Temporal dynamics of natural product biosynthesis in marine cyanobacteria

Eduardo Esquenazi[a,b], Adam C. Jones[a], Tara Byrum[a], Pieter C. Dorrestein[a,c,d], and William H. Gerwick[a,c,1]

[a]Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, [b]Department of Biology, [c]Skaggs School of Pharmacy and Pharmaceutical Sciences, and [d]Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0212

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Sessile marine organisms are prolific sources of biologically active natural products. However, these compounds are often found in highly variable amounts, with the abiotic and biotic factors governing their production remaining poorly understood. We present an approach that permits monitoring of in vivo natural product production and turnover using mass spectrometry and stable isotope (^{15}N) feeding with small cultures of various marine strains of the natural product-rich cyanobacterial genus *Lyngbya*. This temporal comparison of the amount of in vivo ^{15}N labeling of nitrogen-containing metabolites represents a direct way to discover and evaluate factors influencing natural product biosynthesis, as well as the timing of specific steps in metabolite assembly, and is a strong complement to more traditional in vitro studies. Relative quantification of ^{15}N labeling allowed the concurrent measurement of turnover rates of multiple natural products from small amounts of biomass. This technique also afforded the production of the neurotoxic jamaicamides to be more carefully studied, including an assessment of how jamaicamide turnover compares with filament growth rate and primary metabolism and provided new insights into the biosynthetic timing of jamaicamide A bromination. This approach should be valuable in determining how environmental factors affect secondary metabolite production, ultimately yielding insight into the energetic balance among growth, primary production, and secondary metabolism, and thus aid in the development of methods to improve compound yields for biomedical or biotechnological applications.

The secondary metabolites of marine organisms are a valuable and inspirational source for a host of biomedical and technological applications. Since the emergence of marine natural products research as a discipline 40 years ago, approximately 17,000 compounds have been isolated from a variety of prokaryotic and eukaryotic organisms, and several of these have advanced from initial evaluations of bioactivity to preclinical and clinical trials that target specific diseases (1, 2). Of prokaryotic marine natural products identified and evaluated for their biomedical potential, it is estimated that approximately 40% are of cyanobacterial origin (1).

Natural products from marine cyanobacteria have a wide range of bioactivities (2), and in the last decade, considerable advances have been made in elucidating the enzymatic mechanisms used in compound biosynthesis (3, 4). However, the natural functions these molecules serve in the producing organism are comparatively less well understood. Some cyanobacterial secondary metabolites appear to have a protective role (5), acting as feeding deterrents (6) or toxins (7), while others may be involved in microbial communication as quorum sensors (8). As with plants or algae (9, 10), the production rate of a specific cyanobacterial compound may vary based on its effective concentration, genetic or environmental regulatory factors, or the presence of an inducing action. The energetic expense of secondary metabolite production (9) may also lead to variability in its biosynthesis depending on available resources or specific ecological conditions. This variability is often observed during laboratory secondary metabolite isolation efforts, where compound amounts can range from trace (submilligram) levels (11) to instances where a single natural product is the major component of a given extract (12). Low compound yields, in addition to very slow growth in culture (with doubling times of 6 days or longer in some cases) (13), are among the most significant impediments to further pursuit of marine cyanobacterial lead compounds for biotechnological applications.

One strategy to assess the relative importance of a compound to an organism in different environmental conditions, as well as determine if overall production can be augmented, is to develop an effective means to monitor natural product turnover in relation to organism growth. Here, we show that the marine cyanobacterial genus *Lyngbya* provides for a powerful model system with which to conduct further investigation into the dynamics of natural product biosynthesis. *Lyngbya* strains are among the most important sources of cyanobacterial natural products yet discovered (14). Our laboratory has been successful in growing a variety of marine *Lyngbya* strains (13, 15), and many of these have remained stable in laboratory culture for several years (13). By growing *Lyngbya* cultures in ^{15}N-enriched growth media and probing over time using micro-extraction and MALDI mass spectrometry, the dynamics of cyanobacterial natural product biosynthesis can be observed, and conditions that may influence the turnover of these molecules can be tested.

There have been previous reports of using ^{15}N to track the biosynthesis of chlorophyll and its derivatives in cyanobacteria (16) as well as in plants (17). Recently, sub-1,000 Da natural products produced by *Lyngbya* have been visualized directly using matrix-assisted laser desorption ionization (MALDI) imaging techniques (18, 19), overturning the common belief that matrix-related interference during MALDI compromises the effectiveness of small molecule detection. MALDI time-of-flight (TOF) MS is a highly sensitive method that involves minimal sample preparation and straightforward data analysis (20). While liquid chromatography–mass spectrometry (LC-MS) or NMR in conjunction with isotope-based methods have been applied to the investigation of natural product biosynthesis and metabolite dynamics (21, 22), the MALDI-based approach is complementary with particular attributes in the ease, cost efficiency, and rapidity of analysis of very large numbers of crude samples.

Understanding the time course and factors influencing the production of cyanobacterial natural products is important for determining ecological functions of compounds, as well as how to modify yields from laboratory cultures; however, an experimental framework by which to reliably measure their production in vivo has been lacking. By comparing and monitoring the changes in the isotopic profiles of metabolites arising from

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[1]To whom correspondence should be addressed. E-mail: wgerwick@ucsd.edu.

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$[^{15}N]NaNO_3$-enriched cultures of *Lyngbya*, such as pheophytin *a*, a stable breakdown product of chlorophyll *a* (23) and various other metabolites, it is possible to determine the percentage of ^{15}N labeling (representing new biosynthesis) of each compound at various time points and, in turn, gain insight into their turnover rates in vivo. Herein, the results of several studies with *Lyngbya* strains are provided; these data collectively suggest that combining MALDI mass spectrometry and ^{15}N feeding is a powerful way to monitor natural product production rates, including relative differences between primary and secondary metabolites, and provide new means of manipulating their production. The approach is also applied to investigate the timing of compound biosynthesis and complements more traditional in vitro approaches by providing insights of in vivo relevance.

Results

Metabolome Wide Turnover of Nitrogen-Containing Metabolites in Three Different *Lyngbya* Strains. Cultures of *Lyngbya majuscula* 3L (13), *Lyngbya bouillonii*, and *L. majuscula* JHB (15) were inoculated at comparable filament densities with media in which the $NaNO_3$ had been replaced with $[^{15}N]NaNO_3$ and sampled over a 10-day period. On days 1–5, 7, and 10, one to two filaments (approximately 0.5 mg wet weight) of each culture were analyzed by positive mode MALDI MS (see *Materials and Methods*). A variety of known metabolites displayed evidence of ^{15}N labeling (Fig. 1*A*). Other nitrogen-containing metabolites that have not been identified also underwent mass shifts (indicated with a red asterisk). From these spectra, it was possible to accurately calculate and compare the average ^{15}N percent labeling for pheophytin *a* (23) (**1**) as well as the change in the percentage of fully

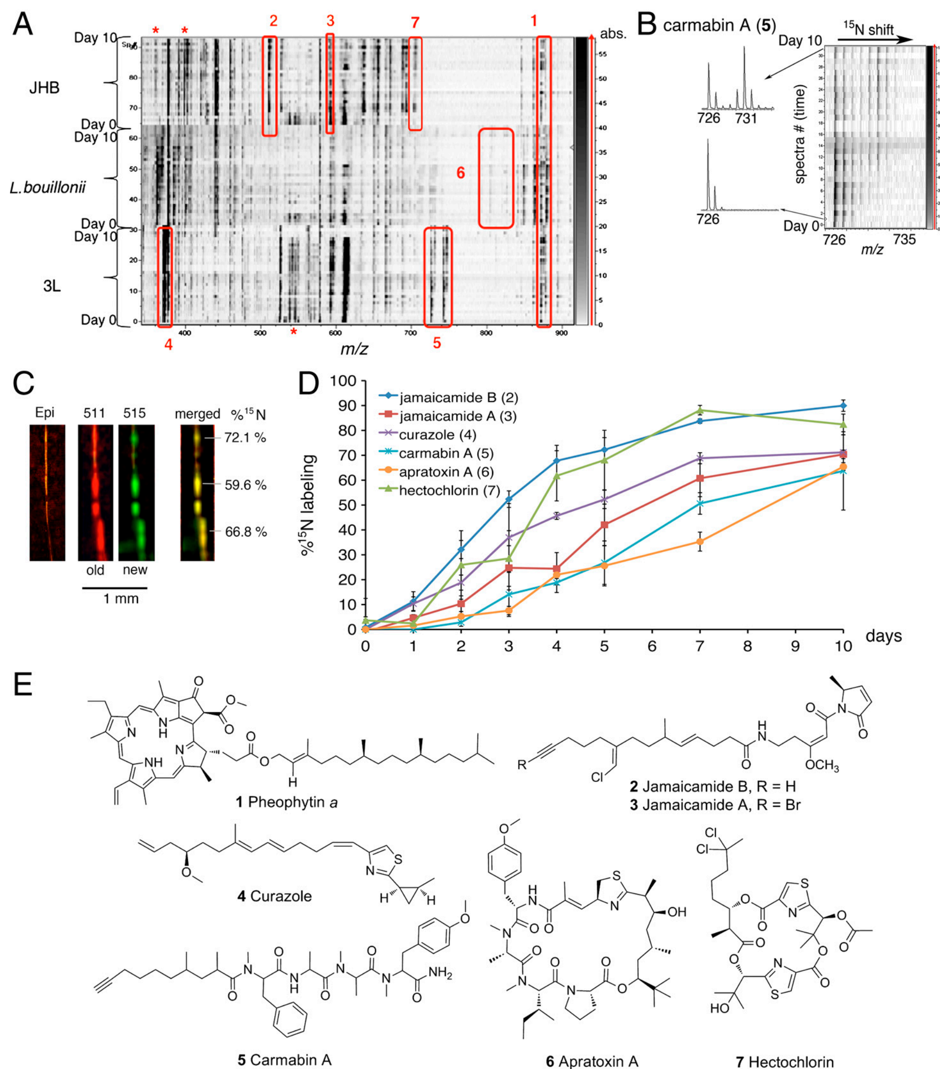

Fig. 1. ^{15}N labeling of nitrogen-containing metabolites from *L. majuscula* 3L, JHB and *L. bouillonii* over time. The culture conditions and time of inoculation were identical for the three species. (*A*) Isotope "heat map" view of *m/z* 300–950 MALDI spectra taken daily, with two technical and two experimental replicates per day, during the course of a 10-day feeding experiment (days 0–5, 7, and 10 shown). Shifting of the vertical spectral bands for each species indicates nitrogen-containing metabolites undergoing production and turnover. The red boxes and corresponding numbers highlight described, known metabolites; unknown metabolites can be also be seen undergoing nitrogen shifts (red asterisk). (*B*) Enlarged heat map showing region pertaining to carmabin A (**5**). The spectra for day 1 and day 10 are also provided, with the shift of 5 Da (pertaining to the 5 nitrogen atoms in carmabin A) appearing as heavier isotopic peaks that correspond to peak shifts in the heat map. (*C*) MALDI imaging of a *L. majuscula* JHB filament after six days of ^{15}N incubation. From left to right: an epifluorescence image of the filament at ex. 590 nm, the distribution of the *m/z* 511 peak (red represents older jamaicamide B) the *m/z* 515 peak (green represents only newly biosynthesized jamaicamide B), the overlayed distribution of the 511 and 515 peaks (yellow) annotated with the calculated percentage of labeling in different areas. (*D*) Percent ^{15}N labeling over the 10-day study for the compounds indicated by the red boxes: jamaicamide A, B, and hectochlorin from *L. majuscula* JHB, carmabin A and curazole from *L. majuscula* 3L, and apratoxin A from *L. bouillonii*. These calculations represent the percentage of fully ^{15}N-labeled molecules for each compound (33). ($N = 2$, error bars are SEM, calculated from two experimental groups, each with two technical replicates. See *Materials and Methods* for calculation.) (*E*) Chemical structures as discussed in text.

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^{15}N-labeled molecules of the natural products jamaicamide B (**2**) and A (**3**) and hectochlorin (**7**) in *L. majuscula* JHB, curazole (**4**) and carmabin A (**5**) in *L. majuscula* 3L, and apratoxin A (**6**) in *L. bouillonii* over time (Fig. 1*D*). Because the percentage of labeled molecule at any time point is a result of both newly biosynthesized ^{15}N-labeled molecules accumulating over the course of the experiment and elimination (via any mechanism, such as catabolism or excretion) of the older ^{14}N-labeled species, and because both the ^{15}N and ^{14}N version of the molecules have equal ionization efficiencies, the combination of these values represents an approximate measure of the net turnover of the metabolite.

MALDI imaging (18) of a *L. majuscula* JHB filament on day 6 of ^{15}N incubation (Fig. 1*C*) revealed that newly labeled jamaicamide B colocalizes with the older ^{14}N molecules and that the percentage of ^{15}N labeling along different parts of the filaments is comparable. Importantly, these results show that *L. majuscula* JHB filaments elongate by replication of cells throughout the length of the filament instead of in particular parts or on the filament ends.

Growth Rate and Turnover of Pheophytin *a* and Jamaicamide B in *L. majuscula* JHB. *L. majuscula* JHB, a strain responsible for producing the jamaicamides, hybrid PKS/NRPS natural products with neurotoxic and ichthyotoxic activity (15), was chosen for more detailed study due to its spectrum of produced metabolites. Jamaicamide B was selected for these comparisons due to its robust ionization and detection by MALDI. Pheophytin *a*, a more stable breakdown product of chlorophyll *a*, was chosen for its role as a primary metabolite critical to energy production in these organisms (it differs from chlorophyll *a* in the lack of a central magnesium atom, removed during the acidic MALDI preparation) (23).

In order to further compare the turnover of selected compounds, the growth rate for JHB filaments was defined under typical culture conditions (Fig. 2*A* and *SI Text*). In Fig. 2*B*, a comparison of the control and experimental isotopic profiles for each molecule over 10 days illustrates the progressive incorporation of ^{15}N into pheophytin *a* and jamaicamide B. The resulting percentage of ^{15}N labeling over time, calculated from weighted isotopic averages (23) is shown in Fig. 2*C*, with comparison to new growth. The average ratio of jamaicamide B to pheophytin *a* ^{15}N labeling during the 10 days was 2.1 ± 0.2 (SEM), indicating that jamaicamide B is turned over at twice the rate of pheophytin *a* in these cultures. To confirm the concentration of $[^{15}N]NaNO_3$ was sufficient to saturate the system with available nitrate throughout the time course of the experiment, a new culture was inoculated with 13-day-old ^{15}N media that had been used in a previous JHB labeling experiment. A comparison of the labeling rates for jamaicamide B was performed for both sets of media, with no significant differences observed when using the partially spent media (Fig. S1).

While the fate of jamaicamide B after biosynthesis was not clear from these experiments, the measured rates of turnover suggested that jamaicamide B is not accumulated within the cells, because by day 10, ^{15}N-labeled (or newly biosynthesized) jamaicamide B represents nearly all of the molecules present. To determine whether jamaicamide B was actively secreted from *L. majuscula* filaments into the culture medium, media from a dense JHB culture maintained in a Petri dish for 17 days was extracted and analyzed by MALDI-TOF MS. Neither jamaicamide B nor any other known natural products from *L. majuscula* JHB were present in sufficient concentrations (approximately 1 μM) to be detected in this media extract.

The utility of the ^{15}N labeling/MALDI-TOF approach was further explored by manipulation of culture conditions and comparing the resulting effect on jamaicamide B and pheophytin *a* turnover. Reducing the available nitrate in the media and exposure to UV light were two factors that showed appreciable impact, reducing the production of these metabolites (Fig. S1, *SI Text*, and *SI Materials and Methods*).

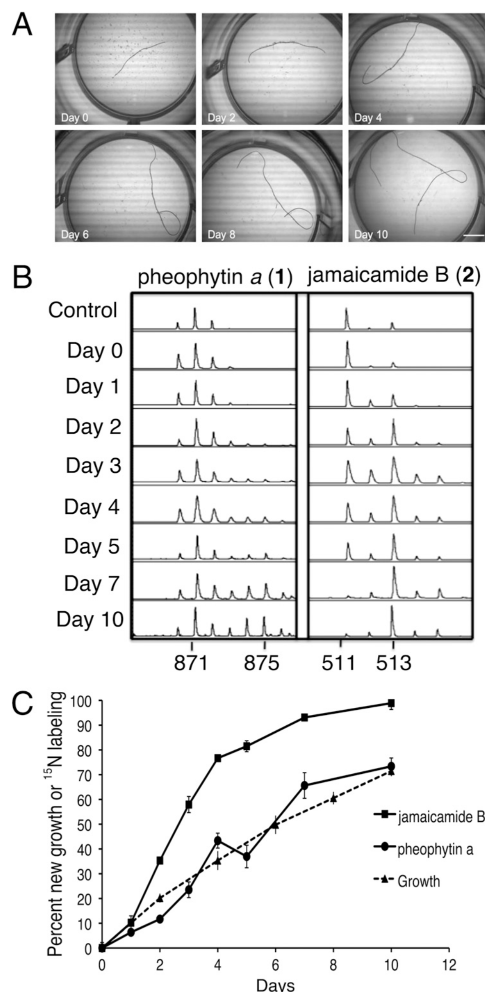

Fig. 2. Comparison of growth, percent ^{15}N labeling of jamaicamide B and pheophytin *a* over 10 days from small cultures of *L. majuscula* JHB. (*A*) Optical images (8x) show the growth of a single filament over the course of 10 days. (*B*) MALDI-MS spectra from micro-extractions showing the relative changes in the pheophytin *a* (left) and jamaicamide B (right) isotope clusters over time. Top panels contain control spectra for each molecule. Because the ionization efficiency is the same for a labeled and unlabeled compound, the decrease in the unlabeled, monoisotopic peaks indicates the reduced abundance of the ^{14}N containing species, while the increase in other peaks is indicative of newly synthesized, ^{15}N labeled species. (*C*) Comparison of growth and percentage of ^{15}N labeling of jamaicamide B and pheophytin *a* over 10 days. [Error bars are SEM. For ^{15}N labeling $N = 5$, for growth $N = 16$ (day 2), 10 (day 4), 7 (day 6), 5 (day 8), 3 (day 10).]

Comparison of ^{15}N Labeling States of Jamaicamide B with the Brominated Natural Product Jamaicamide A over 10 Days. The consistent difference in the rates of ^{15}N labeling of jamaicamide B and pheophytin *a* was surprising and provoked a similar analysis of the related compound jamaicamide A from the datasets depicted in Fig. 2. Jamaicamide A (**3**) is the brominated analog of jamaicamide B (**2**), and the terminal alkynyl bromide of jamaicamide A is thought to be incorporated via an uncharacterized halogenase encoded in the jamaicamide gene cluster (15). Because the isotopic cluster for jamaicamide A in the MALDI spectra overlaps with another chlorophyll breakdown product (likely pheophorbide *a*) (24), calculating an accurate weighted average of the cluster was not possible. Instead, a different but common calculation strategy (25) was employed in order to examine the differences in the rate of ^{15}N incorporation between these molecules. Because both molecules contain only two nitrogen atoms, it was possible to calculate the percentage of unlabeled vs. single, double, and total labeled molecules present at each time point by using only

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the first three peaks of both the jamaicamide A and B parent isotopic clusters (Fig. 3 *A* and *B*).

The results in Fig. 3*C* show that jamaicamide A is turned over more slowly than jamaicamide B. At day 6, approximately 78% of the jamaicamide A present in the sample is labeled whereas nearly 95% of jamaicamide B is labeled. There is a delay of approximately 1.5 days before a sharp increase in ^{15}N labeling of jamaicamide A when compared to jamaicamide B (T_{50} for jamaicamide $B = 2.5$ days, T_{50} for jamaicamide $A = 4.0$ days). One conceivable hypothesis to explain these observations is that the bromination reaction to form jamaicamide A occurs after the biosynthesis of jamaicamide B is completed (15).

The relationship between jamaicamide A and B biosynthesis was further investigated with a series of ^{15}N feeding experiments conducted with *L. majuscula* JHB using small cultures inoculated in 24 well plates. First, to determine if bromide might be a limiting nutrient in the biosynthesis of jamaicamide A, additional sodium bromide was added to the ^{15}N culture media. The simple addition of NaBr to the media in either 0.5 g/L or 1.0 g/L resulted in a significant and stepwise increase in the percentage of ^{15}N-labeled jamaicamide A (Fig. 4*A*), indicating that the concentration of bromide in seawater (*ca.* 65 ppm) is a limiting nutrient in jamaicamide biosynthesis. However, the addition of bromide still did not result in an equal ^{15}N labeling rate to that of jamaicamide B.

To further explore the hypothesis that the bromination of jamaicamide A occurs after the assembly of jamaicamide B, an additional experiment was performed using a similar ^{15}N feeding time course with the inclusion of a dark phase (Fig. 4*B*). In preliminary trials, it was found that *L. majuscula* JHB cultures grown in a completely and continuously dark environment effectively stopped turnover of jamaicamide B and pheophytin *a*, a finding that supports the notion of light regulation of these pathways

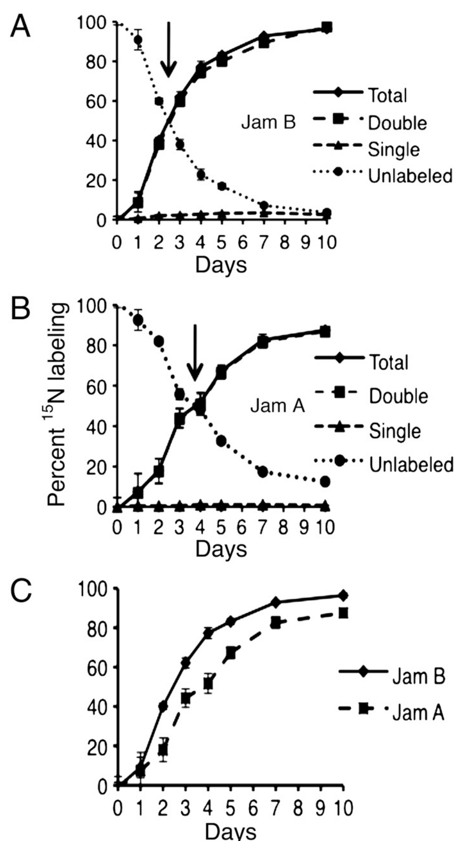

Fig. 3. Different ^{15}N labeling states of jamaicamide B and A over 10 days in *L. majuscula* JHB. (*A*) Time course of single, double, total, and unlabeled jamaicamide B over 10 days, calculated from same data as in Fig. 2*B*. The arrows point to T_{50} (time at which 50% of the molecules are ^{15}N labeled), =2.5 days. (*B*) Time course of single, double, total, and unlabeled jamaicamide A over 10 days from the same dataset. $T_{50} = 3.9$ days (arrow). (*C*) Comparison of total ^{15}N-labeled jamaicamide B and A. The initiation of ^{15}N labeling in jamaicamide A is delayed by approximately 1.5 days when compared to jamaicamide B. (Errors are SEM, $N = 5$.)

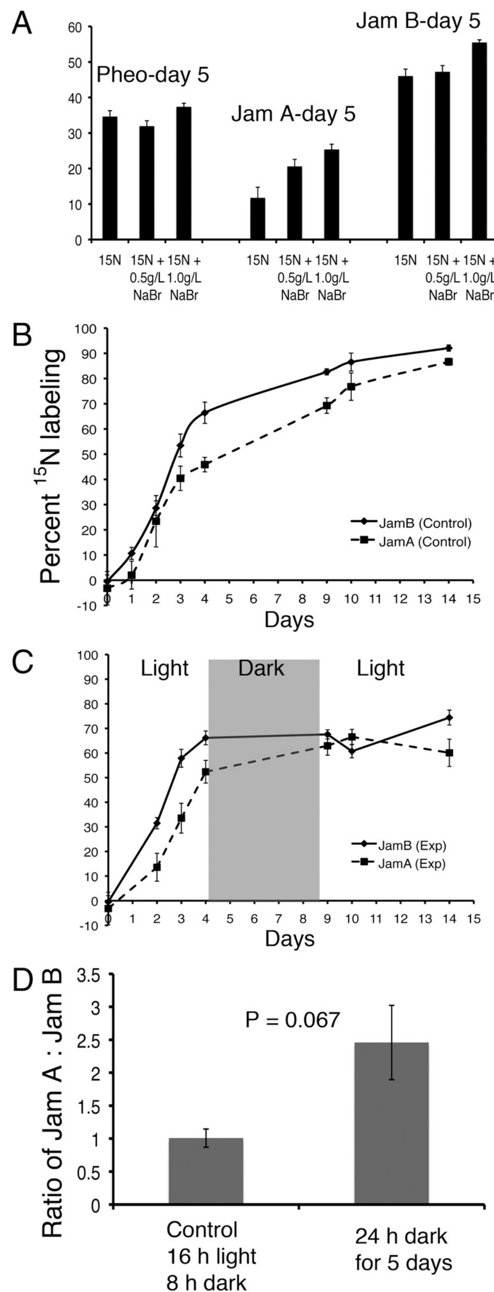

Fig. 4. Further investigation into jamaicamide A and B biosynthesis. (*A*) Total percent ^{15}N labeling of jamaicamide A is enhanced by addition of NaBr to media. Bars indicate percent ^{15}N labeling of pheophytin *a*, jamaicamide A, and jamaicamide B in small *L. majuscula* JHB cultures incubated for five days in media containing 100% $[^{15}N]NaNO_3$ and either 0.5 g/L or 1.0 g/L NaBr ($N = 6$; error bars are SEM). (*B*) Total percentage ^{15}N-labeled jamaicamide B (solid line) and A (dashed line) over 14 days in 16 h light/8 h dark conditions. (*C*) New production (total ^{15}N labeling) is terminated for jamaicamide B (solid line) but continues for jamaicamide A (dashed line) when *L. majuscula* JHB is subjected to continuous dark for a five-day period from day 4 to day 9 (opaque box) ($N = 8$; error bars are SEM). (*D*) Ratio of jamaicamide A to jamaicamide B after six days in regular light conditions (16 h light/8 dark) and continuous dark (24 h dark) from larger scale experiments measured by LC–MS.

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(26). Comparison of ^{15}N incorporation into jamaicamide A and B during this dark phase revealed that production of jamaicamide A continues in the dark, whereas production of jamaicamide B is completely abrogated (Fig. 4*C*). These results again suggest that jamaicamide A bromination likely occurs after the assembly of jamaicamide B, and jamaicamide B is implicated as the substrate for bromination. This prediction of the timing of bromination is consistent with previously reported experiments that demonstrated the inclusion of only nonbrominated substrates during the initial steps of jamaicamide biosynthesis (27). This experiment was repeated using an alternative design (Fig. S2), and these latter results also support the initial experiment with ^{15}N labeling of jamaicamide A continuing in the dark condition, while ^{15}N labeling of jamaicamide B is inhibited.

These MALDI results were reinforced by those of a larger scale LC–MS experiment (see *SI Materials and Methods*). The relative amounts of jamaicamides A and B extracted from cultures grown for six days under either complete darkness or regular light cycle were compared. The ratios of total jamaicamide A to jamaicamide B in each extract are shown in Fig. 4*D* and indicate that while jamaicamide A biosynthesis continues in total darkness, the rate of jamaicamide B turnover drops considerably (T-test, $P = 0.067$).

Discussion

Isolation of natural products from sessile marine organisms for drug discovery or biotechnology applications, either from laboratory cultures or field collections, frequently results in the recovery of variable amounts of a metabolite from a parent extract. Many secondary metabolites are under strict environmental and epigenetic modes of regulation (28), while others are produced constitutively and can represent a large percentage of the harvested biomass (29). The observation that the *Lyngbya* natural products included in this study had variable rates of ^{15}N labeling from $[^{15}N]NaNO_3$ feeding and MALDI analysis, and thus different turnover rates, is therefore not surprising. However, until now there has not been a consistent and robust approach to actually measure these production differences in vivo. Here, the use of MALDI-TOF MS of crude preparations, in conjunction with ^{15}N feeding, has revealed itself to be a valuable approach, allowing the dynamics of biosynthesis of various metabolites in marine *Lyngbya* strains to be examined concurrently. Additionally, viewing the results of 10-day $[^{15}N]NaNO_3$ feeding of three *Lyngbya* species in a "heat map" format (Fig. 1*A*) also allowed us to identify unknown metabolites that are being actively produced and turned over (marked by a red asterisk). The combination of these findings suggests that this is an exceptionally rich spectral dataset and provides temporal insights into the active nitrogen-containing metabolome of these organisms.

To better gain insight into the turnover rates of specific metabolites in marine organisms, it is important to understand how both growth and primary metabolism might be influenced by changes in culture conditions. Using *L. majuscula* JHB as a model organism, several factors were explored using filaments from the same parent culture. Under our typical culture conditions (see *SI Materials and Methods*), the percent production of pheophytin *a* was found to directly parallel the growth rate calculated for single filaments (Fig. 2*C*), suggesting that pheophytin *a* turnover is a reasonable proxy for growth. More importantly, under these conditions pheophytin *a* turnover provides a useful measure of primary metabolic activity to which the turnover of specific natural products can be compared. A surprising observation in these cultures of *L. majuscula* JHB was that a greater percentage of ^{15}N-labeled jamaicamide B was detected throughout the course of the experiments than ^{15}N labeled pheophytin *a*, indicating unequal rates of turnover for these two metabolites. It can be concluded that jamaicamide B is being produced significantly faster than chlorophyll in these cultures and that the rate of its biosynthesis outpaces filament growth. Because of the predicted metabolic cost of natural product biosynthesis (9, 30), variations in the relative rates of turnover for different natural products in the same organism may be indicative of their relative ecological importance under a given set of environmental parameters. Although jamaicamide B was previously found to have sodium channel blocking activity and fish toxicity (15), its true ecological role has yet to be determined, and these results suggest that significant energy and resources are being devoted to its metabolism and turnover even in the absence of macroscopic predators. The possibility exists that its high turnover level is a result of microbial interaction/defense, as the polysaccharide sheath of *Lyngbya* strains tend to harbor various heterotrophic bacteria (19) or, equally plausible, evolution may have simply favored a robust expression level for the jamaicamide B pathway. Manipulation of culture conditions (UV exposure and decreased media nitrate concentration) resulted in proportional decreases in the production of both jamaicamide B and pheophytin *a* (*SI Text*), demonstrating that jamaicamide B expression levels remained higher than pheophytin *a* and confirming that the approach can be used to concurrently explore the effects of culture conditions on the production of multiple metabolites.

Jamaicamide B and the brominated analog jamaicamide A are products of the same PKS/NRPS biosynthetic gene cluster (15). The unexpected finding that the two molecules have significant differences in their ^{15}N labeling rates (Fig. 3) led us to more carefully consider the resources being dedicated to the creation of each molecule as well as the possible timing of bromination in jamaicamide A biosynthesis. One possible scenario is that the composition of the surrounding media defines the arsenal of secondary metabolites produced by *L. majuscula*. In the present case, bromide is a limiting element in the biosynthesis of jamaicamide A, as has been observed for the natural product phormidolide in the mat forming cyanobacterium *Phormidium sp.* (31). Another conceivable explanation for the observed production difference between jamaicamide A and B is that the halogenase required in the bromination of jamaicamide A may not be active in every round of jamaicamide biosynthesis, perhaps due to an additional energetic cost of halogenation that results in relatively fewer jamaicamide A molecules being created and turned over. A third explanation is that jamaicamide B represents an initial, shorter-lived and dynamic pool, whereas jamaicamide A represents the end product of the pathway and is metabolically a more stable molecule. Interestingly, this latter scenario provides a potential insight into an unknown aspect of jamaicamide biosynthesis. Although many features of the biosynthesis of the jamaicamides have been described, (3, 15) the timing, identity, and genomic location of the putative halogenase responsible for incorporation of bromine into jamaicamide A has been a source of speculation but has not yet been defined. Under normal culture conditions, the turnover rate of jamaicamide B occurs approximately 1.4 times faster than jamaicamide A. When the medium is supplemented with NaBr, the amount of ^{15}N-labeled jamaicamide A increases while that of jamaicamide B and pheophytin *a* do not, supporting the hypothesis that bromide is a limiting element in the biosynthesis of jamaicamide A. However, even with additional bromide the amount of total ^{15}N labeling for jamaicamide A remains lower than that of the other two molecules (Fig. 4*A*). Furthermore, when cultures are moved to a completely dark environment, thereby inhibiting photosynthesis, the turnover rate of jamaicamide B sharply decreases, while jamaicamide A turnover continues at a rate nearly comparable to JHB cultures in the light condition (Fig. 4 *B*, *C*, and *D*). These data support the third scenario wherein a percentage of the newly biosynthesized jamaicamide B pool is converted to jamaicamide A by replacement of the alkynyl hydrogen with bromine, potentially catalyzed by the protein product of *jamD* from the jamaicamide biosynthetic pathway (15). In this case, a previously

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expressed and functional halogenase could catalyze the enzymatic addition of bromine in the absence of light-driven metabolism. Given the above findings, it appears that jamaicamide B is only formed during daylight hours, whereas jamaicamide A is produced throughout a light/dark cycle and ultimately accumulates to higher concentrations. Light regulation of jamaicamide biosynthesis would be consistent with previous findings (26) and is a mechanism also reported for the cyanobacterial UV sunscreen molecule scytonemin (32) and freshwater microcystins (33). A larger scale experiment aimed at exploring the observed relationship between jamaicamide A and B was analyzed using more conventional methods (LC–MS), and although the difference in the ratio of jamaicamide B to jamaicamide A between the dark and the light conditions was only marginally significant ($p = 0.067$), the resulting trend further supported our conclusion that in contrast to jamaicamide B, jamaicamide A continues to accumulate in the dark (Fig. 4*D*).

The primary strength of the approach described in this report lies in the multiplex ability of MALDI-TOF MS to track the ^{15}N-labeling of multiple metabolites concurrently in a single experiment and within individual samples using a straightforward preparation technique, thereby avoiding unknown variables that might influence metabolite turnover in separate experiments. This allows for careful measurements of the influence of abiotic and biotic factors on the production of individual metabolites, as well as the temporal relationships between metabolites, providing powerful insight into the origin of chemical variability in marine life forms and the in vivo metabolic processing of natural products. The side-by-side comparison of nitrogen-containing natural products over time with other primary metabolites such as pheophytin *a* also provides a window into resource allocation under specific growth conditions. As a complement to appropriate assays using extracts or pure compounds, this approach may significantly increase the accuracy with which ecological roles for these natural products are assigned and should also be applicable to similar investigations using other natural product-rich organisms.

This MALDI-MS approach could also be a valuable tool in efforts aimed at increasing natural product yields from laboratory cultures of cyanobacteria or other organisms with biomedical and biotechnological relevance, especially in cases where compounds are found in low abundance. Future experiments using these MALDI-based techniques with cyanobacteria could help identify the main drivers of metabolite production, including possible factors leading to harmful cyanobacterial blooms, and continue to improve our understanding of the biosynthesis of nitrogen-containing molecules. This combined MALDI-MS/isotope feeding approach could also be useful for optimizing the output of heterologous expression of natural product gene clusters in other strains, as well as gaining insight into the nutrient exchange between co-occurring or symbiotic species.

Materials and Methods

See *SI Text* for details of cyanobacterial strains and culture maintenance, experimental culture conditions, sampling of cultures for MALDI experiments, cultures with sodium bromide supplementation, MALDI sample preparation, and calculations of ^{15}N incorporation from [^{15}N]$NaNO_3$ feeding experiments.

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