

1 **Lack of methylated hopanoids renders the cyanobacterium *Nostoc punctiforme* sensitive**
2 **to osmotic and pH stress**

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17 Running title: *Nostoc* mutant devoid of methylated hopanoids

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22 **To investigate the function of 2-methylhopanoids in modern cyanobacteria, the *hpnP***
23 **gene coding for the radical SAM methylase protein that acts on the C2 position of**
24 **hopanoids, was deleted from the filamentous cyanobacterium *Nostoc punctiforme* ATCC**
25 **29133S. The resulting $\Delta hpnP$ mutant lacked all 2-methylhopanoids, but was found to**

26 produce much higher levels of two bacteriohopanepentol isomers compared to the wild
27 type. Growth rates of $\Delta hpnP$ mutant cultures were not significantly different from those
28 of the wild type under standard growth conditions. Akinete formation was also not
29 impeded by the absence of 2-methylhopanoids. The relative abundances of the different
30 hopanoid structures in akinete-dominated cultures of the wild-type and $\Delta hpnP$ mutant
31 were similar to those of vegetative cell-dominated cultures. However, the $\Delta hpnP$ mutant
32 was found to have decreased growth rates under both pH and osmotic stress, confirming
33 a role for 2-methylhopanoids in stress tolerance. Evidence of elevated photosystem II
34 yield and NAD(P)H-dependent oxidoreductase activity in the $\Delta hpnP$ mutant under
35 stress conditions, compared to the wild type, suggested that the absence of 2-
36 methylhopanoids increases cellular metabolic rates under stress conditions.

37
38 **Importance:** As the first group of organisms to develop oxygenic photosynthesis,
39 Cyanobacteria are central to the evolutionary history of life on Earth and the subsequent
40 oxygenation of the atmosphere. To investigate the origin of cyanobacteria and emergence of
41 oxygenic photosynthesis, geobiologists use biomarkers, the remnants of lipids produced by
42 different organisms that are found in geologic sediments. 2-Methylhopanes have been
43 considered indicative of cyanobacteria in some environmental settings; with the parent lipids
44 2-methylhopanoids being present in many contemporary cyanobacteria. We have created a
45 *Nostoc punctiforme* $\Delta hpnP$ mutant strain that does not produce 2-methylhopanoids to assess
46 the influence of 2-methylhopanoids on stress tolerance. Increased metabolic activity in the
47 mutant under stress indicates compensatory alterations of metabolism in the absence of 2-
48 methylhopanoids.

50 INTRODUCTION

51 Bacteriohopanepolyols (BHPs) are pentacyclic triterpenoids, hypothesized to be functional
52 analogs of eukaryotic sterols, and widely used as biomarkers for bacteria (1). Hopane
53 hydrocarbons, the diagenetic products of BHPs, are ubiquitous and abundant in ancient
54 sediments (2) and, therefore, potentially useful for evaluating past microbial communities and
55 paleoenvironmental conditions. To this end, it is important to increase our understanding of
56 the sources and functions of specific hopanoid structures. Knowledge about how bacteria
57 adjust their hopanoid production to adapt to different conditions enhances the diagnostic value
58 of fossil lipid assemblages in geologic samples.

59 Hopanoids occur in a wide range of Gram-negative and Gram-positive bacteria, including
60 those that form symbiotic association with plants (3, 4), yet large gaps still exist in our
61 understanding of the breadth of physiological functions of hopanoids. The localization of
62 hopanoids in membranes has been documented in a variety of different bacteria (5-8). It
63 appears that these lipids influence outer-membrane order and integrity by interacting with
64 lipid A, in a manner analogous to that of cholesterol with sphingolipids in eukaryotes (9-13).
65 There is mounting evidence for the role of hopanoids in protecting cells against a variety of
66 stress conditions, such as increased temperature (5, 14-18), pH and osmotic tension (16, 18,
67 19), and desiccation (20).

68 Due to their structural stability, 2-methylhopanoids (2-MeBHPs) in particular are of
69 significant geobiological interest. 2-MeBHPs have been found in significant quantities both in
70 cyanobacterial cultures and cyanobacterial-dominated microbial mats (21, 22), with over
71 twenty strains of cyanobacteria across multiple orders testing positive for 2-MeBHP
72 (reviewed in (23)). Furthermore, episodic spikes in 2-MeBHP abundances in ancient marine
73 sediments and derived oils have been interpreted as possible indicators of modern
74 cyanobacteria thriving during oceanic anoxic events (24, 25). However, a complicating factor
75 is that despite their distribution across all orders of Cyanobacteria, not all nor even a majority

76 of cyanobacteria have the capability to produce 2-MeBHPs ((23), Garby TJ & Neilan BA,
77 unpublished data).

78 2-MeBHPs have also long been known to be produced by bacteria other than cyanobacteria
79 (26-30). It was initially thought that these taxa produced only small amounts, which could not
80 account for the abundance of 2-MeBHP in sediments and petroleum. However, it has recently
81 been shown that the anoxygenic phototroph *Rhodopseudomonas palustris* produces 2-
82 MeBHPs in significant amounts (31). Further to this, phylogenetic analysis of *HpnP*,
83 responsible for the C-2 methylation of BHPs to produce 2-MeBHP, showed a wide
84 distribution in Alphaproteobacteria; this also suggested that it was unlikely that *hpnP*
85 originated in Cyanobacteria (32). This brought into question the validity of using 2-
86 methylhopanes as taxonomic biomarkers for cyanobacteria and led to the proposal that 2-
87 MeBHPs may instead be more generally diagnostic for specific ecological niches (33).

88 In light of these uncertainties, it is important to learn more about the distribution and
89 physiological role(s) of 2-MeBHPs in cyanobacteria and other bacteria where they are
90 present. Apart from acting as membrane rigidifiers (10), which in turn can influence
91 membrane protein function and transport, the specific functions of 2-MeBHPs in modern
92 cyanobacteria are not well-understood. However, it has been shown that 2-MeBHP are most
93 abundant in the outer membranes of certain cell types, suggesting that C-2 methylation may
94 promote stress resistance (34).

95 Hopanoids are produced by *N. punctiforme* ATCC 29133 (PCC 73102), a filamentous
96 cyanobacterium of the order Nostocales. This cyanobacteria was originally isolated from a
97 root section taken from a *Macrozamia* sp. cycad (35), and can form symbiotic associations
98 with a number of different plant genera (36). The relationship between *N. punctiforme* and its
99 environment is particularly interesting, as this species can differentiate into a number of cell
100 types in response to external factors (37). Photosynthetic vegetative cells are the usual cell

101 type under normal conditions, but several specialized cell types can form under stressful
102 conditions. The absence of a nitrogen source, leads to the formation of specialized non-
103 photosynthetic heterocysts capable of fixing atmospheric nitrogen (N_2). Additionally, resting
104 cells called akinetes form in response to nutrient or light limitation. Akinetes are larger and
105 more resistant to stress than vegetative cells, allowing survival until conditions are favorable
106 for growth. Lastly, hormogonia, gliding filaments that are much more motile than vegetative
107 filaments and composed of smaller cells, form in response to a variety of environmental stress
108 signals.

109 The complex lifecycle of *N. punctiforme* makes it particularly suited for studying the role
110 of hopanoids in stress acclimation. Different cell types contain altered hopanoid profiles, with
111 2-MeBHP levels being greatest in the outer membranes of akinetes and vegetative cells in N_2 -
112 fixing cultures (34). The genome of *N. punctiforme* ATCC 29133 has been sequenced (38),
113 allowing for the identification of genes known to be required for BHP biosynthesis, and
114 particularly that of 2-MeBHP (39). Bacteriohopanetetrol and two isomers of
115 bacteriohopanepentol, bacteriohopane-31,32,33,34,35-pentol (BHpentol-1), and
116 bacteriohopane-30,32,33,34,35-pentol isomer (BHpentol-2), along with their 2-methylated
117 counterparts, have previously been detected in *Nostoc muscorum*, *Nostoc* sp. PCC 6720, and
118 *Nostoc* sp. ATCC 27985 (26, 40, 41). BHpentol-2 bearing a hydroxyl group on C30 has not
119 been reported in taxa other than *Nostoc* sp. and this, together with the R configuration of the
120 hydroxyl at C-34, may be unique to this genus (41).

121 In this study, we sought to evaluate proposed links between 2-MeBHP and environmental
122 stress (42, 43) and based on geological evidence that suggests some sediments contain
123 anomalously high levels of 2-methylhopanes and implicating them as important for microbial
124 survival (44-46). Specifically we investigated the role of 2-MeBHPs in a modern
125 cyanobacterium, in conferring adaptations to various environmental conditions. To this end,

the gene coding for the hopanoid C-2 methylase, *hpnP* (39), was inactivated in *Nostoc punctiforme* ATCC 29133S (47). As 2-MeBHPs have previously been linked to a role in both and stress tolerance and akinete formation, it was expected that the absence of 2-methylhopanoid would adversely affect cell survival under a variety of stress conditions and negatively impact the production of akinetes.

131

132 MATERIALS AND METHODS

Knockout plasmid construction. The *hpnP* gene, along with flanking regions of at least 1 kb, was amplified from the genome of *Nostoc punctiforme* ATCC 29133S (UCD 153; a spontaneous ATCC 29133 derivative that grows more rapidly and homogeneously in liquid, producing slow hormogonia (47)). The primers used for amplification (Table 1) incorporated XhoI restriction sites onto the ends of the amplified product. This PCR product was ligated into the pGEM®-T Easy (Promega) plasmid using the incorporated XhoI restriction sites. A central portion of the cloned *N. punctiforme hpnP* gene, consisting of 1113 bp, was removed by digestion with the restriction enzymes MseI and AclI [New England Biolabs (NEB)].

The omega fragment, containing the neomycin-resistance gene (*npt*) and associated *psbA* promoter (PpsbA), of plasmid pSCR9 (48) was isolated by SmaI (NEB) restriction digest. The recovered PpsbA-*npt* cassette from pSCR9 was ligated into the cleaved *hpnP* gene in the pGEM-T plasmid and transformed into competent *Escherichia coli* 5-alpha cells (NEB). Colony PCR was used to screen for inserts containing the PpsbA-*npt* cassette, with the primers hpnP_IF and hpnP_IR (Table 1).

The *N. punctiforme hpnP* gene and flanking regions, with the inserted PpsbA-*npt* cassette, was removed from pGEM-T by digestion with the restriction enzyme XhoI (NEB). The plasmid pRL271 (49), a conjugative cyanobacterial suicide vector carrying a counterselective *sacB* gene for selection of double crossovers, was also digested with XhoI, and the resistance

151 cassette inserted. These plasmids were used to transform competent *E. coli* NEB5-alpha
152 (NEB) cells.

153 To confirm sucrose sensitivity conferred by the *Bacillus subtilis* levansucrase *sacB* gene
154 present in the pRL271 plasmid, *E. coli* colonies were grown in LB medium with 5% sucrose
155 and 34 µg/mL of chloramphenicol, and compared to controls grown without sucrose.

156 Purified pRL271 containing the knockout cassette (referred to as HNKO) was used to
157 transform *E. coli* TOP10 competent cells (Invitrogen).

158 **Measurement of *N. punctiforme* culture density.** Chlorophyll *a* (*chl a*) concentration was
159 used to determine culture density. Duplicate aliquots of 500 µL or 1000 µL were removed,
160 and cells collected by centrifugation. The supernatant was removed, and cells were
161 resuspended in 90% methanol. Cells were lysed using a FastPrep FP120 cell disrupter (MP
162 Biomedicals) instrument at speed 6.0 for 40 s, then left to extract for at least 5 min in the dark.
163 Aliquots were then centrifuged again, and the absorbance of the supernatant measured at 665
164 nm. The concentration of *chl a* was calculated by multiplying the average A_{665} reading by
165 12.7 to obtain µg *chl a* per mL (50).

166 **Triparental conjugation.** The HNKO plasmid was introduced into *Nostoc punctiforme*
167 29133S by triparental conjugation as previously described ((51);
168 <http://microbiology.ucdavis.edu/meeks/xpro3.htm>), with minor modifications as follows.
169 Briefly, *E. coli* TOP10 harboring HNKO plasmid (donor strain) and *E. coli* JCM113 (helper
170 strain; *E. coli* HB101 carrying both pRK2013 and pRL528 plasmids for mobilization and
171 methylation, respectively) were grown in LB medium containing both chloramphenicol and
172 kanamycin, at 30°C, until cultures reached an OD₆₀₀ of about 0.7. Cells were washed, and
173 concentrated to OD₆₀₀ 9-10, gently mixed and incubated at room temperature for ~1 h.

174 *Nostoc punctiforme* ATCC 29133S cells from a dense 500 mL culture were harvested by
175 centrifugation and resuspended in 30 mL of fresh Allen & Arnon medium diluted four-fold

176 (A&A/4). Cultures were disrupted with a Branson digital sonicator in order to fragment
177 filaments to a length of 1-4 cells. Cells were harvested, resuspended in A&A/4 medium
178 containing 5 mM MOPS (pH 7.8) and 2.5 mM NH₄Cl, and placed in low light (~5-10 $\mu\text{E m}^{-2} \text{s}^{-1}$)
179 to recover for at least 4 h, prior to concentration of the cells to a chl *a* concentration of 100
180 $\mu\text{g chl a/mL}$.

181 Sterile conjugation filters (Millipore HATF08250, 0.45 μm , 82 mm) were placed on 1%
182 agar A&A plates containing 5 mM MOPS (pH 7.8), 2.5 mM NH₄Cl and 0.5% LB. Equal
183 amounts (500 μL) of *N. punctiforme* culture and *E. coli* mixture were combined and briefly
184 centrifuged. Supernatant was removed, and the remaining culture was spread onto a
185 conjugation filter. Plates were placed in low light (~5-10 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 25°C overnight. All
186 conjugation filters were then transferred to plain A&A 1% agar plates and incubated for a
187 further 4 days.

188 **Growth and selection of ΔhpnP mutants.** Conjugation filters were transferred to 1% agar
189 A&A plates containing 25 $\mu\text{g/mL}$ neomycin and placed in high light (~25-30 $\mu\text{E m}^{-2} \text{s}^{-1}$) at
190 25°C. *N. punctiforme* colonies that grew on the neomycin selection plates were transferred to
191 0.5 mL of A&A/4 medium with 25 $\mu\text{g/mL}$ of neomycin and incubated for 2 weeks. At this
192 point, to induce resolution and exclusion of the pRL271 plasmid backbone, the cultures were
193 transferred to 4 mL of A&A/4 medium without neomycin and incubated for two weeks. Cells
194 were recovered by centrifugation, resuspended in 0.5 mL of medium and vortexed. Cells were
195 spread onto 1% agar A&A plates containing 5 mM MOPS, 25 $\mu\text{g/mL}$ neomycin, and 5%
196 (w/v) sucrose to select against cells retaining the pRL271 plasmid backbone, which carries the
197 *sacB* gene. Sucrose resistant colonies were screened by PCR to confirm resolution by double
198 crossover (Fig. S1). The ΔhpnP mutant strain was maintained in A&A/4 medium with 25
199 $\mu\text{g/mL}$ neomycin.

200 **Confirmation of 2-methylhopanoid absence/gene inactivation.** Detection and
201 quantification of hopanoids by high-performance liquid chromatography mass spectrometry
202 (LC-MS) was used to confirm the absence of 2-MeBHP production, and assess the production
203 of other BHP compounds in cultures. A fraction of each total lipid extract (TLE) was
204 acetylated with pyridine/acetic anhydride (1:1, v/v) at 70°C for 1 h and left at room
205 temperature overnight. The acetylated TLEs were analyzed by LC-MS. The LC-MS system
206 comprises a 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with
207 an autosampler and a binary pump linked to a Q-TOF 6520 mass spectrometer (Agilent
208 Technologies) via an atmospheric pressure chemical ionization (APCI) interface (Agilent
209 Technologies) operated in positive ion mode. The analytical procedure was adapted from H.
210 M. Talbot, et al. (52). A Poroshell 120 EC-C18 column (2.1 × 150 mm, 2.7 μm; Agilent
211 Technologies) was chosen to provide fast and high-resolution separations at lower pressures.
212 The APCI parameters were as follows: gas temperature 325°C, vaporizer temperature 350°C,
213 drying gas (N₂) flow 6 l min⁻¹, nebulizer (N₂) flow 30 l min⁻¹, capillary voltage 1200 V,
214 corona needle 4 μA, and fragmentor 150 V.

215 Hopanoids were identified on the basis of accurate mass measurements of their protonated
216 molecular ions, fragmentation patterns in MS-MS mode, and by comparison (Fig. 1) of
217 relative retention times (53-55). Quantification was accomplished using an external standard,
218 5β-pregnane-3α,12α-diol-20-one diacetate (PD), chosen because of its structural similarity to
219 hopanoids and because its retention time does not overlap with BHP retention times.
220 However, considerable variability exists in the ionization efficiencies of all hopanoid
221 structures and especially compared to PD (10). Therefore, the relative response factors for
222 BHtetrol and 2-MeBHtetrol were determined using authentic standards and applied to the
223 quantification of all BHP compounds in this study. The relative responses of all BHP, relative
224 to PD, were previously observed to be linear over four orders of magnitude (1). Without

225 equivalent standards for BHpentol and 2-MeBHpentol, however, our quantification can only
226 be considered approximate for those particular compounds. Since this study focuses on the
227 relative abundance of BHPs, precise quantification for all compounds is not required.

228 **Akinete formation and microscopy.** To induce akinete differentiation, cells were
229 transferred to A&A medium without phosphate (56), and placed in very low light ($\sim 1\text{-}2\ \mu\text{E}$
230 $\text{m}^2\ \text{s}^{-1}$). Cells were maintained in these conditions for several weeks. Akinete formation was
231 checked periodically by light microscopy. Sample preparation and electron microscope
232 imaging was performed using an FEI Nova NanoSEM 230 FESEM instrument. Cellular
233 morphology of live *N. punctiforme* cultures was examined by Leica TCS SP5 confocal
234 inverted microscope (Leica Microsystems, Heidelberg). Imaging was performed at excitation
235 by 488 nm line of Argon laser and by 633 nm line of HeNe laser, with emissions at 520-620
236 nm captured in PMT 1 and emissions at 638-720 nm captured in PMT 2, respectively.
237 Fluorescence emissions from photosynthetic pigments in vegetative and akinete cells ($n = 3\text{-}$
238 4), mainly phycobiliproteins and chlorophyll *a* (chl *a*), were microspectrally characterised
239 using the λ scan mode of Leica SP5 at excitation by 488 nm by sequentially imaging a series
240 of images from 500 to 750 nm, each acquired at 10-nm emission detection band-width, with 5
241 nm steps. Spectral data from defined regions of interests (ROIs) inside cells obtained from
242 Leica LAS software was imported into Excel spread-sheet and plotted.

243 **Stress treatments.** *Nostoc punctiforme* ATCC 29133S wild-type and $\Delta hpnP$ mutant
244 cultures were subjected to a variety of stress conditions. Cells were grown in A&A/4 medium
245 (without neomycin) at 25°C on a shaking platform under cool white fluorescent lights ($\sim 20\ \mu\text{E}$
246 $\text{m}^2\ \text{s}^{-1}$) until they reached mid-log phase, then transferred to the stress treatments. Two
247 different experimental designs for growing cells under stress conditions were used, covering a
248 range of temperatures, pH levels, and salt concentrations. The standard growth conditions
249 used for control cultures in all experiments were: 25°C, 1.1 mM NaCl, and pH 7.8.

250 First, cultures were subjected to heat stress (34°C), osmotic stress (100 mM NaCl) and pH
251 stress (6.0 and 9.5). The media for control, salt and heat stress treatments media were buffered
252 to pH 7.8 with 10 mM HEPES. The medium for the pH 9.5 treatment was buffered with 10
253 mM sodium carbonate – sodium bicarbonate, and the medium for the pH 6.0 treatment was
254 buffered with 10 mM MES. Three biological replicates were maintained for each treatment,
255 under continuous light on a shaking platform. At the beginning of the trial, 130 mL of each
256 type of medium was inoculated with cells at an initial density of approximately 0.1 µg chl *a*
257 per mL. Growth was measured over the course of several weeks by chl *a* extraction as
258 described above. The maximum quantum yield of photosystem II (PSII) (F_v/F_m) of each
259 culture was also measured at various times over the course of the trial as described below.

260 Second, a range of pH levels and NaCl concentrations were evaluated concurrently. The
261 range of pH levels was from 4.8 to 10.0, and the range of NaCl concentrations was 100-180
262 mM. Four biological replicates were maintained for each treatment, under continuous light on
263 a shaking platform. Cultures were grown in a 24-well plate, where 2 mL of medium in each
264 well was inoculated with cells at an initial density of approximately 0.6 µg chl *a* per mL.
265 Media for cultures grown at pH levels from 6.0-6.5 were buffered with 10 mM MES, media
266 for cultures grown at pH levels from 6.8-8.2 were buffered with 10 mM HEPES, and media
267 for cultures grown at pH levels from 8.5-9.0 were buffered with 10 mM Tris. Growth was
268 measured over the course of several weeks by chl *a*, and MTT [3-(4,5-dimethylthiazol-2-yl)-
269 2,5-diphenyltetrazolium bromide] assays were performed as described below.

270 Cells were also subjected to stress caused by freezing. Cultures were concentrated by
271 centrifugation to approximately 12 µg chl *a* per mL, then 1 mL aliquots were vortexed to
272 homogenize cultures. These 1 mL aliquots were placed in a freezer at -20°C, in two sets of
273 biological triplicates. One set was thawed and refrozen twice over one week, and the second
274 set thawed and refrozen three times over two weeks. After final removal from the freezer,

275 thawed cells were used to inoculate 50 mL of A&A/4 medium, and left to recover under
276 control conditions. Growth was measured over the course of several weeks by chl *a*
277 extraction.

278 **PAM fluorometry.** A portable Pulse Amplitude Modulation (PAM) fluorometer 2500
279 (Walz) was used to measure F_v/F_m . F_v/F_m is an indicator of PSII efficiency, which can in turn
280 be used as an indicator of overall cell health. Aliquots of 1 mL were removed from cultures
281 and dark adapted for a minimum of 10 min, then placed under a weak red measuring light.
282 Cells were exposed to a strong red saturation pulse of 100 ms in duration to measure the
283 maximum (dark adapted) yield of PSII (F_v/F_m). Slow kinetics curves were determined for
284 cells from each treatment by applying red actinic light for 250 s, with a saturation pulse every
285 20 s.

286 **MTT assays.** The MTT assay was used to assess cell proliferation based on the NAD(P)H-
287 dependent oxidoreductase enzyme activity of cells. Cell densities were standardized to the
288 lowest density present according to chl *a* measurements. Cells were resuspended in 100 μ L of
289 medium in a 96-well plate, using four biological replicates for each treatment. A 20 μ L
290 solution of 5 mg/mL MTT (Thiazolyl Blue Tetrazolium Blue, Sigma Aldrich) was added, and
291 samples were incubated at 37°C for 2 h. Plates were centrifuged, medium was removed from
292 the wells, and 200 μ L of acidic isopropanol (0.04 M HCl) was added to each well and mixed.
293 Absorbance was measured at 570 nm, with higher absorbance readings corresponding to a
294 higher level of metabolic function.

295 **Statistical analysis.** Slopes of the exponential phase of growth curves for cultures treated
296 by heat stress, pH 6.0, pH 9.5, 100 mM NaCl and freezing were calculated in Prism v. 6.0,
297 and one-way ANOVA tests were performed to compare slopes.

298 For cell densities of cultures grown in a range of pH levels and NaCl concentrations,
299 values were adjusted to account for differences between cultures of wild-type and $\Delta hpnP$

mutant cells under control conditions. Assuming exponential growth, growth rates of treatment cultures were normalized to average control culture growth rates at each time point following the formula:

$$\text{density}A_x \times (\text{density}B_{\text{control_average}}/\text{density}A_{\text{control_average}}),$$

where A is the slower-growing culture under optimal conditions and B is the faster-growing culture under optimal conditions. Densities of cultures in each treatment at each time point were compared separately by one-way ANOVA tests.

For MTT assays, values were adjusted to account for differences between cultures of wild-type and $\Delta hpnP$ mutant cells under control conditions. Assuming a linear relationship between NAD(P)H-dependent enzyme activity and the A_{570} values observed, values for treatment cultures were normalized to average control culture values at each time point following the formula:

$$\text{density}A_x + (\text{density}B_{\text{control_average}} - \text{density}A_{\text{control_average}}),$$

A and B are defined as above. Absorbance readings of assays from each treatment at each time point were compared separately by one-way ANOVA tests.

RESULTS

Confirmation of gene inactivation. PCR amplification and sequencing of *hpnP* and flanking loci showed the successful substitution of the central region of the *hpnP* gene with the neomycin-resistance cassette of pSCR9. Additionally, PCRs performed with primers internal to the deleted section of the *hpnP* gene failed to amplify any product from the genomes of mutated cells.

Hopanoid profiles. Hopanoid analyses of *N. punctiforme* wild-type and $\Delta hpnP$ mutant vegetative and akinete cells revealed six BHP compounds (Fig. 1). Bacteriohopanetetrol (BHtetrol) and methylated counterpart, 2-methylbacteriohopanetetrol (2-MeBHtetrol) were

325 present in wild-type vegetative and akinete cell types. BHTetrol was present while 2-
326 MeBHTetrol was absent in all $\Delta hpnP$ mutants. Bacteriohopanepentol (BHpentol) was
327 identified, as well as a putative isomer, denoted BHpentol-1 and BHpentol-2. 2-
328 Methylbacteriohopanepentol (2-MeBHpentol) was also identified, as well as a putative
329 isomer, denoted 2-MeBHpentol-1 and 2-MeBHpentol-2. 2-MeBHpentol-1 and -2 were the
330 most abundant compounds in wild-type vegetative and akinete cell types and were absent in
331 all $\Delta hpnP$ mutant cell types. BHpentol-2 and 2-MeBHpentol-2 were not detected by GC-MS,
332 and required LC-MS techniques for identification.

333 A clear change was observed in the diversity and abundance of BHPs produced by wild-
334 type *N. punctiforme* and $\Delta hpnP$ mutant strain cell types (Fig. 2). LC-MS analysis using
335 authentic standards enabled the robust quantification of BHP compounds (Fig. 1). Total BHP
336 is greater in vegetative cells as compared to the akinete cell type in both wild type and $\Delta hpnP$
337 mutants. Total BHP is greater in wild type cells (vegetative and akinete) as compared to
338 $\Delta hpnP$ mutant cells. While $\Delta hpnP$ vegetative and akinete cell mutants do not produce 2-
339 MeBHP, the abundance of BHpentol-1, BHpentol-2, and BHTetrol increase, possibly
340 compensating for the lack of 2-MeBHPs produced. Both wild type vegetative and akinete
341 cells contained an order of magnitude more methylated BHPs as compared to desmethyl
342 BHPs. Total BHP is ~75% greater in wild-type vegetative cells than wild-type akinetes.
343 However, total BHP is only ~35% greater in $\Delta hpnP$ mutant vegetative cells compared to
344 $\Delta hpnP$ akinetes. Wild-type cells still produce ~30% more BHPs than $\Delta hpnP$ mutants due to
345 their high abundance of non-methylated compounds.

346 **Akinete formation.** Cultures were induced to form akinetes to determine if the lack of 2-
347 methylhopanoids impeded this process. Akinete formation was not affected by the lack of 2-
348 methylhopanoids, with the $\Delta hpnP$ mutant culture forming filaments of akinetes as readily as
349 the wild-type culture (Fig. 3, 4). Confocal imaging revealed no differences in the cell shape,

350 the size or fluorescence emission intensity between wild-type and $\Delta hpnP$ mutant *N.*
351 *punctiforme*. Akinete dominated cultures had more rounded cells and many were larger in size
352 that the vegetative cells (Fig. 4A, B). It was also observed that hormogonia filaments formed
353 alongside the akinetes in the wild-type culture, whereas in the $\Delta hpnP$ mutant culture
354 hormogonia filaments were rare.

355 Fluorescence emissions were highest in the orange and the red wavebands due to
356 phycobilins and chlorophyll (Fig. 4). Microspectral confocal imaging identified prominent in
357 vivo emission peaks due to the photosynthetic pigments typical to *Nostoc* spp. These were due
358 to phycoerythrin (PE), with emission maximum (em_{max}) at 575 nm, phycocyanin (PC) with
359 em_{max} = 625-645 nm and allophycocyanin (ALP), em_{max} = 660-665nm, with shoulders at 675-
360 690 (chlorophyll a photosystem II, chl PSII) and far-red emissions at 710 nm (chl
361 photosystem I, PSI) (Fig. 4C).

362 There was a distinct increase in the PE emissions relative to other peaks in akinetes, which
363 was most prominent in $\Delta hpnP$ mutant cells, and lowest in the $\Delta hpnP$ mutant vegetative cells
364 (Fig. 4C). Akinetes were also observed to have reduced levels of ALP, as well as of chl *a* PSII
365 and PSI emissions, compared to the vegetative cells (Fig. 4C). The differences were
366 especially prominent in mutant $\Delta hpnP$ cells. Since 2-methylhopanoids are not known to be
367 functionally linked to oxygenic photosynthesis, the cause of these differences in pigment
368 expression in mutant $\Delta hpnP$ cells is presently unknown.

369 **Mutant response to temperature stress.** *N. punctiforme* ATCC 29133S wild-type and
370 $\Delta hpnP$ cultures were subjected to stress caused by elevated temperature and freezing/thawing.
371 Based on chl *a* levels, cultures of *N. punctiforme* $\Delta hpnP$ mutant cells grown at 34°C did not
372 grow significantly more slowly than wild-type cultures (Fig. 5). Cultures of *N. punctiforme*
373 $\Delta hpnP$ mutant cells frozen and thawed over a period of 2 weeks and then used to inoculate
374 media and incubated at 25°C failed to grow in two out of three replicates, as opposed to

375 cultures from similarly treated *N. punctiforme* wild-type cultures, where all three replicates
376 recovered and resumed growth. F_v/F_m was measured for control cultures and heat-treated
377 cultures over the course of five weeks to obtain a measurement of photosynthetic efficiency as
378 an indication of cell health (Fig. S3A). F_v/F_m values for both heat-stressed cultures were
379 consistently higher than F_v/F_m values for both control condition cultures. There was no
380 significant difference in F_v/F_m values between *N. punctiforme* wild-type and $\Delta hpnP$ cells in
381 either control or heat-stress conditions.

382 **Mutant response to variable pH.** *N. punctiforme* ATCC 29133 wild-type and $\Delta hpnP$
383 cultures were subjected to different pH values in two separate experimental designs. At pH
384 9.5 the growth rate of both the wild-type and $\Delta hpnP$ mutant did not significantly vary from
385 the control condition (Fig. 5). Growth rates for both treated cultures began to decline after 10
386 days, however the wild-type did not vary considerably from the control over the course of the
387 experiment, indicating a faster stress adaptation. Culture density of the $\Delta hpnP$ mutant was
388 significantly lower than that of the wild-type after 23 days, supporting a faster adaptation of
389 the wild-type to the high pH stress condition.

390 When a range of pH values were tested over several weeks, there was a significant
391 difference in culture density between the wild-type and $\Delta hpnP$ strains grown in pH levels of
392 6.0, 6.2, 6.5, 6.8 and 7.0 after 5 weeks (Fig. 6A). Cultures grown at higher pH values (7.8-9.0)
393 did not survive the duration of the experiment.

394 F_v/F_m was measured for control cultures and cultures grown at pH 6.0 and pH 9.5 over the
395 course of 5 weeks (Fig. S3B). F_v/F_m values for cultures grown at pH 6.0 were no different
396 from those grown in control conditions, and there was also no difference between wild-type
397 and $\Delta hpnP$ mutant cultures. The F_v/F_m values for cultures grown at pH 9.5 varied over time,
398 with a drop in F_v/F_m around days 13-30 corresponding to a decreased growth rate over this

399 period. F_v/F_m values of the $\Delta hpnP$ mutant culture remained higher than those of the wild-type
400 culture over this period.

401 An MTT assay was performed on *N. punctiforme* wild-type and $\Delta hpnP$ cells that had been
402 growing at pH levels from 6.0 to 7.8 for 4 weeks. Values were significantly higher for the
403 mutant cells across all pH values from 6.0 to 7.0 (Fig. S4).

404 **$\Delta hpnP$ mutant response to osmotic stress.** *N. punctiforme* ATCC 29133S wild-type and
405 $\Delta hpnP$ cultures were subjected to a variety of different concentrations of NaCl in two
406 different experimental designs. There was a significant difference in culture density between
407 the wild-type and $\Delta hpnP$ strains grown in concentrations of NaCl of 100 mM, 120 mM, 140
408 mM, 160 mM and 180 mM for 5 weeks (Fig. 6B).

409 F_v/F_m was measured for control cultures and cultures grown at 100 mM NaCl over the
410 course of 5 weeks. F_v/F_m values of both NaCl-treated cultures were consistently higher than
411 those of the control cultures, and F_v/F_m values of the $\Delta hpnP$ mutant cells grown in 100 mM
412 NaCl were significantly higher than those of the wild-type cells grown in the same conditions
413 (Fig. S3C).

414 An MTT assay was performed on *N. punctiforme* wild-type and $\Delta hpnP$ cells that had been
415 growing at concentrations of NaCl from 1.1 mM to 180 mM for 4 weeks. Absorbance values
416 were significantly higher for the mutant cells across all NaCl concentrations from 120 mM to
417 180 mM (Fig. S4).

418

419 DISCUSSION

420 **Hopanoid production in the $\Delta hpnP$ mutant.** It has previously been shown that in *Nostoc* sp.
421 PCC 6720, 2-MeBHPs are produced in far greater abundance than their non-methylated
422 analogs (41). The results presented here were consistent with this. In the $\Delta hpnP$ mutant cells,
423 the abundance of non-methylated pentols increased by 1-2 orders of magnitude, resulting in

424 the combined level of hopanoids being similar for the wild-type and $\Delta hpnP$ mutant cells. The
425 increased production of BHpentol-1 and BHpentol-2 in the $\Delta hpnP$ mutant is likely a response
426 to the inability to produce methylated hopanoids.

427 The lack of 2-MeBHPs in the $\Delta hpnP$ cultures did not impede the cells' ability to form
428 akinetes, which indicates these lipids are not essential for the formation of resting cells under
429 normal conditions. However, as akinetes can survive extreme conditions (57, 58) that were
430 not tested here, it may be that 2-MeBHP is more important for akinete survival against
431 external stresses than for their formation, however this is beyond the scope of the current
432 study.

433 The present examination of akinete-dominated cultures of wild-type and $\Delta hpnP$ mutant
434 cells followed the results of Doughty et al. (34), who found a large increase in the amount of
435 2-methylhopanoids, especially 2-MeBHtetrol, in akinete outer membranes of *N. punctiforme*
436 compared to other cell membrane types. In the present study, no change in the relative
437 abundances of 2-MeBHPs/BHPs was detected between vegetative cell and akinete-dominated
438 cultures (Fig. 2). In comparing the quantitative aspects of the data, however, it is important to
439 note that Doughty et al. (34) used GC-MS and conducted semiquantitative analyses of the
440 lipid contents of isolated membrane fractions as opposed to the data reported here which is
441 based on LC-MS and is for whole cells. More significantly, the bulk of BHP in this strain of
442 *Nostoc punctiforme* is in the form of the two BHpentols and their methylated counterparts.
443 These have longer retention times than BHtetrol and are likely less thermally stable. Thus, it
444 is probable that a significant amount decomposed during the GC-MS analyses reported by
445 Doughty et al. (2009), as evidenced by the presence of a single BHpentol GC peak and
446 thereby rendering the quantification less reliable in that earlier study. In this work we also
447 improved the quantitative rigor by employing an internal standard PD and authentic standards

448 of BHTetrol and 2-MeBHTetrol (10) to determine the relative APCI response factors for these
449 compounds which were slightly but measurably different.

450 **Response of the $\Delta hpnP$ mutant to physiological stress.** Cyanobacteria flourish in a
451 variety of environments where factors such as temperature, pH and salt concentrations are
452 considered outside the optimal range for most bacteria. Cyanobacterial cells consequently
453 have developed adaptations in order to protect themselves against these conditions. Culture
454 conditions used here simulate those found in many extreme environments where
455 cyanobacteria thrive, such as desert soil crusts, hot springs, hypersaline pools, and Antarctic
456 melt water pools.

457 The $\Delta hpnP$ mutant was less resistant to pH and osmotic stress than the wild type. The role
458 of 2-MeBHPs in stress tolerance against pH and osmotic stress is consistent with the findings
459 of Kulkarni et al. (18) in *Rhodopseudomonas*, except that they found a regulatory effect on
460 *hpnP* only in the presence of non-ionic osmolytes.

461 The influence of hopanoids on the tolerance of temperature stresses has been investigated
462 in *Rhodopseudomonas* (18) and *Nostoc punctiforme* (42). Our findings were in agreement
463 with these studies as there was no significant difference between the growth rates or the PSII
464 efficiency of wildtype and $\Delta hpnP$ strains under elevated temperature. Studies of microbial
465 mats from Antarctic melt water ponds (59) and Arctic soils (60) suggest that hopanoids may
466 play a role in cryoprotection. We investigated this by subjecting the wildtype and $\Delta hpnP$
467 mutant to freezing in the absence of an exogenous cryoprotectant. Survival of wildtype
468 cultures was greater than the $\Delta hpnP$ mutant, indicating that the absence of 2-MeBHPs
469 decreases the tolerance of cells to freezing; however, this should be investigated more
470 thoroughly at various temperatures and time scales.

471 F_v/F_m values reflect the efficiency of PSII, while MTT absorbance readings reflect the
472 activity of NAD(P)H-dependent cellular oxidoreductases. Under control culture conditions,

473 no difference in growth rate or F_v/F_m values was observed between wild-type and $\Delta hpnP$
474 mutant cultures. However, when differences (albeit not statistically significant) in growth
475 rates between the two strains became apparent, these were reflected in differing F_v/F_m values
476 and oxidoreductase activity (MTT assays). F_v/F_m for the slower-growing $\Delta hpnP$ mutant
477 cultures were higher than those for the wild-type cultures. From the MTT assays, absorbance
478 readings were higher for $\Delta hpnP$ mutant cells under salt and pH stress than for wild-type cells
479 under the same stress. Taken together, the fact that values were consistently higher for $\Delta hpnP$
480 mutant cells than for wild-type cells under stress indicates an increased metabolic rate in the
481 mutant strain, possibly as a compensatory stress response to reduced protection by hopanoids.
482 This likely resulted in an increase of other protective strategies, that require energy to
483 maintain homeostasis rather than for growth. In addition, our finding of differences in the
484 emission signals from the photosynthetic pigments in $\Delta hpnP$ mutant cultures, with substantial
485 increases of phycoerythrin emissions in mutant akinete-dominated cultures, compared to wild-
486 type akinetes (and both vegetative cultures), may similarly be related to compensatory
487 strategies in cells lacking hopanoids.

488 The presence of 2-MeBHPs does not appear to be necessary for *N. punctiforme* cell
489 function under standard laboratory growth conditions. 2-MeBHPs do, however, appear to aid
490 cyanobacterial cells to resist a variety of stress conditions including pH and osmotic stress.
491 Similar observations have been made in hopanoid mutants of *Burkholderia cenocepacia* (61)
492 and *Rhodopseudomonas palustris* (19) that was attributed to a decrease in outer membrane
493 integrity. While Doughty et al (2009) found that 2-MeBHPs were highest in akinetes, they are
494 also present in vegetative cells being localised between the cells of the filament (6). We
495 observed that cells within $\Delta hpnP$ filaments were not as closely packed (Fig. 3), with this
496 decreased filament integrity at the cell-cell junction potentially increasing the sensitivity of
497 the $\Delta hpnP$ mutant to stressors. Contrary to our original hypothesis, 2-MeBHPs were not

498 necessary for *N. punctiforme* cells to form akinetes under phosphate-limitation and low-light
499 conditions. Akinete outer membrane integrity is not impaired by the absence of 2-MeHPNs,
500 however the total absence of hopanoids (Δshc mutant) results in a substantial decrease of
501 akinete resilience, as determined by lysozyme treatment (42). Although the resistance of
502 akinetes lacking 2-MeBHPs to other stresses is yet to be tested.

503 **Conclusions.** Given the importance of 2-MeBHPs in the adaptation to environmental
504 stressors such as pH, and their dominance in the resting cell-type, the presence of 2-MeBHPs
505 in geological sediment samples are indicative of periods of climate disturbances necessitating
506 adaptation or senescence. As *hpnP* diversity was quite high in desert environments sampled,
507 testing the *N. punctiforme* $\Delta hpnP$ mutant's resistance to UV radiation and desiccation under
508 conditions similar to those experienced by desert biological soil crust communities would be
509 quite relevant (62). The preliminary evidence for a role for 2-MeBHPs in *N. punctiforme* in
510 protection against freezing/thawing stress found here should be followed up with trials over
511 different temperatures, cycles and durations. Unfortunately, we cannot distinguish between
512 the effects of lower amounts of BHPs vs the loss of 2-MeBHP, however complementary
513 experiments utilizing a hopanoid-null mutant may further clarify their role. Overall, the
514 *Nostoc punctiforme* $\Delta hpnP$ mutant created here provides a useful tool for investigating the
515 role of 2-methylhopanoids in cyanobacteria.

516

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525

Supplemental information for this article is available on the Internet.

527

528 REFERENCES

- 529 1. Sáenz JP, Waterbury JB, Eglinton TI, Summons RE. 2012. Hopanoids in marine
530 cyanobacteria: probing their phylogenetic distribution and biological role. *Geobiology*.
531 10:311-319. 10.1111/j.1472-4669.2012.00318.x
- 532 2. Ourisson G, Albrecht P. 1992. Hopanoids. 1. Geohopanoids: The most abundant natural
533 products on Earth? *Accounts of Chemical Research*. 25:398-402. 10.1021/ar00021a003
- 534 3. Kannenberg EL, Perzl M, Müller P, Härtner T, Poralla K. 1996. Hopanoid lipids in
535 *Bradyrhizobium* and other plant-associated bacteria and cloning of the *Bradyrhizobium*
536 *japonicum* squalene-hopene cyclase gene. *Plant Soil*. 186:107-112. 10.1007/BF00035063
- 537 4. Kannenberg EL, Poralla K. 1999. Hopanoid biosynthesis and function in bacteria.
538 *Naturwissenschaften*. 86:168-176. 10.1007/s001140050592
- 539 5. Doughty DM, Coleman ML, Hunter RC, Sessions AL, Summons RE, Newman DK. 2011.
540 The RND-family transporter, HpnN, is required for hopanoid localization to the outer
541 membrane of *Rhodopseudomonas palustris* TIE-1. *Proceedings of the National Academy*
542 *of Sciences*. 108:E1045-1051. 10.1073/pnas.1104209108
- 543 6. Doughty DM, Dieterle M, Sessions AL, Fischer WW, Newman DK. 2014. Probing the
544 subcellular localization of hopanoid lipids in bacteria using NanoSIMS. *PLoS ONE*.
545 9:e84455. 10.1371/journal.pone.0084455
- 546 7. Jürgens UJ, Simonin P, Rohmer M. 1992. Localization and distribution of hopanoids in
547 membrane systems of the cyanobacterium *Synechocystis* PCC 6714. *FEMS Microbiology*
548 *Letters*. 71:285-288. 10.1016/0378-1097(92)90723-2
- 549 8. Simonin P, Jürgens UJ, Rohmer M. 1996. Bacterial triterpenoids of the hopane series from
550 the prochlorophyte *Prochlorothrix hollandica* and their intracellular localization.
551 *European Journal of Biochemistry*. 241:865-871. 10.1111/j.1432-1033.1996.00865.x
- 552 9. Poralla K, Kannenberg E, Blume A. 1980. A glycolipid containing hopane isolated from
553 the acidophilic, thermophilic *Bacillus acidocaldarius*, has a cholesterol-like function in
554 membranes. *FEBS Letters*. 113:107-110. 10.1016/0014-5793(80)80506-0
- 555 10. Wu C-H, Bialecka-Fornal M, Newman DK. 2015. Methylation at the C-2 position of
556 hopanoids increases rigidity in native bacterial membranes. *eLife*. 4. 10.7554/eLife.05663
- 557 11. Silipo A, Vitiello G, Gully D, Sturiale L, Chaintreuil C, Fardoux J, Gargani D, Lee H-I,
558 Kulkarni G, Busset N, Marchetti R, Palmigiano A, Moll H, Engel R, Lanzetta R, Paduano
559 L, Parrilli M, Chang W-S, Holst O, Newman DK, Garozzo D, D'Errico G, Giraud E,
560 Molinaro A. 2014. Covalently linked hopanoid-lipid A improves outer-membrane
561 resistance of a *Bradyrhizobium* symbiont of legumes. *Nature Communications*. 5:5106.
562 10.1038/ncomms6106
- 563 12. Komaniecka I, Choma A, Mazur A, Duda KA, Lindner B, Schwudke D, Holst O. 2014.
564 Occurrence of an unusual hopanoid-containing lipid A among lipopolysaccharides from
565 *Bradyrhizobium* species. *J Biol Chem*. 289:35644-35655. 10.1074/jbc.M114.614529

- 566 13. Sáenz JP, Grosser D, Bradley AS, Lagny TJ, Lavrynenko O, Broda M, Simons K. 2015.
567 Hopanoids as functional analogues of cholesterol in bacterial membranes. Proceedings of
568 the National Academy of Sciences. 112:11971-11976. 10.1073/pnas.1515607112
- 569 14. Caron B, Mark AE, Poger D. 2014. Some like it hot: the effect of sterols and hopanoids on
570 lipid ordering at high temperature. The journal of physical chemistry letters. 5:3953-3957.
571 10.1021/jz5020778
- 572 15. Joyeux C, Fouchard S, Llopiz P, Neunlist S. 2004. Influence of the temperature and the
573 growth phase on the hopanoids and fatty acids content of *Frateuria aurantia* (DSMZ
574 6220). FEMS Microbiol Ecol. 47:371-379. 10.1016/S0168-6496(03)00302-7
- 575 16. Poralla K, Htrtnr T, Kannenberg E. 1984. Effect of temperature and pH on the hopanoid
576 content of *Bacillus acidocaldarius*. FEMS Microbiology Letters. 23:253-256.
577 10.1111/j.1574-6968.1984.tb01073.x
- 578 17. Schmidt A, Bringer-Meyer S, Poralla K, Sahm H. 1986. Effect of alcohols and
579 temperature on the hopanoid content of *Zymomonas mobilis*. Applied Microbiology and
580 Biotechnology. 25:32-36. 10.1007/BF00252509
- 581 18. Kulkarni G, Wu C-H, Newman DK. 2013. The general stress response factor EcfG
582 regulates expression of the C-2 hopanoid methylase HpnP in *Rhodopseudomonas*
583 *palustris* TIE-1. Journal of Bacteriology. 195:2490-2498. 10.1128/JB.00186-13
- 584 19. Weland PV, Hunter RC, Zhang L, Sessions AL, Summons RE, Newman DK. 2009.
585 Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas*
586 *palustris* TIE-1. Journal of Bacteriology. 191:6145-6156. 10.1128/JB.00460-09
- 587 20. Poralla K, Muth G, Härtner T. 2000. Hopanoids are formed during transition from
588 substrate to aerial hyphae in *Streptomyces coelicolor* A3(2). FEMS Microbiology Letters.
589 189:93-95. 10.1111/j.1574-6968.2000.tb09212.x
- 590 21. Jahnke LL, Embaye T, Hope J, Turk KA, M VZ, Des Marais DJ, Summons RE. 2004.
591 Lipid biomarker and carbon isotopic signatures for stromatolite-forming, microbial mat
592 communities and *Phormidium* cultures from Yellowstone National Park. Geobiology.
593 2:31-47. 10.1111/j.1472-4677.2004.00021.x
- 594 22. Summons RE, Jahnke LL, Hope JM, Logan GA. 1999. 2-Methylhopanoids as biomarkers
595 for cyanobacterial oxygenic photosynthesis. Nature. 400:554-557. 10.1038/23005
- 596 23. Talbot HM, Summons RE, Jahnke LL, Cockell CS, Rohmer M, Farrimond P. 2008.
597 Cyanobacterial bacteriohopanepolyol signatures from cultures and natural environmental
598 settings. Organic Geochemistry. 39:232-263. 10.1016/j.orggeochem.2007.08.006
- 599 24. Knoll AH, Summons RE, Waldbauer JR, Zumberge J. 2007. The geological succession of
600 primary producers in the oceans, p 133-163. In Falkowski PG, Knoll AH (ed), Evolution
601 of primary producers in the sea. Elsevier, Boston.
- 602 25. Kuypers MMM, van Breugel Y, Schouten S, Erba E, Sinninghe Damsté JS. 2004. N₂-
603 fixing cyanobacteria supplied nutrient N for Cretaceous oceanic anoxic events. Geology.
604 32:853. 10.1130/G20458.1
- 605 26. Bissert P, Zundel M, Rohmer M. 1985. Prokaryotic triterpenoids 2. 2 β -methylhopanoids
606 from *Methylobacterium organophilum* and *Nostoc muscosum*, a new series of prokaryotic
607 triterpenoids. European Journal of Biochemistry. 150:29-34. 10.1111/j.1432-
608 1033.1985.tb08982.x
- 609 27. Bravo JM, Perzl M, Härtner T, Kannenberg EL, Rohmer M. 2001. Novel methylated
610 triterpenoids of the gammacerane series from the nitrogen-fixing bacterium
611 *Bradyrhizobium japonicum* USDA 110. European Journal of Biochemistry. 268:1323-
612 1331. 10.1046/j.1432-1327.2001.01998.x
- 613 28. Renoux JM, Rohmer M. 1985. Prokaryotic triterpenoids. New bacteriohopanetetrol
614 cyclitol ethers from the methylotrophic bacterium *Methylobacterium organophilum*.
615 European Journal of Biochemistry. 151:405-410. 10.1111/j.1432-1033.1985.tb09116.x

- 616 29. Stampf P, Herrmann D, Bisseret P, Rohmer M. 1991. 2 α -Methylhopanoids: First
617 recognition in the bacterium *Methylobacterium organophilum* and obtention via sulphur
618 induced isomerization of 2 β -methylhopanoid. An account for their presence in sediments.
619 Tetrahedron. 47:7081-7990. 10.1016/s0040-4020(01)96162-9
- 620 30. Vilcheze C, Llopiz P, Neunlist S, Poralla K, Rohmer M. 1994. Prokaryotic triterpenoids:
621 new hopanoids from the nitrogen-fixing bacteria *Azotobacter vinelandii*, *Beijerinckia*
622 *indica* and *Beijerinckia mobilis* Microbiology. 140:2749-2753. 10.1099/00221287-140-
623 10-2749
- 624 31. Rashby SE, Sessions AL, Summons RE, Newman DK. 2007. Biosynthesis of 2-
625 methylbacteriohopanepolyols by an anoxygenic phototroph. Proceedings of the National
626 Academy of Sciences. 104:15099-15104. 10.1073/pnas.0704912104
- 627 32. Ricci JN, Michel AJ, Newman DK. 2015. Phylogenetic analysis of HpnP reveals the
628 origin of 2-methylhopanoid production in Alphaproteobacteria. Geobiology. 13:267-277.
629 10.1111/gbi.12129
- 630 33. Ricci JN, Coleman ML, Welander PV, Sessions AL, Summons RE, Spear JR, Newman
631 DK. 2014. Diverse capacity for 2-methylhopanoid production correlates with a specific
632 ecological niche. ISME J. 8:675-684. 10.1038/ismej.2013.191
- 633 34. Doughty DM, Hunter RC, Summons RE, Newman DK. 2009. 2-Methylhopanoids are
634 maximally produced in akinetes of *Nostoc punctiforme*: geobiological implications.
635 Geobiology. 7:524-532. 10.1111/j.1472-4669.2009.00217.x
- 636 35. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic
637 assignments, strain histories and properties of pure cultures of cyanobacteria.
638 Microbiology. 111:1-61. doi:10.1099/00221287-111-1-1
- 639 36. Meeks JC. 1998. Symbiosis between nitrogen-fixing cyanobacteria and plants. Bioscience.
640 48:266-276. 10.2307/1313353
- 641 37. Meeks JC, Campbell EL, Summers ML, Wong FC. 2002. Cellular differentiation in the
642 cyanobacterium *Nostoc punctiforme*. Archives of Microbiology. 178:395-403.
643 10.1007/s00203-002-0476-5
- 644 38. Meeks JC, Elhai J, Thiel T, Potts M, Larimer F, Lamerdin J, Predki P, Atlas R. 2001. An
645 overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic
646 cyanobacterium. Photosynthesis Research. 70:85-106. 10.1023/A:1013840025518
- 647 39. Welander PV, Coleman ML, Sessions AL, Summons RE, Newman DK. 2010.
648 Identification of a methylase required for 2-methylhopanoid production and implications
649 for the interpretation of sedimentary hopanes. Proceedings of the National Academy of
650 Sciences. 107:8537-8542. 10.1073/pnas.0912949107
- 651 40. Rohmer M, Bouviernave P, Ourisson G. 1984. Distribution of hopanoid triterpenes in
652 prokaryotes. J Gen Microbiol. 130:1137-1150. 10.1099/00221287-130-5-1137
- 653 41. Zhao N, Berova N, Nakanishi K, Rohmer M, Mougnot P, Jürgens UJ. 1996. Structures of
654 two bacteriohopanoids with acyclic pentol side-chains from the cyanobacterium *Nostoc*
655 PCC 6720. Tetrahedron. 52:2777-2788. 10.1016/j.orggeochem.2014.02.004
- 656 42. Ricci JN, Morton R, Kulkarni G, Summers ML, Newman DK. 2016. Hopanoids play a
657 role in stress tolerance and nutrient storage in the cyanobacterium *Nostoc punctiforme*.
658 Geobiology. 15:173-183. 10.1111/gbi.12204
- 659 43. Ricci JN, Coleman ML, Welander PV, Sessions AL, Summons RE, Spear JR, Newman
660 DK. 2013. Diverse capacity for 2-methylhopanoid production correlates with a specific
661 ecological niche. The ISME Journal. 8:675-684. 10.1038/ismej.2013.191
- 662 44. Sepúlveda J, Wendler J, Leider A, Kuss H-J, Summons RE, Hinrichs K-U. 2009.
663 Molecular isotopic evidence of environmental and ecological changes across the
664 Cenomanian-Turonian boundary in the Levant Platform of central Jordan. Organic
665 Geochemistry. 40:553-568. 10.1016/j.orggeochem.2009.02.009

- 666 45. Sepúlveda J, Wendler JE, Summons RE, Hinrichs K-U. 2009. Rapid resurgence of marine
667 productivity after the Cretaceous-Paleogene mass extinction. *Science*. 326:129-132.
668 10.1126/science.1176233
- 669 46. French KL, Tosca NJ, Cao C, Summons RE. 2012. Diagenetic and detrital origin of
670 moretane anomalies through the Permian-Triassic boundary. *Geochim Cosmochim Acta*.
671 84:104-125. 10.1016/j.gca.2012.01.004
- 672 47. Campbell EL, Summers ML, Christman H, Martin ME, Meeks JC. 2007. Global gene
673 expression patterns of *Nostoc punctiforme* in steady-state dinitrogen-grown heterocyst-
674 containing cultures and at single time points during the differentiation of akinetes and
675 hormogonia. *Journal of Bacteriology*. 189:5247-5256. 10.1128/JB.00360-07
- 676 48. Cohen MF, Meeks JC. 1997. A hormogonium regulating locus, *hrmUA*, of the
677 cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its response to an extract of a
678 symbiotic plant partner *Anthoceros punctatus*. *Molecular Plant-Microbe Interactions*.
679 10:280-289. 10.1094/MPMI.1997.10.2.280
- 680 49. Cai YP, Wolk CP. 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC
681 7120 to select for double recombinants and to entrap insertion sequences. *Journal of*
682 *Bacteriology*. 172:3138-3145. 10.1128/jb.172.6.3138-3145.1990
- 683 50. Meeks JC, Castenholz RW. 1971. Growth and photosynthesis in an extreme thermophile,
684 *Synechococcus lividus* (Cyanophyta). *Arch Mikrobiol*. 78:25-41. 10.1007/BF00409086
- 685 51. Cohen MF, Wallis JG, Campbell EL, Meeks JC. 1994. Transposon mutagenesis of *Nostoc*
686 sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular
687 differentiation alternatives. *Microbiology* 140:3233-3240. 10.1099/13500872-140-12-
688 3233
- 689 52. Talbot HM, Watson DF, Murrell JC, Carter JF, Farrimond P. 2001. Analysis of intact
690 bacteriohopanepolyols from methanotrophic bacteria by reversed-phase high-performance
691 liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry.
692 *Journal of chromatography A*. 921:175-185. 10.1016/S0021-9673(01)00871-8
- 693 53. Talbot HM, Rohmer M, Farrimond P. 2007. Rapid structural elucidation of composite
694 bacterial hopanoids by atmospheric pressure chemical ionisation liquid
695 chromatography/ion trap mass spectrometry. *Rapid Commun Mass Spectrom*. 21:880-892.
696 10.1002/rcm.2911
- 697 54. Talbot HM, Summons R, Jahnke L, Farrimond P. 2003. Characteristic fragmentation of
698 bacteriohopanepolyols during atmospheric pressure chemical ionisation liquid
699 chromatography/ion trap mass spectrometry. *Rapid Commun Mass Spectrom*. 17:2788-
700 2796. 10.1002/rcm.1265
- 701 55. Welander PV, Doughty DM, Wu CH, Mehay S, Summons RE, Newman DK. 2012.
702 Identification and characterization of *Rhodopseudomonas palustris* TIE-1 hopanoid
703 biosynthesis mutants. *Geobiology*. 10:163-177. 10.1111/j.1472-4669.2011.00314.x
- 704 56. Wong FCY, Meeks JC. 2002. Establishment of a functional symbiosis between the
705 cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires
706 genes involved in nitrogen control and initiation of heterocyst differentiation.
707 *Microbiology*. 148:315-323. 10.1099/00221287-148-1-315
- 708 57. Olsson-Francis K, de la Torre R, Towner MC, Cockell CS. 2009. Survival of akinetes
709 (resting-state cells of cyanobacteria) in low earth orbit and simulated extraterrestrial
710 conditions. *Origins of life and evolution of the biosphere : the journal of the International*
711 *Society for the Study of the Origin of Life*. 39:565-579. 10.1007/s11084-009-9167-4
- 712 58. Yamamoto Y. 1975. Effect of desiccation on the germination of akinetes of *Anabaena*
713 *cylindrica*. *Plant and Cell Physiology*. 16:749-752. 10.1093/oxfordjournals.pcp.a075195

- 714 59. Jungblut AD, Allen MA, Burns BP, Neilan BA. 2009. Lipid biomarker analysis of
715 cyanobacteria-dominated microbial mats in meltwater ponds on the McMurdo Ice Shelf,
716 Antarctica. *Organic Geochemistry*. 40:258-269. 10.1016/j.orggeochem.2008.10.002
717 60. Rethemeyer J, Schubotz F, Talbot HM, Cooke MP, Hinrichs K-U, Mollenhauer G. 2010.
718 Distribution of polar membrane lipids in permafrost soils and sediments of a small high
719 Arctic catchment *Organic Geochemistry*. 41:1130-1145.
720 10.1016/j.orggeochem.2010.06.004
721 61. Schmerk CL, Bernards MA, Valvano MA. 2011. Hopanoid production is required for
722 low-pH tolerance, antimicrobial resistance, and motility in *Burkholderia cenocepacia*.
723 *Journal of Bacteriology*. 193:6712-6723. 10.1128/JB.05979-11
724 62. Rajeev L, da Rocha UN, Klitgord N, Luning EG, Fortney J, Axen SD, Shih PM, Bouskill
725 NJ, Bowen BP, Kerfeld CA, Garcia-Pichel F, Brodie EL, Northen TR, Mukhopadhyay A.
726 2013. Dynamic cyanobacterial response to hydration and dehydration in a desert
727 biological soil crust. *ISME J*. 7:2178-2191. 10.1038/ismej.2013.83
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729

730 **FIGURE LEGENDS**

731 **Figure 1.** Abundance of hopanoids as analyzed by LC-MS. The lipid content of wild-type
732 (WT) and $\Delta hpnP$ cultures dominated by late exponential phase vegetative cells or akinetes
733 was characterized. Hopanoids (mg BHPs/g cellular material) detected in each sample is given
734 below the cellular type. Quantification was completed using authentic BHtetrol and 2-
735 MeBHtetrol standards. Values were averaged from 2 LC-MS runs (June 4, 2014 and June 16,
736 2015). Error bars represent 1 standard deviation.

737

738 **Figure 2.** Typical LC chromatograms showing the elution patterns of acetylated hopanoids
739 from wild-type (WT) and $\Delta hpnP$ cultures dominated by vegetative cells or akinetes.
740 BHpentol-1 is tentatively identified as bacteriohopane-31,32,33,34,35-pentol based on its
741 elution position relative to that compound in other samples. BHpentol-2 is assigned as
742 bacteriohopane-30,32,33,34,35-pentol previously identified by Zhao et al. (1996). The 2-
743 methyl analogs of these compounds are identified, similarly, by their relative retention times.
744 BHT is bacteriohopane-32,33,34,35-tetrol and 2-MeBHT is its 2-methyl analog.

745

746 **Figure 3.** Differential interference contrast micrographs (A-D) and SEM images (E, F) of
747 cultures of wild-type *N. punctiforme* (A, C, E) and the $\Delta hpnP$ mutant (B, D, F), under control
748 conditions (A, B) showing vegetative cell filaments with heterocysts (Het), and sixteen weeks
749 after transfer to phosphate-free medium and low light conditions (C-F), showing formation of
750 akinetes (Ak) amongst vegetative filaments (Veg).

751

752 **Figure 4.** Confocal imaging of fluorescence emissions of *N. punctiforme* with emissions from
753 PMT1 (520-620 nm) and PMT 2 (638-700 nm) combined - (A) akinete-dominated culture of
754 wild-type; (B) the $\Delta hpnP$ mutant stages; and (C) confocal microspectral detection of
755 photosynthetic pigments in wild-type vegetative stages (red solid line) and akinete stages (red

756 dashed line); of the $\Delta hpnP$ mutant vegetative (green solid line) and mutant akinete (dashed
757 green line) stages. The spectral emission peak at 575 nm is for phycoerythrin (PE), at 645 nm
758 - phycocyanin (PC), at 660 nm - allophycocyanin (APC), the shoulder at 680 nm - chlorophyll
759 a photosystem II (Chl PSII) and at 710 nm - Chl photosystem I (Chl PSI).

760

761 **Figure 5.** Growth curves of *N. punctiforme* wild-type and $\Delta hpnP$ cultures grown under
762 control conditions (pH 7.8, 25°C), heat stress (34°C), and high pH stress (pH 9.5), based on
763 chl *a* concentration. Error bars represent SE from the mean of three independent cultures.

764

765 **Figure 6.** Density of *N. punctiforme* wild-type and $\Delta hpnP$ cultures grown at a range of pH
766 levels from 7.8 to 6.0 (A) or a range of NaCl concentrations from 1.1 mM to 180 mM (B)
767 after five weeks. Measurements are relative to the density of cultures grown in control
768 conditions (pH 7.8, 1.1 mM NaCl), based on chl *a* concentration. Error bars represent SE
769 from the mean of three to four independent cultures. Culture density at weeks 3 and 4
770 are shown in Fig. S2.

Table 1. Primers used for knockout mutant construction.

Primer name	Sequence 5'-3'	Function
hpnP_NKOF	CTCGAGGGTGCAAGAGTCCTAATG GCGGG	Amplify <i>hpnP</i> and flanking regions from <i>N. punctiforme</i> ATCC 29133S genome, and introduce XhoI restriction site for cloning into plasmid
hpnP_NKOR	CTCGAGAGTACAGTCTTGGCTTCAC TGCCA	Amplify <i>hpnP</i> and flanking regions from <i>N. punctiforme</i> ATCC 29133S genome, and introduce XhoI restriction site for cloning into plasmid
hpnP_IF	TAATCAAATGATGCCCCACA	Confirm removal of middle of <i>hpnP</i> gene in plasmid
hpnP_IR	ATTAATGAGCTTGCCCATCG	Confirm removal of middle of <i>hpnP</i> gene in plasmid
NptIntF	TTGGTGATCTCGCCTTTCACCTA	Confirm presence of neomycin-resistance gene in plasmid
NptIntR	GGATGATCTGGACGAAGAGCATC	Confirm presence of neomycin-resistance gene in plasmid
271F1	TGGAGTGAATACCACGACGA	Check sequence of knockout cassette in plasmid
271R1	TTGCCGGGAAGCTAGAGTAA	Check sequence of knockout cassette in plasmid
HNKO_seqF	GGCTTGACACAGGAATGGCGGTGG	Sequence <i>hpnP</i> and surrounding region from <i>N. punctiforme</i> $\Delta hpnP$ genome
HNKO_seqR	GGCTGGTTAGCGCATATTGGCGTGG	Sequence <i>hpnP</i> and surrounding region from <i>N. punctiforme</i> $\Delta hpnP$ genome
HNKO_seqF2	TGCCAGGAGCAAACCAGCAAC	Sequence <i>hpnP</i> and surrounding region from <i>N. punctiforme</i> $\Delta hpnP$ genome
HNKO_seqR2	CGCTACTCGTTGTGTAATTCCGGT	Sequence <i>hpnP</i> and surrounding region from <i>N. punctiforme</i> $\Delta hpnP$ genome
NRT_hpnPF1	TCGGGTTAGATACAGATACACCA	Confirm absence of middle of <i>hpnP</i> gene from <i>N. punctiforme</i> $\Delta hpnP$ genome
NRT_hpnPF2	GGCATGGAAGTAGTATCAGGAAT	Confirm absence of middle of <i>hpnP</i> gene from <i>N. punctiforme</i> $\Delta hpnP$ genome
NRT_hpnPR1	CCATAACGGAGTTCTAGGCAAA	Confirm absence of middle of <i>hpnP</i> gene from <i>N. punctiforme</i> $\Delta hpnP$ genome
NRT_hpnPR2	GTTCTAGGCAAAGCATGAAGTAG	Confirm absence of middle of <i>hpnP</i> gene from <i>N. punctiforme</i> $\Delta hpnP$ genome











