Nostopeptolide plays a governing role during cellular differentiation of the symbiotic cyanobacterium *Nostoc punctiforme*

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Nostoc punctiforme is a versatile cyanobacterium that can live either independently or in symbiosis with plants from distinct taxa. Chemical cues from plants and *N. punctiforme* were shown to stimulate or repress, respectively, the differentiation of infectious motile filaments known as hormogonia. We have used a polyketide synthase mutant that accumulates an elevated amount of hormogonia as a tool to understand the effect of secondary metabolites on cellular differentiation of *N. punctiforme*. Applying MALDI imaging to illustrate the reprogramming of the secondary metabolome, nostopeptolides were identified as the predominant difference in the *pks2*[−] mutant secretome. Subsequent differentiation assays and visualization of cell-type-specific expression of nostopeptolides via a transcriptional reporter strain provided evidence for a multifaceted role of nostopeptolides, either as an autogenic hormogonium-repressing factor or as a chemoattractant, depending on its extracellular concentration. Although nostopeptolide is constitutively expressed in the free-living state, secreted levels dynamically change before, during, and after the hormogonium differentiation phase. The metabolite was found to be strictly down-regulated in symbiosis with *Gunnera manicata* and *Blasia pusilla*, whereas other metabolites are up-regulated, as demonstrated via MALDI imaging, suggesting plants modulate the fine-balanced cross-talk network of secondary metabolites within *N. punctiforme*.

nonribosomal peptide | cell differentiation | symbiosis | MALDI imaging | signaling

The filamentous cyanobacterium *Nostoc punctiforme* displays a very complex and unique life cycle (1). When experiencing changes in environmental conditions or in response to chemical cues, individual vegetative cells in the filaments or entire trichomes are capable of entering distinct differentiation routes. Specifically, *N. punctiforme* can develop vegetative cells and nitrogen-fixing heterocysts that are regularly spaced within filaments, resting stages designated as akinetes, and motile hormogonia (1) (SI Appendix, Fig. S1). Remarkably, hormogonia can be internalized by plants from three distant taxa; namely, the bryophytes *Blasia* and *Anthoceros*, the angiosperm *Gunnera*, and cycads such as *Macrozamia* spp. (2). A given strain of *N. punctiforme* is capable of infecting all distinct plant partners, yet the plant hosts undergo very different morphogenetic changes on symbiotic interactions (2). In hornworts and *Gunnera* species, the major changes occur in contact cells of specialized cavities (auricles) and glands, respectively, both of which are preexisting symbiotic organs. Cycads, in contrast, develop so-called coralloid roots on infection (3). These symbiont-induced changes on the plant side indicate a rather long coevolution process, yet no gene transfer, genome reduction, or dual-partner gene products have been documented for plant–*Nostoc* symbioses (3). The partnership is considered mutualistic or commensal, with plants

providing fixed carbon in exchange for fixed nitrogen from the cyanobacteria (3).

Still, the interaction is only facultative for *Nostoc*, suggesting that under specific conditions, the benefit gained is outweighed by the costs. Hence, the infection process needs to be tightly balanced. The tug-of-war between plants and symbiotic cyanobacteria is exemplified by the reciprocal influence of both partners on hormogonia differentiation (4). Plant hosts secrete yet-unidentified hormogonium-inducing factors (HIF) (5) to stimulate development of the infectious filaments that remain motile for 48–72 h (6). Once the *Nostoc* culture has completed a cycle of HIF-induced hormogonium formation, there is a lengthy delay before hormogonium formation can occur again (6). Meeks has introduced the term “immunity period” for this phase, during which no secondary infection can be initiated, even in the presence of HIFs (5). *Nostoc* apparently contributes autogenic secretory factors to this hormogonia repression, as an exchange of growth medium stimulates hormogonia formation (7). Similarly, hormogonia are repressed inside plant hosts by an unidentified hormogonium-repressing factor (HRF), whereas vegetative filaments with a high abundance of heterocysts are maintained.

Sequencing of *N. punctiforme* ATCC (American Type Culture Collection) 29133 has paved the way for a substantial molecular analysis of the complex lifestyle of this symbiotic strain (8). Array data showing the response to different environmental stimuli have provided insight into the complex regulatory network of *N. punctiforme* (9, 10). Several gene loci were shown to modulate

Significance

Nostoc symbioses with plants represent one of the most versatile and ancient types of symbioses. The infection process is a tug-of-war between the plant host and the cyanobacterial symbiont, with a reciprocal influence of both partners on the differentiation of the infectious motile filaments, hormogonia. In the present study, we have uncovered a major hormogonium-repressing factor of *Nostoc punctiforme*, nostopeptolide. To our knowledge, the nonribosomal peptide is the first complex secondary metabolite of cyanobacteria for which a governing role during cellular differentiation could be demonstrated. Different plant partners were shown to strictly downregulate the factor in symbiosis, thereby unveiling a complex cross-talk network between plants and *Nostoc*.

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Fig. 2. Comparison of microscopic features of *N. punctiforme* wild-type and *pks2*⁻ mutant with and without nostopeptolide addition under diazotrophic conditions (liquid culture). (A) Microscopic pictures of *pks2*⁻ mutant (Left), wild-type (Right), and *pks2*⁻ mutant treated with 500 ng/mL nostopeptolide (+Nos, mid) after 14 d of cultivation. H, hormogonia; V, vegetative cells; P, primordia; arrows, heterocysts. (B) Graphic representation of the median percentage of hormogonia after exchange of medium with and without addition of 100–1,500 ng/mL nostopeptolide A in three replicates of *N. punctiforme* wild-type. Shown are quantitative data for cultures grown under diazotrophic conditions (N₂) or with ammonium as nitrogen source (NH₄). Please see *SI Appendix, Table S2* and *Fig. S8* for statistics on further filament types and representative micrographs obtained with 1,000 ng/mL nostopeptolide, respectively.

supplementation were analyzed after exchange of the medium to fresh BG11₀ or BG11₀+NH₄, respectively, with or without the addition of 100–1,500 ng/mL nostopeptolide A. Medium exchange generally triggered hormogonium formation with a maximum after 2 d, where up to 75% of the filaments were hormogonia in the untreated cultures (Fig. 2B and *SI Appendix, Fig. S8*). The addition of nostopeptolide led to a gradually increasing hormogonium repression effect, and the addition of 1,500 ng/mL nostopeptolide A completely abolished hormogonium formation in all cultures. In diazotrophic medium, the HRF activity was clearly visible with 500 ng/mL nostopeptolide A, whereas in cultures grown with ammonium, 1,000 ng/mL nostopeptolide A was required to get a noticeable repression effect. These data provide very clear evidence that nostopeptolide represents an autogenic HRF.

To test whether nostopeptolide addition can complement the *pks2*⁻ phenotype on diazotrophic agar plates, a *pks2*⁻ colony was placed in 1 cm distance to another *pks2*⁻ colony, a wild-type colony, or a paper disk soaked with different concentrations of nostopeptolide A. When a *pks2*⁻ colony or a paper disk containing only solvent was placed at 1 cm distance, *pks2*⁻ colonies showed a very even distribution of hormogonia surrounding the colonies (Fig. 3A and C). Placing a wild-type colony in the neighborhood of a *pks2*⁻ colony, in contrast, led to a reduction of the amount of hormogonia in the *pks2*⁻ mutant on the side facing the wild-type colony (Fig. 3B). Similarly, when 50 μg nostopeptolide A was spotted on the paper disk, the hormogonia differentiation and spreading on the side facing the nostopeptolide paper disk was reduced. In addition, the colony showed a darker appearance compared with the untreated

control (Fig. 3E). Strikingly, spotting of only 10 μg nostopeptolide A on the paper disk led to a chemoattractive response, with hormogonia moving toward the paper disk (Fig. 3D). When *N. punctiforme* wild-type or *pks2*⁻ mutant were directly grown on top of paper discs soaked with nostopeptolide A, 10 μg was sufficient to repress hormogonium formation over a period of up to 6 wk (*SI Appendix, Fig. S9*). These results substantiate the hypothesis that nostopeptolide is a major hormogonium-repressing factor. We also aimed to construct a knockout strain completely impaired in nostopeptolide production. However, numerous attempts with two independent constructs targeting the *nosA* and the *nosC* genes did not yield a single mutant clone.

Nostopeptolide Is Not Differentially Regulated on the Transcriptional Level, but on the Level of Secretion During the Differentiation Cycle.

Cell-type-specific expression of nostopeptolide biosynthesis genes was evaluated using a transcriptional reporter construct in which the 500-bp-long 5' UTR of the *nosA* gene was fused to a cyan fluorescence protein (CFP) gene (*P-nosA*-CFP) and expressed from an autonomously replicating plasmid. The *N. punctiforme* wild-type strain revealed a low background in the CFP channel (Fig. 4). The *P-nosA*-CFP mutant yielded specific fluorescence signals in vegetative cells, hormogonia, and akinetes; however, it did not do so in heterocysts (Fig. 4A–I). No major differences could be observed in the intensity of *P-nosA*-CFP signals in different stages of the differentiation cycle. This is in agreement with microarray data that have evaluated transcriptional variations of the *nosA-G* genes during the hormogonium differentiation phase (10). To test the hypothesis that nostopeptolide is regulated on the level of secretion, nostopeptolide A was quantified from cell extracts and supernatants. In agreement with the transcriptional data, the amount of cell-bound nostopeptolides (constituting up to 99% of the nostopeptolide pool) was constantly increasing parallel to growth (Fig. 4J). Subcellular fractionation clearly demonstrated that this cell-bound nostopeptolide portion was localized in the extracellular sheath (*SI Appendix, Fig. S10*). In contrast, the amount of released nostopeptolide A showed clear variations with a minimum between day 2 and 5 after a transfer to fresh diazotrophic medium (hormogonium differentiation phase). The concentration of secreted nostopeptolide A increased to around 500 ng/mL at day 7 and thereafter (Fig. 4K), equaling the amount that had resulted in the hormogonium repression effect (Fig. 2B). This could indicate that the typical immunity period after an initial round of hormogonium formation is safeguarded by secreted nostopeptolides. Further support for a regulation of nostopeptolides on the level of

Fig. 3. Comparison of macroscopic features of *N. punctiforme* wild-type and the *pks2*⁻ mutant with or without nostopeptolide addition under diazotrophic conditions on plate. *pks2*⁻ mutant colonies were spotted at a 1 cm distance to (A) a second *pks2*⁻ colony and (B) a wild-type colony. (C–E) Paper discs soaked with 0–50 μg nostopeptolide A, as indicated.

Fig. 4. Fluorescence micrographs of a P-nosA-CFP transcriptional reporter strain under diazotrophic conditions and dynamics of nostopeptolide production and secretion. (A and D) P-nosA-CFP strain visualized using phase contrast microscopy. (B and E) P-nosA-CFP strain visualized in the CFP channel. (C and F) Red autofluorescence of P-nosA-CFP strain. (G–I) Control micrographs of ATCC29133 wild-type. (H) Hormogonia; P, primordia; V, vegetative filaments; A, akinetes; arrows, heterocysts. (J) Quantitative amount of cell-bound nostopeptolide A (NPL A) detected in *N. punctiforme* at different stages of growth under diazotrophic conditions. Shown is the median of two biologically independent cultures. (K) Quantitative amount of secreted nostopeptolide A (NPL A) in the same cultures. (L, Top) Macroscopic picture of *N. punctiforme* wild type and *pks2*[−] mutant in coculturing experiments. (Middle) False color intensity representations of selected nostopeptolide analogs from MALDI imaging experiments showing asymmetric secretion patterns. −N diazotrophic conditions; +N, nitrogen-replete conditions.

secretion comes from MALDI imaging analyses. When wild-type colonies were placed in the direct neighborhood of *pks2*[−] colonies under either nitrogen-deplete (−N) or nitrogen-replete (+N) conditions (Fig. 4L), nostopeptolides showed asymmetric secretion patterns around the wild-type colony. Under −N conditions, secretion of nostopeptolides was increased at the colony side facing the *pks2*[−] colony, and under +N conditions, secretion of nostopeptolides was more pronounced on the side opposite the *pks2*[−] colony. When two wild-type colonies were placed in direct neighborhoods, nostopeptolides showed symmetric secretion patterns around the colonies (SI Appendix, Fig. S11). This can be taken as an indication that, depending on the availability of solute nitrogen, metabolites released by the *pks2*[−] mutant are either stimulating or repressing nostopeptolide secretion.

Nostopeptolide Is Down-Regulated in Planta and in Response to Blasia Signals. To gain insight into the expression of nostopeptolides and other secondary metabolites in symbiotic interactions, seedlings of *G. manicata* were infected with the wild-type and the *pks2*[−] strain and cocultivated for 9 mo. Thin slices of the shoot basis of *G. manicata* plants hosting either wild-type or mutant were analyzed by MALDI imaging (Fig. 5). In parallel, *N. punctiforme* grown in the free-living state was spotted on the same MALDI slide. The majority of metabolites detected in planta could be assigned to the cyanobacterial biomass in the glands. Whereas a considerable number of metabolites were expressed both in the free-living state and in planta, several metabolites were either up- or down-regulated (SI Appendix, Fig. S12). None of the up-regulated metabolites could be identified or

assigned to a known metabolite family (Fig. 5E and F). The specific masses and fragment ions of nostopeptolides could not be detected in *G. manicata* infected with *N. punctiforme* wild-type and *pks2*[−] mutant (Fig. 5D), indicating that the peptide is down-regulated in symbiosis or altered by plant cells in the direct proximity.

We selected *B. pusilla* as a second symbiosis host in our analysis. The fragile liverwort tissue, however, was not suitable for MALDI imaging experiments. As an alternative approach, the host was infected with the P-nosA-CFP reporter strain. No specific CFP signal could be detected inside the liverwort, even though the cyanobacterial autofluorescence was perfectly visible (Fig. 6J–L). This is a clear hint that nostopeptolide is strictly down-regulated in symbiosis. A P-nosA-CFP culture grown in parallel in the free-living state showed the regular pattern of nostopeptolide gene expression (Fig. 6G–I). To see whether *B. pusilla* exudate is sufficient to repress nostopeptolide expression, the P-nosA-CFP strain was cultivated in parallel either with or without *B. pusilla* medium supplementation. Remarkably, the P-nosA-CFP signal was strongly reduced in the presence of the *B. pusilla* medium (Fig. 6D–F) compared with the control without supplementation (Fig. 6A–C). Thus, there are different lines of evidence in two different symbiosis hosts, indicating that nostopeptolide is down-regulated in planta and in response to plant exudates.

Discussion

It has long been suggested that the complex lifestyle of *N. punctiforme* is influenced by autogenic secretion factors that act at least partly antagonistic to plant factors that stimulate the infection process (1). The present study uncovers the identity of one of those factors and demonstrates that nostopeptolide plays a crucial role in the orchestration of cellular differentiation under diazotrophic conditions.

Remarkably, different concentrations of released nostopeptolide had completely different effects on cellular differentiation and the direction of motility of *N. punctiforme*. A possible explanation for this observation is that other factors produced by *N. punctiforme* interfere with the signaling process triggered by nostopeptolides, and that the ratio between the different factors finally determines the route of differentiation and the direction

Fig. 5. Effect of symbiotic interaction with *G. manicata* on the secondary metabolome of *N. punctiforme* and the *pks2*[−] mutant. (A) Macroscopic picture of the shoot basis of *G. manicata* infected with *Nostoc* 9 mo postinfection. (B) Microscopic picture of cyanobacteria inside host cells in the *G. manicata* glands. (C, Top) Macroscopic picture of symbiotic tissue of *G. manicata* harboring *N. punctiforme* wild-type and *pks2*[−] mutant. 1, *N. punctiforme* wild-type in BG11₀ liquid medium; 2, *N. punctiforme* wild-type in *Gunnera* gland; 3, *pks2*[−] mutant in BG11₀ liquid medium; 4, *pks2*[−] mutant in *G. manicata* gland. (D–F) MALDI imaging false color intensity representation of selected metabolites. Intensities are reflected by a heat map (Right). For a detailed overview, see SI Appendix, Fig. S12. (D) Selected nostopeptolides that are down-regulated in symbiosis. (E) Selected peptide only produced in symbiosis by the *pks2*[−] mutant. (F) Selected metabolites that are only produced in symbiosis. (G) Selected metabolites that are produced both in the free-living state and in symbiosis by both wild-type and *pks2*[−] mutant.

Fig. 6. Fluorescence micrographs of a P-*nosA*-CFP transcriptional reporter strain treated with supernatant of *B. pusilla* or in symbiosis. (A–F) Comparative analysis of P-*nosA*-CFP strain-treated *Blasia* exudate and untreated control. (A–C) Control micrographs showing P-*nosA*-CFP strain without *Blasia* exudate addition. (D–F) Micrographs showing P-*nosA*-CFP strain treated with *Blasia* exudate. (G–L) Comparative analysis of P-*nosA*-CFP strain inside and outside of symbiotic host. (G–I) Control micrographs of P-*nosA*-CFP strain grown in the free-living state. (J–L) Micrographs showing P-*nosA*-CFP strain in symbiosis with *B. pusilla*.

of motility. Evidence for an interconnection of secondary metabolites in *N. punctiforme* comes from the metabolomic comparison of the wild-type and the *pks2*[−] mutant strains, using MALDI imaging. Loss of the cryptic *pks2* product has led to a pronounced reprogramming of the secondary metabolome. A knockout mutant impaired in nostopeptolide production could have strengthened this view. However, despite numerous attempts, we could not generate a mutant. Although we cannot rule out the possibility that this failure is a result of technical problems, this could be a hint that nostopeptolide is essential for the strain in the free-living state. Further support for a metabolite cross talk in *N. punctiforme* is provided by the MALDI imaging analysis of neighboring wild-type and *pks2*[−] colonies. Nostopeptolide secretion was apparently influenced by other factors released by the *pks2*[−] mutant.

Strikingly, MALDI images suggested that the entire nostopeptolide fraction is located outside cells, contradicting the fact that nostopeptolides were primarily detected in cellular extracts. Subcellular fractionation studies, however, could solve this discrepancy and demonstrate that the cell-bound fraction is localized in the extracellular polysaccharide sheath. Together, these findings suggest that nostopeptolide is regulated neither on the level of transcription nor at the level of membrane transport in the free-living state. The dynamics observed are apparently the result of a dynamic release and reuptake of nostopeptolide from and into the sheath. The metabolite cross-talk indicated on the MALDI neighboring images may thus reflect a reciprocal influence of different secretion factors on nostopeptolide release from the extracellular polysaccharide matrix. The identity of the factors that bind nostopeptolides in this matrix remains elusive. Lectin-binding studies have revealed that the sugar composition

of the *N. punctiforme* extracellular polysaccharide is changing during the hormogonium differentiation phase and the following immunity phase (24). Different types of extracellular polysaccharide may have different capacities to “hold” nostopeptolides, and may thus interfere with the secondary metabolite cross-talk.

Nostopeptolide was down-regulated in two completely different symbiotic hosts, as shown using MALDI imaging and a transcriptional reporter strain, respectively. This is particularly surprising, as nostopeptolide is expressed under all conditions in the free-living state. In sharp contrast, the exudate of the *B. pusilla* host led to complete down-regulation of the *nos* cluster. This could be an indication that nostopeptolide is one of the primary targets of the *B. pusilla* signal or signals. Apparently, nostopeptolide is not essential for the lifestyle of *N. punctiforme* in symbiosis. In contrast, several low-molecular-weight metabolites detected inside *G. manicata* were never observed in the free-living state. The “plant-induced” metabolites could potentially result from a biotransformation of *N. punctiforme* metabolites by plant cells in close proximity or may result from tissue effects that may lead to an altered ionization. The lack of nostopeptolides, however, corresponds well to the down-regulation of the biosynthetic gene cluster on the transcriptional level in the second symbiosis host. One may therefore speculate that other metabolite families are being up-regulated as response to plant factors. This observation can inspire the development of novel genome mining strategies for plant-induced secondary metabolites from *Nostoc* species. Similar observations were made for the interaction of Streptomyces and fungi. Only the intimate physical interaction of the partners could stimulate the production of certain types of metabolites, including the archetypical metabolite lecanoric acid that was previously known from lichen (25).

Considering the complexity of the lifestyle of *N. punctiforme* and the multitude of distinct secondary metabolite families that can be estimated for the strain, one can speculate that nostopeptolide represents only the tip of an iceberg. Future studies have to show the nature of other factors balancing the effects of nostopeptolide, to solve the structure of the cryptic *pks2* metabolite, and to identify the nostopeptolide-signaling cascade and the mechanism regulating nostopeptolide secretion. Understanding the lifestyle of symbiotic cyanobacteria and the role of secondary metabolites in the process can provide fascinating insight into one of the most versatile and ancient partnerships on earth.

Materials and Methods

Organisms and Cultivation Conditions. Cyanobacteria were maintained under permanent white light at the illumination intensity of 30 $\mu\text{mol photons}/\text{M}^2\text{s}^{-1}$ at 23 °C in 50 mL BG11₀ for diazotrophic growth or in BG11 for nitrogen-supplemented cultures (26). Ammonium chloride was added at a concentration of 2.5 mM when used as alternative nitrogen source. In all assays on agar plates, the media contained 0.8% agar. The medium for the *pks2*[−] mutant strain was supplemented with 12.5 $\mu\text{g}/\text{mL}$ neomycin, and the P-*nosA*-CFP mutant was maintained on media with 2 $\mu\text{g}/\text{mL}$ streptomycin. The antibiotics were omitted when strains were cocultivated with other strains or organisms. Cell density was measured by estimation of Chl *a* content, according to ref. 27.

The hepatic *B. pusilla* was grown under the same light and temperature conditions in 1/5 BG11 or 1/5 BG11₀. To obtain active supernatant, the culture of *B. pusilla* was transferred to nitrogen-free medium and starved for 5 wk, as described earlier (28). *G. manicata* Linden was propagated from fresh seeds collected in the greenhouses of the Department of Botany, University of Stockholm, Sweden. The propagation procedure and media used were according to Johansson and Bergman (26). Seedlings with one true leaf were transferred to sterile plastic boxes filled with vermiculite soaked with the medium and were grown hydroponically under sterile conditions.

Symbiosis and Differentiation Assays. A loopful of cyanobacterial culture was transferred to the nitrogen-starved *B. pusilla* liquid culture and grown for 3 wk. The effect of plant exudates was tested in medium composed of one part filtered plant-free supernatant from starved *B. pusilla* and one part

BG11₀. Media for control cultures was made by mixing 1/5 BG11₀ and BG11₀. *G. manicata* seedlings were infected by the addition of 10 μ L cyanobacterial suspension to the apex. Reinoculations were repeated every 4 wk. For differentiation assays, cyanobacterial cultures were grown to a cell density of $\sim 5 \mu\text{g/mL}$ Chla. The cultures were washed three times with fresh medium and then inoculated into the respective medium to a cell density of 0.2–0.3 $\mu\text{g/mL}$ Chla. Nostopeptolide was added to a final concentration of 10–1,500 ng/mL. The respective volume of solvent [50% (vol/vol) methanol] was added to the control cultures. Samples for microscopic observations were taken after 2, 5, and 14 d. For each sample, 1,000 filaments were counted in focus plane in randomly taken snapshots. Microscopic analyses were performed on a Leitz DMRBE microscope equipped with Leica DFC420 camera and filters N2.1 for autofluorescence and CGFP for detection of CFP. Image acquisition was done in related LAS Version 3.4.1 software (Leica Microsystems).

Assays on Agar Plates. For testing of mutual influence on motility and growth, 20 μ L cell suspension from late exponential cultures was pipetted on the surface of agar at a 1-cm distance. The 6-mm filter paper disks with or without nostopeptolide were applied in the same way. Incubation time was 3 wk.

MALDI Imaging. Thin-layer BG11 and BG11₀ agar plates of *N. punctiforme* wild-type and *pks2*[−] mutant monocultures or bacterial interactions were inoculated from fresh liquid cultures and incubated for 10 d (29). Sample preparation, matrix application, and dehydration were conducted as previously described (30). For MALDI imaging experiments of plant cyanobacteria symbiosis, *G. manicata* plants harboring the symbiotic glands were cut into thin slices. The stem slices were immobilized on conductive glass slides. Universal MALDI matrix in methanol was sprayed onto the plant samples. Data were acquired on a Bruker UltrafleXtreme MALDI TOF mass spectrometer equipped with smart beam technology in positive mode. Method optimization was conducted using FlexControl3.0 (Bruker Daltonic GmbH). Data were analyzed with FlexImaging3.0 (Bruker Daltonic GmbH). False colors were assigned to masses of interest and superimposed onto the optical reference picture taken before matrix application. Single spectra analysis on different spatial localizations for spectral comparison was performed using FlexAnalysis3.0 (Bruker Daltonic GmbH).

Generation of a Reporter Mutant for the Nostopeptolide Biosynthesis 5' UTR.

The 5' UTR of Npun_F2181 (*P-nosA*) was amplified by PCR, using the primer pair *NosA_Prom_SacI_FW* and *NosA_Prom_NdeI_RV*, thereby introducing a *SacI* and an *NdeI* site. The *cfp* gene was obtained from the pECFP-C1 vector (Clontech Laboratories) by PCR, using the primer pair *E-CYFP-FW* and *ECFP-RV*, thereby introducing *NdeI* and *BamHI* sites for subsequent ligation into the vector pRL1049 and introducing a stop codon for the *cfp* gene. The resulting construct pRL1049-Npun_F2181CFP was transferred into *N. punctiforme* by electroporation at 1.5 kV in a Bio-Rad-MicroPulser. The reaction mix was immediately diluted with BG11 medium supplemented with 2.5 mM NH_4Cl + 5 mM Mops + 20 mM MgCl_2 and plated onto HATF filters (Millipore) on plates containing BG11 medium. After initial growth, the filters were transferred to selective plates containing 2 $\mu\text{g/mL}$ streptomycin. The obtained mutant was tested by PCR analysis, using a primer pair targeting the construct-flanking region, and the resulting amplicon was sequenced.

Nostopeptolide Quantification. Strains were precultured for 1 wk in BG11₀ with 2.5 mM NH_4Cl and divided in cultures of 50 mL BG11₀. Cells were harvested by centrifugation after 0–14 d. Supernatants were purified on C18-Sep-Pak cartridges (Waters). Cell pellets were lysed by sonication and finally dissolved in methanol. HPLC was conducted on a Shimadzu HPLC unit comprising the system controller CBM-20A, the pump LC-20AD, the auto-sampler SIL-20AC HT, and the photo diode array detector SPD-M-20A. Separation was carried out on a SymmetryShield RP18 column (Waters). HPLC profiles were monitored at the wavelength 199 nm, and peaks were integrated and calculated using a quantitative nostopeptolide reference.

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