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Extracellular Polysaccharide Production in a Scytonemin-Deficient Mutant of *Nostoc punctiforme* Under UVA and Oxidative Stress

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Abstract Some cyanobacteria can protect themselves from ultraviolet radiation by producing sunscreen pigments. In particular, the sheath pigment scytonemin protects cells against long-wavelength UVA radiation and is only found in cyanobacteria which are capable of extracellular polysaccharide (EPS) production. The presence of a putative glycosyltransferase encoded within the scytonemin gene cluster, along with the localization of scytonemin and EPS to the extracellular sheath, prompted us to investigate the relationship between scytonemin and EPS production under UVA stress. In this study, it was hypothesized that there would be a relationship between the biosynthesis of scytonemin and EPS under both UVA and oxidative stress, since the latter is a by-product of UVA radiation. EPS production was measured following exposure of wild-type *Nostoc punctiforme* and the non-scytonemin-producing strain SCY59 to UVA and oxidative stress. Under UVA, SCY59 produced significantly more EPS than the unstressed controls and the wild type, while both strains produced more EPS under oxidative stress compared to the controls. The results suggest that EPS secretion occurs in response to the oxidative stress by-product of UVA rather than as a direct response to UVA radiation.

Abbreviations

EPS	Extracellular polysaccharides
RPS	Released polysaccharides
UVR	Ultraviolet radiation
UVA	Ultraviolet A radiation
UVB	Ultraviolet B radiation
ROS	Reactive oxygen species
PSA	Phenol-sulfuric acid test
TE	Total extractable carbohydrates
EP	Ethanol-precipitable carbohydrates

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Introduction

Many microorganisms are able to secrete extracellular polysaccharides (EPS) that can be in the form of either a tight capsule or secreted as a loose slime that does not reflect the shape of the cell [25]. These loose slime layers are composed of a mucilaginous material that is often sticky in nature due to the large contribution of water mixed with carbohydrate polymers of different subunits including glucose, fructose, sucrose, and related compounds [37]. In environments with limited water and nutrient availability, such as arid deserts, EPS can sequester scarce metals and nutrients from the environment and provide them to the cell for general cell metabolism [7]. EPS can also protect cells from harmful ultraviolet radiation (UVR) in exposed environments by attenuating the solar rays before they can reach the cell, reacting with some of the toxic reactive oxygen species (ROS) that are formed as a by-product of UVR, or by harboring the UV-absorbing sunscreen pigment scytonemin [14–16].

Photosensitization is the chemical modification of a molecule by light. In the presence of oxygen, UVA (320–400 nm) can photosensitize pigments and other molecules to generate harmful forms of oxygen, such as superoxide and hydrogen peroxide, which can nonspecifically react with and damage other molecules [22, 38]. For

cyanobacteria, which produce oxygen during photosynthesis and contain several UVA-absorbing photosensitizing pigments, UVA can account for much of the damage from solar radiation [13]. In the cyanobacterium *Nostoc punctiforme* ATCC 29133, for example, acclimation to UVA can potentially involve the differential regulation of 573 genes, or about 8.3 % of all genes in the genome [31].

Among the strategies to cope with UVR damage, some cyanobacteria are able to synthesize and accumulate EPS and photo-protective compounds that can act as either UVR-absorbing sunscreens or as antioxidant scavengers of the ROS generated from UVR [4]. For instance, the cyanobacterial sunscreen scytonemin is a lipid-soluble, yellow-brown, indole-alkaloid pigment that absorbs strongly in the UVA range, effectively absorbing >95 % of the UVA radiation before it reaches the cell [14]. The production of this pigment is primarily induced by UVA radiation, although in some strains other stress factors, particularly desiccation, osmotic stress, and poor nutritional status, have played a role in modulating its production [9, 12].

Since scytonemin is deposited into the extracellular sheath, along with the EPS, it is hypothesized that there is some relationship between scytonemin and EPS biosynthesis, especially since scytonemin can accumulate to large concentrations of up to 5 % of the cellular dry weight in laboratory cultures. Only EPS-containing cyanobacteria produce scytonemin, although not universally [4], and it has also been found in cell-free, extracellular fractions of rehydrated cells of the EPS-producing *Nostoc commune* [40]. In *N. punctiforme*, scytonemin biosynthesis is encoded by an 18-gene polycistronic operon (Npun_R1276 to Npun_R1259) [32, 34]. Some genes in the cluster are unique to scytonemin biosynthesis [2, 3]. Others are involved in the biosynthesis of the aromatic amino acids tryptophan and tyrosine, which are the metabolic precursors of scytonemin [19]. Among the remaining genes is a putative glycosyltransferase encoded by Npun_R1270. The specific function of this glycosyltransferase is unknown, although its location within the scytonemin gene cluster suggests that it may play a role in exporting the pigment to the extracellular sheath [33]. Moreover, glycosyltransferases have been shown to be involved with EPS biosynthesis [26, 36].

Scytonemin not only absorbs incident UVA radiation, but the oxidative stress experienced by the cell as a by-product of UVA radiation is also indirectly reduced when less UVA is absorbed [14]. Furthermore, scytonemin extracts have been shown to quench radical scavenging activity in vitro [23]. Given that EPS production is one of the mechanisms which enables the cyanobacterial cell to cope with UVA and oxidative stress, and that the sunscreen scytonemin accumulates within the EPS with its own ability to provide protection against these stress conditions, we hypothesized that there would be a positive relationship between scytonemin and EPS production under both UVA and oxidative stress.

This study addresses the relationship between UVA and oxidative stress on scytonemin and EPS production by comparing the amount of EPS released from cells of the wild-type *N. punctiforme* strain to that of a scytonemin-deficient mutant strain following exposure to UVA and oxidative stress. The scytonemin-deficient mutant, SCY59, was previously constructed through transposon mutagenesis with a transposon insertion into the fourth gene of the polycistronic scytonemin gene cluster (*scyD*; Npun_R1273). SCY59 is characterized as a biosynthetic, as opposed to regulatory, mutant and the phenotypic effects of this mutation appear to be restricted to the inhibition of scytonemin biosynthesis [34]. Furthermore, the mutation in SCY59 occurs upstream of the glycosyltransferase encoded by Npun_R1270 described above, eliminating any potential contribution made by this putative glycosyltransferase in scytonemin or EPS secretion.

Materials and Methods

Strains, Culture Conditions, and Pigment Analysis

Cultures of wild-type *N. punctiforme* ATCC 29133 and the scytonemin-deficient mutant, SCY59 [34], were grown under white light (40 μmol photons m^{-2} s^{-1}) in sterile flasks containing AA growth medium [1] supplemented with neomycin (25 μg ml^{-1}) and streptomycin (1 μg ml^{-1}) for strain SCY59. For scytonemin induction, white light was supplemented with UVA (8 W m^{-2}) for 6 days. Self-shading of the cultures was minimized by filtering cells onto 90-mm membrane filters (EMD Millipore, Billerica, MA, USA) that were allowed to float on top of liquid growth media in glass Petri plates [32]. To induce oxidative stress, filtered cultures were exposed to 2 μM methylene blue and to minimize methylene blue consumption, filters were transferred to fresh media with 2 μM methylene blue daily for all 6 days of the experiment [9, 21]. All experiments were done in triplicate and control cultures for each variable were also grown under standard conditions in the absence of UVA and methylene blue.

To verify scytonemin production, lipid-soluble pigments from cultures exposed to 6 days of continuous UVA were extracted in acetone. Scytonemin was confirmed by absorption at 384 nm [14]. Chlorophyll *a* was measured for biomass normalization at 665 nm from 90 % methanol extracts [24].

Quantification and Visualization of Extracellular Polysaccharides

To quantify the EPS from each culture, the phenol–sulfuric acid (PSA) test was used [10, 39]. For this, cells were gently sonicated in 0.1 M EDTA with pulsing for 1 min to help release the EPS, as released polysaccharides (RPS), from the cell surface. While this technique was not meant to capture all of the RPS, it allowed us to apply a consistent approach for comparison across all samples. Cell integrity was confirmed by microscopy to ensure that cells were not lysed following sonication. Sonicated cells were incubated at 20 °C for 10 min and then centrifuged for 10 min to collect the supernatant. The total extractable (TE) carbohydrates in the supernatant were quantified immediately by adding 1 ml of each sample to 1 ml phenol and 5 ml sulfuric acid and incubating at room temperature for 10 min followed by incubation in a waterbath at 25 °C for 15 min. Carbohydrates were measured colorimetrically at 488 nm against a glucose standard curve and normalized to biomass based on chlorophyll *a* content. An aliquot of each sample (4.5 ml) was also used for an overnight precipitation at 4 °C in 70 % ethanol (total volume 15 ml) to obtain the ethanol-precipitable (EP), higher molecular weight carbohydrates [29]. The pellet from this precipitate was resuspended in sterile water and the EP carbohydrates were quantified using the PSA test as outlined above. Normalized values were compared with a one-way analysis of variance (ANOVA) followed by a post hoc analysis using Tukey's HSD to identify between treatment differences.

To visualize the RPS, cells were stained with 1 % alcian blue for 30 min following UVA stress exposure. Stained cells were allowed to absorb into a thin layer of 1 % molecular grade agarose (in sterile water) on a microscope slide in order to minimize issues with viewing aggregates of filamentous cells. All stained cell preparations were observed on an Olympus CX22LED brightfield microscope and captured with a Leica DFC295 camera (Leica Microsystems, Buffalo Grove, IL, USA). Those images that best represented the RPS and EPS distribution were chosen, although all images consistently showed the same patterns by strain and treatment.

Gene Expression of the Putative Glycosyltransferase

Since expression of the putative glycosyltransferase gene Npun_R1270 in the scytonemin biosynthetic gene cluster could interfere with interpreting our results, we tested the expression of this gene in both strains under UVA and white light using triplicate samples as described above. Following 48 h of stress, as determined previously for maximal expression of the scytonemin biosynthetic genes [32], cells were harvested from the membrane filters by vortexing into cold AA medium. The cells were then concentrated by centrifugation at 4 °C and the pellets were frozen at −80 °C until RNA extraction [32]. RNA extraction, DNase treatment, and cDNA synthesis were done as previously described [18], with the exception that the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used. Each 20 µl cDNA synthesis reaction included 4 µl 5X iScript reaction mix, 1 µl iScript reverse transcriptase, and 0.5 µg RNA. The cDNA reactions were done in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories) at 25 °C for 10 min, 42 °C for 15 min, and 85 °C for 5 min. Quantitative-PCR was performed on a Bio-Rad CFX Connect^{TM} Real-Time PCR Detection System in 20 µl reaction mixtures consisting of 10 µl 2X iTaq Universal SYBR Green Supermix (Bio-Rad), 0.5 µM of each primer, and 1 µl of cDNA. Primers for Npun_F1270 were the same as described in Soule et al. [32], while those for the reference gene DNA gyrase (Npun_F0025) were the same as described in Sorrels et al. [30]. Controls included wild-type *N. punctiforme* genomic DNA along with a no template (distilled water) control. Thermal cycling conditions and data analysis were performed as described previously [18].

Results

To study the relationship between scytonemin and EPS, the PSA test was used to measure the RPS captured from stressed cells of wild-type *N. punctiforme* as well as the scytonemin-deficient mutant strain, SCY59, following gentle sonication. In this reaction carbohydrates are hydrolyzed and measured from the liquid phase. It should be noted that while this assay is sensitive to a wide range of carbohydrates, such as methylated sugars and neutral and acidic carbohydrates, it is not used to measure amino or acetylated sugars [10]. Although the specific values obtained in this study are an underestimation of the total RPS, the relative amounts used for comparison should be consistent.

The scytonemin mutant strain SCY59 exhibited a substantial increase in RPS production (in both EP and TE carbohydrates) relative to controls when given a UVA treatment (Fig. 1a). The wild type under UVA also exhibited a small, but significant, increase in TE carbohydrates compared with the control under white light. Interestingly, the SCY59 mutant under white light also displayed the same slight increase. While the amount of EP carbohydrates detected was significantly lower than the TE carbohydrates for SCY59 under UVA, it was still greater than the amount of EP carbohydrates detected from all other cells in this experiment. These cells were also stained at the end of the experiment with alcian blue, a dye specific

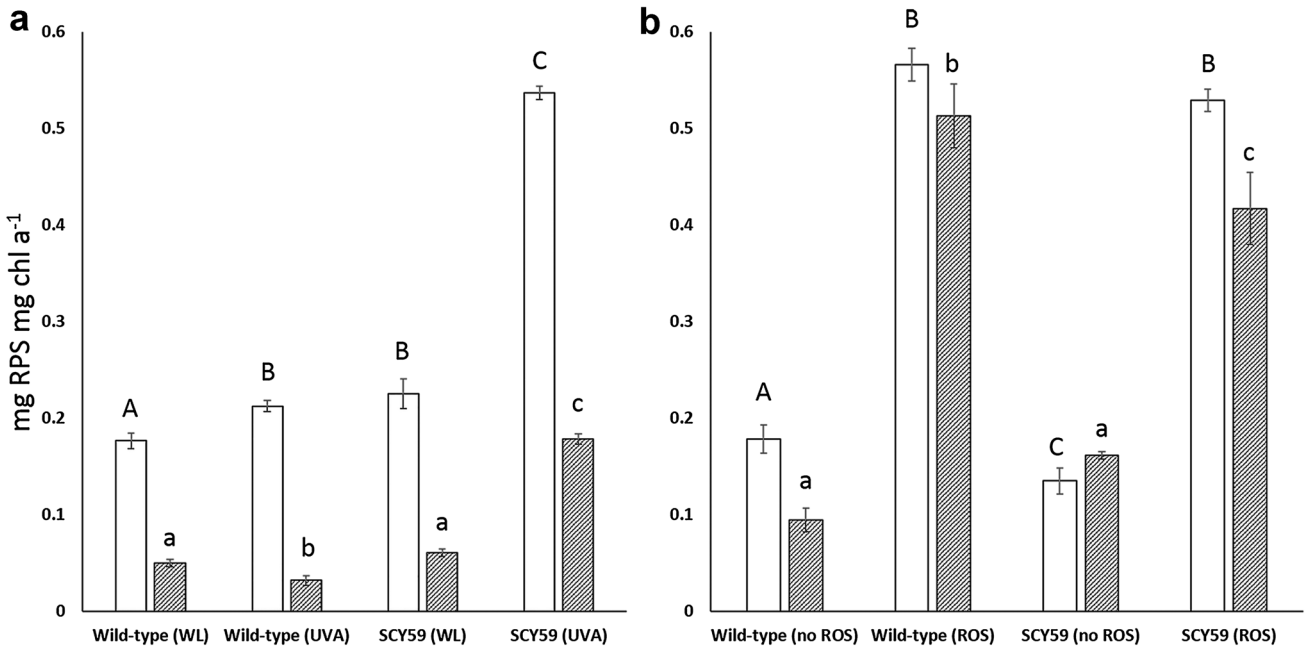

Fig. 1 RPS production of wild-type *N. punctiforme* and SCY59 cells following (**a**) white light (WL) versus UVA stress and (**b**) no reactive oxygen species (no ROS) vs ROS stress. *Open bars* TE carbohydrates; *dashed bars* EP carbohydrates. *Error bars* represent the standard deviation of triplicate samples with *letters* denoting values that are statistically similar ($P \leq 0.001$) for each treatment. *Capital letters* refer to TE and *lower-case letters* refer to EP carbohydrate significance between strains and treatments within each panel

for mucosubstances and acetic mucins [35]. Staining of the cells post-UVA exposure with alcian blue visually confirmed the increase in EPS, specifically as RPS, in SCY59 as evidenced by a more dispersed blue background compared to wild-type *N. punctiforme* under UVA (blue in Fig. 2d vs c). However, a small increase in the overall EPS cannot be discounted for wild-type *N. punctiforme* under UVA, as opposed to the unstressed control (Fig. 2c vs 2a),

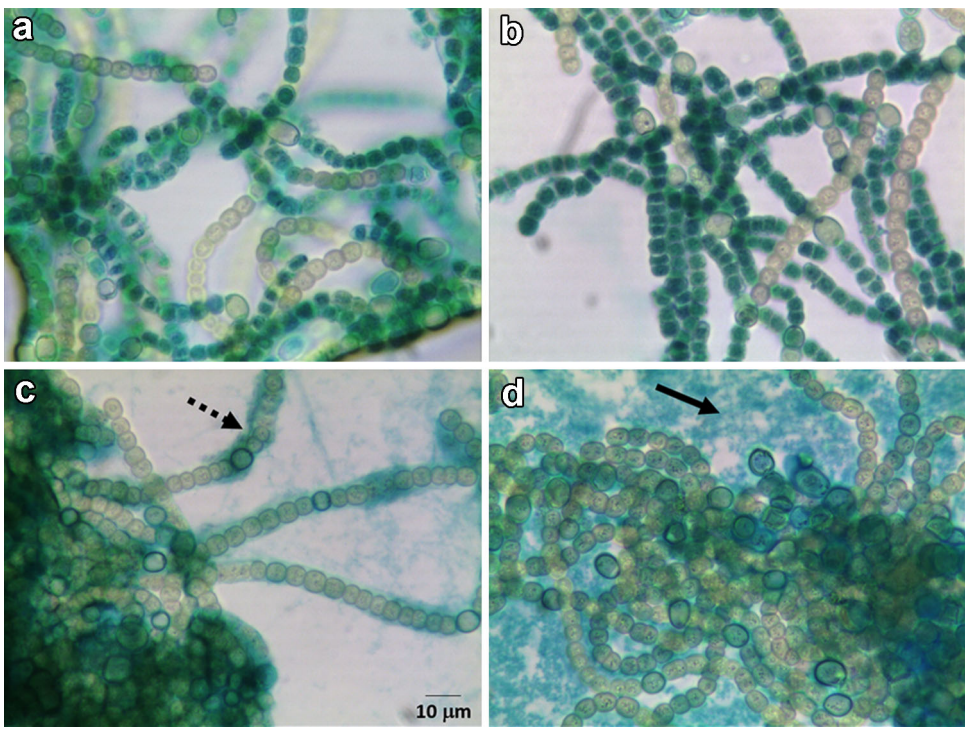

Fig. 2 RPS visualized by alcian blue staining for the (**a**) wild-type *N. punctiforme* and (**b**) SCY59 unstressed controls, and the (**c**) wild-type *N. punctiforme* and (**d**) SCY59 cultures exposed to UVA stress. The *solid arrow* shows RPS dispersed in the *blue background*, while the *dashed arrow* shows EPS bound to the sheath of the cells

since the dark blue staining near the sheath of these cells might suggest that wild-type *N. punctiforme* may have just resisted releasing the EPS into the surrounding medium. Indeed, this is reflected in a slight, but statistically significant, increase in RPS from wild-type *N. punctiforme* cells exposed to UVA versus the unstressed control (Fig. 1a).

To determine if the response in SCY59 was due to the oxidative stress generated by UVA radiation, both strains were exposed to oxidative stress via a methylene blue supplement in the absence of UVA, and the RPS was measured as previously described. Both strains produced statistically similar levels of TE carbohydrates per unit biomass following oxidative stress (Fig. 1b), which was also significantly more than the unstressed controls. The EP carbohydrates also significantly increased under ROS stress, although with more variability between strains than the TE carbohydrates. Slight variability was also seen for the TE carbohydrates in the unstressed controls, although the overall levels of RPS remained significantly lower than the ROS-stressed cells. None of the cultures in the oxidative stress experiment produced scytonemin (data not shown).

In order to better inform our interpretation of the data, we wanted to ensure that the putative glycosyltransferase (Npun_R1270) was not differentially transcribed in SCY59. UVA stress was applied to both strains for 48 h to obtain maximal expression of the scytonemin biosynthetic genes as previously determined [32]. Using quantitative-PCR, we were able to determine the fold-change of expression of Npun_F1270 for each strain under UVA versus white light only controls. As hypothesized, Npun_R1270 was not up-regulated under UVA stress in SCY59; in fact, it was down-regulated about 8.8-fold ($P < 0.01$) compared to the unstressed controls. We also confirmed that this gene was up-regulated in the wild-type strain under UVA (about 92.7-fold, $P < 0.01$). While this is much higher than the differential regulation previously reported [32], it could be because the cells were also under a higher intensity of UVA (8 W m^{-2}) than in the previous study (5 W m^{-2}).

Discussion

It was hypothesized that since scytonemin and EPS both occur in the extracellular sheath and are capable of remediating UVA and oxidative stress, their production perhaps is coupled under these conditions. An increase in EPS under UVA stress versus unstressed controls was hypothesized for both the wild-type *N. punctiforme* strain capable of producing scytonemin and the scytonemin-deficient mutant strain SCY59. This is because both strains would experience oxidative stress as a by-product of UVA radiation, resulting in an increase in EPS production as one of the mechanisms enabling the cell to cope with the elevated ROS. The difference in EPS production between each strain, however, is more difficult to predict. If the production of scytonemin in the wild type under UVA reduces the ROS received by the cells, then the wild type should produce less EPS than SCY59 under UVA stress. Alternatively, if the localization of scytonemin to the extracellular sheath requires an increase in EPS to accommodate the sunscreen, then the wild type is expected to produce more EPS than SCY59 under UVA stress. Under just oxidative stress, however, both strains should respond similarly by increasing EPS production since neither one would produce scytonemin as an additional mechanism of protection when oxidative stress is provided without supplemental UVA radiation (Fig. 3).

The EPS did not substantially increase in the scytonemin-producing wild type exposed to UVA as hypothesized. Although this is supported by a previous study which did not see an increase in EPS production under UVA (less than 2 W m^{-2}) in *N. commune* DRH1 [11], the UVA intensity in our study was much higher at 8 W m^{-2}. Furthermore, oxidative stress was not examined in the previous study, and the availability of a scytonemin-deficient mutant allowed us the unique opportunity to study the

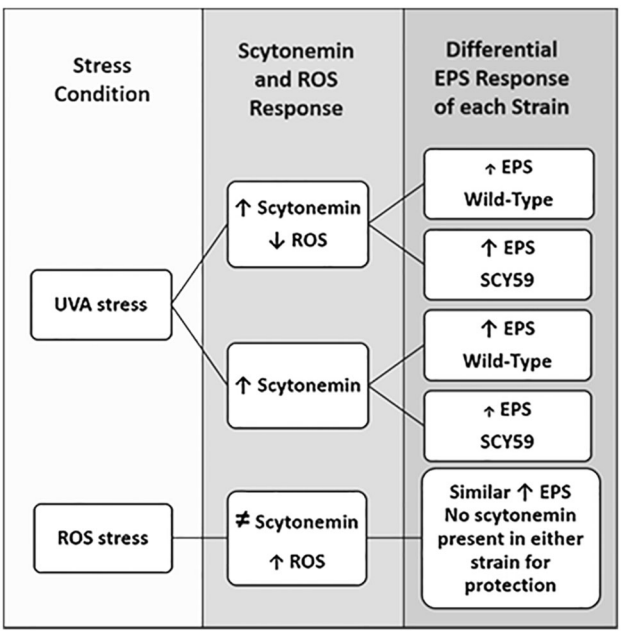

Fig. 3 Hypothesized differential EPS production response in each strain to UVA and ROS stress. *Up* and *down arrows* indicate an increase or decrease in the associated response, with the *smaller* and *larger arrow* size representing the magnitude of the change compared to the other strain, and (*not-equal to symbol*) means that scytonemin is not present under this condition

effect of UVA in the absence of scytonemin in *N. punctiforme*.

The localization of EPS and scytonemin to the extracellular sheath should provide opportunities for EPS constituents and scytonemin to cooperatively aid in UVA attenuation. For instance, a noncovalent interaction between scytonemin and the secreted water stress protein WspA was observed in *N. commune* upon UVR and desiccation stress. The authors suggested that this interaction could lead to the formation of stable complexes in the extracellular matrix [40]. Thus, it would be reasonable to hypothesize that, while maybe not essential, a scytonemin-WspA complex contributes to either the stability of the EPS or to the stability of scytonemin within the EPS, especially during desiccation or UVR stress. Indeed, scytonemin is retained during periods of desiccation and *N. punctiforme* has a higher scytonemin content under desiccation than when continuously hydrated [12]. While WspA homologs are not found in *N. punctiforme*, a similar stabilizing complex, such as with Npun_F1748, a *N. punctiforme* ortholog of a WspA-immunoreactive protein from *N. commune*, may be formed [40]. Furthermore, in a genomic microarray study of *N. punctiforme* exposed to UVA stress, approximately 20 contiguous genes on native Plasmid A were strongly up-regulated throughout the duration of the experiment, 24–96 h of UVA stress. Some of these were up-regulated as high as 90-fold over unstressed cells by 96 h. While most of these genes were of unknown function, two of these genes, Npun_AR040 and Npun_AR048, encode putative VirD4 and VirB4 proteins, respectively [31]. These proteins are part of a typical type IV secretion pathway (T4S) which are generally associated with pilus formation and conjugation, although some have been involved with the formation of sheathed structures [6].

Since the SCY59 mutant does not produce scytonemin and yet produced significantly more RPS than the wild type under UVA, it was hypothesized that the EPS in SCY59 served to provide protection against the ROS produced as a by-product of UVA radiation [17]. If indeed the increased RPS production in SCY59 was in response to ROS and not specifically to UVA or scytonemin, then both SCY59 and the wild type exposed to just oxidative stress should have an increase in RPS. This is because without UVA there will be no scytonemin in the wild type, and therefore no phenotypic difference between the two strains, since the phenotypic difference is likely limited to scytonemin biosynthesis [34]. As hypothesized, both strains responded similarly by an increase in RPS production following oxidative stress as compared to unstressed controls. While it is possible that unknown phenotypic differences between the wild-type and SCY59 mutant strain may have affected a consistent release of these polysaccharides in each strain, the data suggest that similar levels of RPS were obtained under the same conditions for both strains (unstressed in Fig. 1a, b and ROS stressed in Fig. 1b). It is also important to note that since the specific carbohydrates were not evaluated, we cannot discount the effect that different carbohydrate constituents may have had on the overall amount of RPS detected in each strain or condition tested.

The results from this study suggest that the cells may not be responding so much to the direct effects of UVA, but rather to the indirect effects of the ROS generated by UVA. Indeed, a microarray analysis of genes differentially expressed in response to UVA revealed eleven up-regulated genes associated with oxidative stress in the same *N. punctiforme* strain used in this study [31]. While scytonemin is one of the major mechanisms by which *N. punctiforme* protects itself from UVA stress [14], increased EPS has also been shown to protect cyanobacteria from ROS [8]. In particular, the polysaccharides from *Spirulina maxima* have been shown to have radical scavenging activity, particularly against the hydroxyl radical [41]. Additionally, the thick EPS of *Nostoc cordubensis* heterocysts was found to be essential for protecting the oxygen-sensitive enzyme nitrogenase from inhibition by oxygen [27]. While a thick EPS layer (heterocyst envelope polysaccharide) is essential for protecting nitrogenase from oxygen in all heterocyst-forming cyanobacteria, it may also play an important role in providing protection from oxidative stress as seen in this study. Even in nonheterocystous cyanobacteria, EPS seems to play a role in protecting nitrogenase, as seen in the unicellular aerobic nitrogen-fixing cyanobacterium *Cyanothece* BH68 [28]. While this seems to be the case for the strains mentioned here, it is worth noting that protection from oxidative stress by EPS may vary from strain to strain. As such, no added protection for nitrogenase activity was conferred upon a sheathed strain of *Gloeothece* as compared to a sheathless mutant strain [20]. Nonetheless, the data here demonstrate that oxidative stress results in an increase in EPS for both wild-type *N. punctiforme* and the scytonemin-deficient mutant strain SCY59, while UVA alone significantly increases the EPS in just the SCY59 mutant strain lacking scytonemin. Similarly, it was found that UVB radiation resulted in a significant increase in reducing sugars in the non-scytonemin-producing cyanobacterium *Microcoleus vaginatus* [5].

To our knowledge, this is the first study to evaluate the relationship between scytonemin and EPS production in cyanobacteria with a scytonemin-deficient mutant. By comparing the response of wild-type *N. punctiforme* to the scytonemin-deficient mutant SCY59, under both UVA and oxidative stress, it appears that EPS production is more closely related to oxidative stress than UVA radiation. Even the ROS produced as a by-product of UVA radiation was sufficient to significantly increase the amount of EPS

in SCY59 over the wild type and unstressed controls, further demonstrating the effect of ROS on UVA-stressed cells.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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