNA after digestion with EcoRI. By using a probe specific to the transposon (Materials and Methods), a single band (corresponding to the expected fragment size of about 11 kb) was detected, confirming that a single copy of the transposon was present in the genome of SCY 59 (data not shown).

In order to confirm that the phenotypic differences between the wild type and SCY 59 were restricted to scytonemin synthesis, we characterized some physiological and morphological traits of both strains. Growth rates of the wild type and SCY 59 under white light were not significantly different ($n = 3$ cultures; $P < 0.05$). The number of doublings per day, represented as μ (24), in exponential phase was 0.994 ± 0.17 for the wild type, while the value for SCY 59 was 0.959 ± 0.06. Even upon exposure to UV-A irradiation, the growth rates of the wild type and SCY 59 (0.972 ± 0.06 and 0.905 ± 0.19 doublings per day, respectively) were not significantly different ($n = 3$; $P < 0.05$).

With respect to the lipid-soluble pigment complement, HPLC analyses confirmed that the only major pigment difference between the two strains was the lack of scytonemin in SCY 59 under conditions of induction by exposure to UV-A irradiation. All other pigments, including chlorophyll *a* and the carotenoids, myxoxanthophyll, echinenone, and β-carotene, were present in the wild type as well as in SCY 59, and their specific contents were not significantly different ($n = 3$; $P < 0.05$) under white light or white light supplemented with UV-A irradiation (Table 2; Fig. 2). In water-soluble extracts from the wild type and SCY 59 exposed to white light or white light supplemented with UV-A irradiation, no significant difference ($n = 3$; $P < 0.05$) in the phycobilin contents or compositions was found (Table 2). Additionally no obvious differences between the strains in colony morphology, vegetative cell shape or size, filament formation, or the ability to form heterocysts were detected.

Reconstruction of the mutation. The evidence presented above strongly suggests that the mutant phenotype of SCY 59 was restricted to an inability to produce scytonemin and that this characteristic coincided with the interruption of a partic-

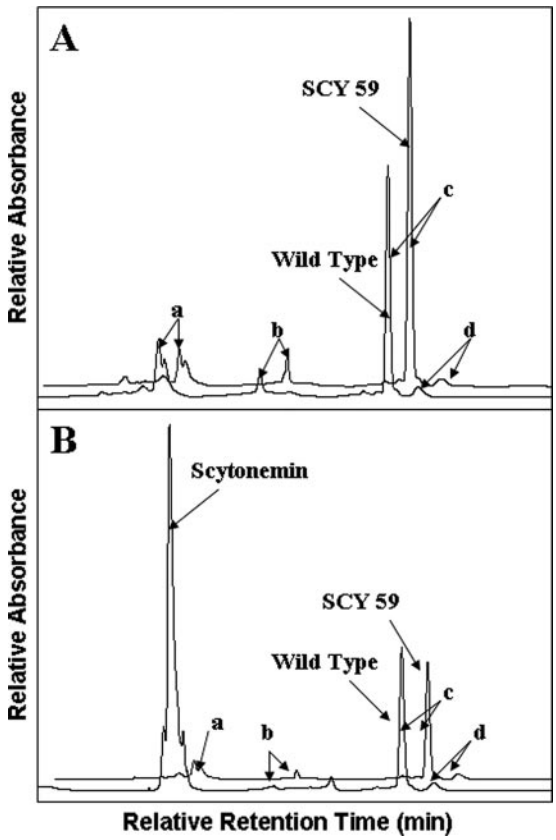

FIG. 2. HPLC chromatograms for the *N. punctiforme* wild-type and mutant SCY 59 lipid-soluble pigment extracts under white light (A) and under white light supplemented with UV-A irradiation (B). The mutant SCY 59 chromatograms in both panels are right shifted and up shifted 3% relative to those of the wild type to facilitate differentiation between the data for the two strains. Pigments are labeled as follows: a, myxoxanthophyll; b, echinenone; c, chlorophyll *a*; and d, β-carotene.

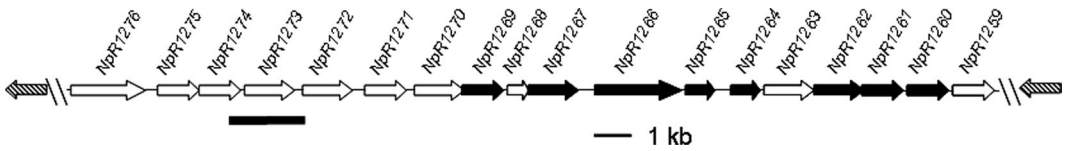

FIG. 3. Genomic region in *N. punctiforme* associated with scytonemin biosynthesis. Arrows denote ORFs and indicate transcriptional orientations. The ORFs are labeled according to annotations in the *N. punctiforme* database (Table 3). NpR1273 was the ORF interrupted by the transposon in SCY 59, and the ORFs involved in the biosynthesis of the aromatic amino acids are represented as solid arrows. The thick line delimits the region that was sequenced in this study. The nearest ORFs outside of this region are indicated by gray arrows, and the hatch marks indicate a break in the distance scale.

ular region in the genome of *N. punctiforme* by a single insertion of the transposon. However, it was still a possibility that the two findings were coincident but independent and that the phenotype was the result of a spontaneous mutation elsewhere in the genome. To establish a cause-effect relationship, it was then necessary to reconstruct the mutation in the wild type at the same location of the original mutation found in SCY 59. This reconstruction was accomplished by ligating a DNA fragment encompassing the transposon and its flanking region (pTD59a) (Table 1) to a positive-selection vector to create a plasmid with regions complementary to the original insertion found in the genome of SCY 59. This plasmid was then recombined into the wild type strain by triparental conjugation (Materials and Methods). Double-crossover recombinants were then selected by the antibiotic resistance encoded in the transposon. The double-crossover recombinant strain resulting from this site-directed mutation (SCY M7301) was incapable of synthesizing scytonemin upon UV-A irradiation induction, thus reproducing the phenotype of SCY 59 and confirming the cause-effect relationship between this mutation and the scytoneminless phenotype.

Characterization of the ORF involved in scytonemin biosynthesis and the surrounding genomic region. Primer walking on one of the plasmid clones obtained previously from the SCY 59 transposon insertion region (pTD59a) (Table 1) yielded 2,495 bp of the genomic sequence flanking the insertion. This fragment is 98% identical to a region of the *N. punctiforme* genome that includes a fragment of ORF NpR1272, the complete ORF NpR1273, and a fragment of ORF NpR1274 as annotated in the *N. punctiforme* genomic database. The original mutation involved the interruption of NpR1273, which is the fourth in a series of 18 ORFs that are in relatively close proximity (NpR1276 to NpR1259) (Fig. 3) and are transcribed in the same direction. The nearest ORF upstream from NpR1276, NpF1277, is transcribed in the opposite direction, as is the nearest ORF downstream from NpR1259, NpF1258. Therefore, the region from NpR1276 to NpR1259 constitutes a potentially functional genetic unit and was selected for more-detailed analyses.

Most of the ORFs in the downstream region of this cluster (NpR1269 to NpR1260) are predicted to encode a set of enzymes involved in the shikimic acid and aromatic amino acid biosynthesis pathways (Fig. 3) (2). Copies of most of these ORFs are also found elsewhere in the genome at dispersed loci, as is the case for most other cyanobacterial aromatic amino acid biosynthesis genes. In fact, these dispersed genes are the only copies that exist in most other cyanobacterial lineages (49). Most of the upstream ORFs within this cluster (NpR1276 to NpR1270) encode products annotated as hypothetical proteins and have relatively similar syntenic orthologues in *Anabaena* sp. strain PCC 7120, a related cyanobacterium. Aside from the products of these orthologues, the putative proteins in the public databases have less than 36% identity to the proteins encoded by these ORFs. The NpR1274, NpR1273, NpR1272, and NpR1271 products all contain NHL repeats (Table 3) that resemble the WD-40 repeat (a unit of about 40 residues with tryptophan and aspartic acid at defined positions), which would classify them as conserved hypothetical proteins in the beta propeller clan (33). However, this structural resemblance does not imply a functional similarity, since the protein family is functionally heterogeneous.

NpR1273, the mutated ORF, encodes a putative protein of 423 amino acid residues with a predicted signal peptide (amino acids 1 to 25) and a putative cleavage site between amino acids 26 and 27 (37). This predicted protein sequence has 71% identity to an uncharacterized protein in *Anabaena* strain PCC 7120. The next best protein sequence match for the NpR1273 product is another hypothetical protein from *Anabaena* strain PCC 7120, at 40% identity, but there are no other protein matches in the pubic databases with an identity greater than 30%. It is interesting that NpR1273 and NpR1272 are approximately 67% similar to one another, which may suggest an ancestral duplication event. Characteristics of putative genes in this cluster as determined by genomic analyses are found in Table 3.

Although we do not have the evidence to conclude that these genes constitute an operon, they are in relatively close proximity to one another, with no more than a 479-bp gap between any two adjacent ORFs from NpR1276 to NpR1259. A search for obvious promoter sequences (48) within this region was not successful, although this result is not surprising since promoter consensus sequences in cyanobacteria are generally not well defined (13).

DISCUSSION

We have shown that the *N. punctiforme* mutant SCY 59 is incapable of producing the lipid-soluble UV-A irradiation-induced sunscreen scytonemin as a result of the insertion of the transposon Tn*5*-1063a into ORF NpR1273. Furthermore, since neither the growth rates, pigment complements (aside from scytonemin), nor cellular morphologies differ between SCY 59 and the wild type, the mutation seems to be specific for scytonemin production. The fact that the growth rates of the mutant and the wild type under UV-A irradiation were indistinguishable indicates that photoprotective mechanisms other

TABLE 3. *N. punctiforme* ORF annotations

ORF	Length (aa)[a] of product	Database identification of product based on predicted function	Gene name[b]	% Amino acid identity of product to closest orthologue[c]	Designation and name of orthologue(s) in *Anabaena* strain PCC 7120 (% identity)	Predicted feature of product	Additional information
NpR1276	624	Thiamine pyrophosphate-requiring enzyme	*ilvB*	74	all0427 (74)	Transmembrane domain	
NpR1275	353	Leucine dehydrogenase	*leuDH*	79	all0426, *leuDH* (79)		
NpR1274	322	Hypothetical protein		61	all0425 (61)		Product contains NHL repeats[d]
NpR1273	423	Hypothetical protein		71	all0424 (71)	Protein export signature	ORF interrupted by transposon; product contains NHL repeats
NpR1272	432	Hypothetical protein		47	all0423 (47)	Protein export signature	Product contains NHL repeats
NpR1271	398	Hypothetical protein		55	all0422 (55)	Protein export signature	Product contains NHL repeats
NpR1270	429	Putative glycosyltransferase		75	None		
NpR1269	360	Prephenate dehydrogenase	*tyrA*	72	all0418, *tyrA* (72)		Involved in tyrosine biosynthesis
NpR1268	214	Dithiol-disulfide isomerase	*frnE*	35	None		Involved in periplasmic protein folding
NpR1267	395	3-Dehydroquinate synthase	*aroB*	71	all0417, *aroB* (71)		Involved in shikimic acid pathway
NpR1266	735	Anthranilate synthase	*trpE*	81	all0414, *trpE* (81)		Involved in tryptophan biosynthesis
NpR1265	276	Indole-3-glycerol phosphate synthase	*trpC*	79	all0413, *trpC* (79)		Involved in tryptophan biosynthesis
NpR1264	276	Tryptophan synthase (α subunit)	*trpA*	85	all0411, *trpA* (85)		Involved in tryptophan biosynthesis
NpR1263	408	Putative tyrosinase	$tyrP_1$	31	None	Transmembrane domain	Involved in polyphenolic compound biosynthesis
NpR1262	410	Tryptophan synthase (β subunit)	*trpB*	88	all0410, *trpB* (88)		Involved in tryptophan biosynthesis
NpR1261	364	Anthranilate phosphoribosyltransferase	*trpD*	81	all0409, *trpD* (81)		Involved in tryptophan biosynthesis
NpR1260	366	DAHP synthase[e]	*aroG*	84	all0408, *aroG* (84)		Start of shikimic acid pathway; site of feedback inhibition
NpR1259	314	Hypothetical protein		54	all0407 (54)	2 transmembrane domains	

[a] aa, amino acids.
[b] Annotated as determined from the closest blastp result.
[c] Data are based on blastp results (2).
[d] The NHL repeat is found in the copper type II ascorbate-dependent monooxygenase family, which catalyzes the C-terminal alpha-amidation of peptides (23).
[e] DAHP stands for 3-deoxy-D-arabino-heptolosonate-7-phosphate; the corresponding gene is referred to as *aroG* for consistency, as there is more than one isozyme in cyanobacteria (21).

than scytonemin can fully accommodate the absence of the sunscreen in the mutant, a finding consistent with the expected fitness value of scytonemin preferentially under conditions of metabolic inactivity (17, 19). The mutation did not affect a secretory function, since this effect would have resulted in an intracellular accumulation of scytonemin, which would have been detected in our extraction procedures. Although the precise nature of the mutation, whether biosynthetic or regulatory, cannot be determined at this point, the evidence from genomic analyses of the mutated region supports a biosynthetic role for the mutated ORF. For instance, NpR1273, the interrupted ORF, encodes a putative signal peptide with a typical Ala-X-Ala cleavage site (34), indicating that the gene product is probably exported across the plasma membrane, not a likely characteristic for a transcriptional regulatory protein. This predicted feature is shared by the products of ORFs NpR1272 and NpR1271, both of which are immediately downstream. In addition, the product of NpR1276, the first ORF in the cluster, is likely a membrane protein as suggested by the presence of a putative transmembrane helix domain (28). These observations are consistent with the fact that scytonemin is an extracellular compound (17) and may indicate that its assembly occurs in the periplasmic space.

We also regard as significant the fact that the ORFs downstream from the mutation likely encode proteins involved in the biosynthesis of the aromatic amino acids (22). In fact, feedback inhibition and radiotracer experiments have implicated both tyrosine and tryptophan as biosynthetic precursors of scytonemin in *N. punctiforme* (30), as the chemical structure of the molecule also suggests (Fig. 1) (36). This cluster includes genes encoding some of the enzymes of the shikimic acid pathway (*aroB* and *aroG*) and all of the enzymes required for tryptophan biosynthesis (*trpE*, *trpC*, *trpA*, *trpB*, and *trpD*) and *tyrA* (Table 3), which is involved in the biosynthesis of tyrosine (22). Interestingly, all of these genes have at least one copy elsewhere in the genome of *N. punctiforme* at dispersed loci, and it has been suggested, on the basis of genomics, that the genes described here have a novel physiological function (49). We contend that this novel function is to support the dedicated biosynthesis of scytonemin by providing additional pathways leading to its precursor amino acids. This extra copy of clustered aromatic amino acid biosynthetic genes seems to be unique to *N. punctiforme* and *Anabaena* strain PCC 7120 among those cyanobacteria with fully sequenced genomes (49). Interestingly, similar clusters of additional *trp* genes are found in *Streptomyces coelicolor*, where they are involved in the biosynthesis of antibiotic secondary metabolites (39).

Since *N. punctiforme* is the only scytonemin-producing organism whose genome has been fully sequenced, we had originally anticipated that genes involved in scytonemin production would not have orthologues in the databases. This was, however, not the case. ORFs NpR1274 to NpR1271 have clear orthologues in *N. punctiforme*'s close relative *Anabaena* strain PCC 7120 (Table 3). They are also organized in a similar, if not identical, manner. We confirmed the inability of *Anabaena* strain PCC 7120 to produce scytonemin, and it is our opinion that the *Anabaena* strain PCC 7120 region containing the orthologous genes is a relic, rendered nonfunctional by genetic rearrangement events. These rearrangements may have taken place during prolonged (more-than-25-year) cultivation in the absence of UV-A irradiation exposure. Indeed, other than those of the orthologous proteins in *Anabaena* strain PCC 7120, no protein sequences in the public databases, including those from other cyanobacteria, have clear homology to the sequences of the products of NpR1274 to NpR1271.

We propose that NpR1274, NpR1273, NpR1272, and NpR1271 be recognized for their significance in scytonemin biosynthesis. We have experimentally shown that NpR1273 is directly involved in scytonemin biosynthesis. Genomic analyses suggest that the ORFs NpR1274 to NpR1271 are not associated with any known function, nor have their corresponding proteins been detected by proteomic analyses of vegetative cells and colonies at various developmental stages in the absence of exposure to UV irradiation (3; J. C. Meeks, unpublished data). The analysis of the *N. punctiforme* mutant SCY 59 has paved the way for future studies concerning the molecular genetics of scytonemin biosynthesis and regulation. Knowledge of scytonemin biosynthesis at the molecular level has the potential to enhance the applicability of the molecule in the biotechnological realm. For instance, constitutive synthesis of scytonemin, either in *N. punctiforme* or in another organism, may increase the rate of the production of scytonemin for use as a natural sunscreen and further the study of its antiproliferative and anti-inflammatory properties (43, 44). To complement this study, we hope to eventually establish a sound model for the molecular genetics of scytonemin biosynthesis and regulation.

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REFERENCES

1. **Allen, M., and D. I. Arnon.** 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen-fixation by *Anabaena cylindrica*. Plant Physiol. **30:** 366–372.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Anderson, D. C., E. L. Campbell, and J. C. Meeks.** 2006. A soluble 3D LC/MS/MS proteome of the filamentous cyanobacterium *Nostoc punctiforme*. J. Proteome Res. **5:**3096–3104.
4. **Bebout, B. M., and F. Garcia-Pichel.** 1995. UV-B-induced vertical migrations of cyanobacteria in a microbial mat. Appl. Environ. Microbiol. **61:**4215–4222.
5. **Black, T. A., and C. P. Wolk.** 1994. Analysis of a Het$^-$ mutation in *Anabaena* sp. strain PCC 7120 implicates a secondary metabolite in the regulation of heterocyst spacing. J. Bacteriol. **176:**2282–2292.
6. **Black, T. A., Y. Cai, and C. P. Wolk.** 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. **9:**77–84.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
8. **Bryant, D. A., G. Guglielmi, N. Tandeau de Marsac, A.-M. Castets, and G. Cohen-Bazire.** 1979. The structure of phycobilisomes: a model. Arch. Microbiol. **123:**113–127.
9. **Cai, Y., and C. P. Wolk.** 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. J. Bacteriol. **172:**3138–3145.
10. **Castenholz, R. W.** 1997. Multiple strategies for UV tolerance in cyanobacteria. Spectrum **10:**10–16.
11. **Castenholz, R. W., and F. Garcia-Pichel.** 2000. Cyanobacterial responses to UV radiation, p. 591–611. *In* B. A. Whitton and M. Potts (ed.), The ecology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. **Cohen, M. F., J. C. Meeks, Y. A. Cai, and C. P. Wolk.** 1998. Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. Methods Enzymol. **297:**3–17.
13. **Curtis, S. E.** 1994. The transcription apparatus and the regulation of tran-

scription initiation, p. 613–639. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. **Ehling-Schulz, M., W. Bilger, and S. Scherer.** 1997. UV-B-induced synthesis of photoprotective pigments and extracellular polysaccharides in the terrestrial cyanobacterium *Nostoc commune*. J. Bacteriol. **179:**1940–1945.
15. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
16. **Garcia-Pichel, F., and J. Belnap.** 1996. Microenvironments and microscale productivity of cyanobacterial desert crusts. J. Phycol. **32:**774–782.
17. **Garcia-Pichel, F., and R. W. Castenholz.** 1991. Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. J. Phycol. **27:**395–409.
18. **Garcia-Pichel, F., C. E. Wingard, and R. W. Castenholz.** 1993. Evidence regarding the UV sunscreen role of a mycosporine-like compound in the cyanobacterium *Gloeocapsa* sp. Appl. Environ. Microbiol. **59:**170–176.
19. **Garcia-Pichel, F., N. D. Sherry, and R. W. Castenholz.** 1992. Evidence for an ultraviolet sunscreen role of the extracellular pigment scytonemin in the terrestrial cyanobacterium *Chlorogloeopsis* sp. Photochem. Photobiol. **56:**17–23.
20. **Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
21. **Hall, G. C., M. B. Flick, R. L. Gherna, and R. A. Jensen.** 1982. Biochemical diversity for biosynthesis of aromatic amino acids among cyanobacteria. J. Bacteriol. **149:**65–78.
22. **Herrmann, K. M., and R. L. Somerville.** 1983. Amino acids: biosynthesis and gene regulation. Addison-Wesley Publishing Company, London, United Kingdom.
23. **Husten, E. J., and B. A. Eipper.** 1991. The membrane-bound bifunctional peptidoglycine α-amidating monooxygenase protein. J. Biol. Chem. **266:** 17004–17010.
24. **Ingraham, J. L., O. Maaloe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Associates, Sunderland, MA.
25. **Jagger, J.** 1967. Introduction to research in ultraviolet biology. Prentice-Hall, Englewood Cliffs, NJ.
26. **Jurgens, U. J., and J. Weckesser.** 1985. Carotenoid-containing outer membrane of *Synechocystis* sp. strain PCC 6714. J. Bacteriol. **164:**384–389.
27. **Kost, H.-P. (ed.).** 2000. CRC handbook of chromatography, vol. 1. Plant pigments. CRC Press, Boca Raton, FL.
28. **Krogh, A., B. Larsson, G. von Heijne, and E. L. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. **305:**567–580.
29. **Levine, E., and T. Thiel.** 1987. UV-inducible DNA repair in the cyanobacteria *Anabaena* spp. J. Bacteriol. **169:**3988–3993.
30. **Mandalka, A.** 1999. Synthese und regulation des UV-schutzpigmentes scytonemin an modellmikroorganismen. University of Bremen, Bremen, Germany.
31. **Meeks, J. C., and R. W. Castenholz.** 1971. Growth and photosynthesis in an extreme thermophile, Synechococcus lividus (cyanophyta). Arch. Microbiol. **78:**25–41.
32. **Meeks, J. C., J. Elhai, T. Thiel, M. Potts, F. Larimer, J. Lamerdin, P. Predki, and R. Atlas.** 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth. Res. **70:**85–106.
33. **Murzin, A. G.** 1992. Structural principles for the propellar assembly of beta-sheets: the preference for seven-fold symmetry. Proteins **14:**191–201.
34. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1–6.
35. **Portwich, A., and F. Garcia-Pichel.** 2000. A novel prokaryotic UVB photoreceptor in the cyanobacterium *Chlorogloeopsis* PCC 6912. Photochem. Photobiol. **71:**493–498.
36. **Proteau, P. J., W. H. Gerwick, F. Garcia-Pichel, and R. Castenholz.** 1993. The structure of scytonemin, an ultraviolet sunscreen pigment from the sheaths of cyanobacteria. Experientia **49:**825–829.
37. **Richardson, P., J. Meeks, J. Elhai, M. Potts, and T. Thiel.** April 2000, posting date. *Nostoc punctiforme* PCC 731 02 draft genome. [Online.] http://www.jgi.doe.gov/.
38. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories, and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
39. **Ryding, J. J., T. B. Anderson, and W. C. Champness.** 2002. Regulation of the *Streptomyces coelicolor* calcium-dependent antibiotic *absA*, encoding a cluster-linked two-component system. J. Bacteriol. **184:**794–805.
40. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed., vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
41. **Shibata, H., K. Baba, and H. Ochiai.** 1991. Near-UV induces shock proteins in *Anacystis nidulans* R-2: possible role of active oxygen. Plant Cell Physiol. **32:**771–776.
42. **Shirkey, B., D. P. Kovarcik, D. J. Wright, G. Wilmoth, T. F. Prickett, R. F. Helm, E. M. Gregory, and M. Potts.** 2000. Active Fe-containing superoxide dismutase and abundant *sodF* mRNA in *Nostoc commune* (cyanobacteria) after years of desiccation. J. Bacteriol. **182:**189–197.
43. **Stevenson, C. S., E. A. Capper, A. K. Roshak, B. Marquez, C. Eichman, J. R. Jackson, M. Mattern, W. H. Gerwick, R. S. Jacobs, and L. A. Marshall.** 2002. The identification and characterization of the marine natural product scytonemin as a novel antiproliferative pharmacophore. J. Pharmacol. Exp. Ther. **303:**858–866.
44. **Stevenson, C. S., E. A. Capper, A. K. Roshak, B. Marquez, K. Grace, W. H. Gerwick, R. S. Jacobs, and L. A. Marshall.** 2002. Scytonemin, a marine natural product inhibitor of kinases key in hyperproliferative inflammatory diseases. Inflamm. Res. **51:**112–114.
45. **Tandeau de Marsac, N., and J. Houmard.** 1988. Complementary chromatic adaptation: physiological conditions and action spectra. Methods Enzymol. **167:**318–328.
46. **Van Baalen, C.** 1968. The effects of ultraviolet radiation on a coccoid blue-green alga: survival, photosynthesis, and photoreactivation. Plant Physiol. **43:**1689–1695.
47. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc. Natl. Acad. Sci. USA **88:**5355–5359.
48. **Wright, D. J., S. C. Smith, V. Joardar, S. Scherer, J. Jervis, A. Warren, R. F. Helm, and M. Potts.** 2005. UV-irradiation and desiccation modulate the 3-D extracellular matrix of *Nostoc commune* (cyanobacteria). J. Biol. Chem. **280:**40271–40281.
49. **Xie, G., C. A. Bonner, T. Brettin, R. Gottardo, N. O. Keyhani, and R. A. Jensen.** 2003. Lateral gene transfer and ancient paralogy of operons containing redundant copies of tryptophan-pathway genes in *Xylella* species and in heterocystous cyanobacteria. Genome Biol. **4:**R14.