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Widespread production of plant growth-promoting hormones among marine bacteria and their impacts on the growth of a marine diatom

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Abstract

Background Reciprocal exchanges of metabolites between phytoplankton and bacteria influence the fitness of these microorganisms which ultimately shapes the productivity of marine ecosystems. Recent evidence suggests that plant growth-promoting hormones may be key metabolites within mutualistic phytoplankton-bacteria partnerships, but very little is known about the diversity of plant growth-promoting hormones produced by marine bacteria and their specific effects on phytoplankton growth. Here, we aimed to investigate the capacity of marine bacteria to produce 7 plant growth-promoting hormones and the effects of these hormones on *Actinocyclus* sp. growth.

Results We examined the plant growth-promoting hormone synthesis capabilities of 14 bacterial strains that enhance the growth of the common diatom *Actinocyclus*. Plant growth-promoting hormone biosynthesis was ubiquitous among the bacteria tested. Indeed all 14 strains displayed the genomic potential to synthesise multiple hormones, and mass-spectrometry confirmed that each strain produced at least 6 out of the 7 tested plant growth-promoting hormones. Some of the plant growth-promoting hormones identified here, such as brassinolide and trans-zeatin, have never been reported in marine microorganisms. Importantly, all strains produced the hormone indole-3 acetic acid (IAA) in high concentrations and released it into their surroundings. Furthermore, indole-3 acetic acid extracellular concentrations were positively correlated with the ability of each strain to promote *Actinocyclus* growth. When inoculated with axenic *Actinocyclus* cultures, only indole-3 acetic acid and gibberellic acid enhanced the growth of the diatom, with cultures exposed to indole-3 acetic acid exhibiting a two-fold increase in cell numbers.

Conclusion Our results reveal that marine bacteria produce a much broader range of plant growth-promoting hormones than previously suspected and that some of these compounds enhance the growth of a marine diatom. These findings suggest plant growth-promoting hormones play a large role in microbial communication and broaden our knowledge of their fuctions in the marine environment.

Keywords Symbiosis, Bacteria-phytoplankton interactions, Plant growth promoting hormones, Marine diatom, Marine bacteria

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Introduction

Ecological interactions between phytoplankton and bacteria play a key role in structuring the base of aquatic food webs [1]. Mutualistic relationships between these two groups of microorganisms often involve tight metabolic linkages that are underpinned by reciprocal exchanges of metabolites [2–4]. These include the provision of diverse forms of dissolved organic carbon (DOC) from phytoplankton to bacteria, in return for the delivery of re-mineralised micronutrients, vitamins or complex secondary metabolites [2, 4, 5]. For instance, marine Alphaproteobacteria from the Roseobacter clade provide vitamin B12 to the diatom *Thalassiosira*, while benefiting from the provision of diatom-produced 2,3-dihydroxypropane-1-sulfonate (DHPS) [6].

Another group of metabolites called plant growth-promoting hormones (PGPHs) may also be key to phytoplankton-bacteria interactions, with Indole-3 acetic acid (IAA) [5, 7] and gibberellic acid (GA) [8] recently reported in cultures. Previously, GA and IAA were only known to affect plants in terrestrial systems, where GA aids the germination of seed into seedling [8] and IAA promotes plant stem and root elongation towards nutrients [9–11]. However, IAA can be secreted by a range of marine Alpha and Gammaproteobacteria associated with phytoplankton, and this molecule can enhance the growth of the diatom *Pseudo-nitzschia multiseries* [5], the coccolithophore *Emiliania huxleyi* [12, 13] and the dinoflagellates *Symbiodinium microadriaticum* and *Breviolum minutum* [7]. Similarly, GA secreted by bacteria within the Pseudomonadales, Xanthomonadales and Burkholderiales orders induced growth enhancement in the microalgae *Scenedesmus* sp. [8]. Several marine phytoplankton species have recently been shown to benefit from PGPH supplementation [5, 14, 15]. For instance, inoculation of auxins in *Nannochloropsis oceanica* resulted in an increase in growth rates, lipid production and the production of omega-3 polyunsaturated fatty acid and eicosapentaenoic acid [16]. Additionally, Jasmonic acid (JA) increased growth rates, influenced the production of fatty acids and alleviated stress in *Chlorella vulgaris* [17–19]. These findings suggest that different phytoplankton species may be sensitive to a range of PGPHs. Notably, further emerging evidence suggests that, beyond interactions featuring IAA and GA, a wider range of PGPHs may be involved in mutualistic interactions between phytoplankton and bacteria [17, 20].

In terrestrial systems, almost all aspects of plant development and responses to their environment are regulated by PGPHs [21]. Six major classes of PGPHs have been identified: Auxins, Gibberellins, Cytokinin, Ethylene, Jasmonates and Abscisic acid, and each has been linked to specific functions [11, 22–27]. While there is evidence for the presence of PGPHs, such as IAA and GA [5, 8, 28] within phytoplankton and bacteria interactions, we still lack a clear understanding of the diversity of bacteria capable of producing PGPHs and the range of PGPHs they are able to produce.

Given the emerging evidence that PGPHs impact phytoplankton health and productivity, it is important to determine how many of these molecules can be produced by phytoplankton-associated bacteria and which ones affect phytoplankton. Here, we examined the PGPH-production capacity of 14 bacterial isolates that have previously been demonstrated to enhance the growth of *Actinocyclus* [29], a common marine diatom [30, 31]. We subsequently measured the effects of different PGPHs produced by these bacteria on *Actinocyclus* growth.

Materials and methods

Diatom–*Bacteria* model system

We examined the potential role of PGPHs within a model diatom-microbiome system, whereby we have previously identified the growth-promoting benefits of a set of 14 diatom-associated bacteria [29]. *Actinocyclus* sp. was isolated from an oceanographic reference station (Port Hacking — 34° 05.00 S, 151° 15.00 E) on the east coast of Australia [29, 32]. *Actinocyclus* sp. cultures were maintained in sterile seawater supplemented with f/2 medium [33], and grown under light conditions of 55–65 µE, 12:12 day/night at 20–22 °C. We previously demonstrated that 14 unique bacterial strains, spanning five different families and 14 genera (Supplementary Table S1), promote the growth of *Actinocyclus* sp. in co-culture conditions [29]. Here, we screened the capacity of these beneficial diatom-associated bacteria to synthesise PGPHs.

Genome sequencing and genomic survey of PGPH biosynthesis pathway genes in bacterial isolates

To confirm the presence of genes involved in PGPH synthesis within the 14 isolated bacterial strains, whole genome sequencing was conducted. Each isolate was grown in 20 mL of Marine Broth (Difco Marine Broth 2216). Cultures grown to the exponential phase were centrifuged at 4000 rpm for 10 min, and the pellet was used for DNA extraction, using a physical lysis extraction technique [34]. Briefly, lysis of the cells was achieved by adding a solution of 0.0215 g/mL KOH and 0.008 g/mL dithiothreitol, followed by a freeze–thaw cycle. Free DNA was then purified and eluted using AMPure XP magnetic beads (Beckman Coulter) [35].

Genome libraries were prepared and sequenced on the Illumina MiSeq platform at the Australian Genomic Research Facility (AGRF). Raw sequences were quality checked and adapters trimmed using trimmomatic v0.35 [36] before de novo genome assembly was

performed using SPAdes [37]. The resulting contigs were binned using metabat2 [38], with the quality of the assembled genomes calculated using checkM2 [39]. Open reading frames (ORFs) were identified with Prodigal [40], and taxonomy was assigned using Genome Taxonomy Database (GTDB-tk v. 2.2.4). To assess the presence of PGPH biosynthesis pathways for the isolated bacteria, protein sequences, predicted using prodigal, were blasted (e-value $< 10^{-10}$) against a custom reference database that contained all genes for IAA, GA and TZ biosynthesis listed in prior literature [13, 23, 41–43], which were retrieved from NCBI. For the analysis of JA and Brassinosteroid (BSN), for which no bacterial biosynthetic pathways are currently known, we leveraged information from the eukaryotic (plant) alpha-Linoleic and Brassinosteroid reference pathway deposited in the KEGG database. Pathways for Indole-3-butyric acid (IBA) biosynthesis are still completely uncharacterised and were therefore not included in this analysis. A database containing the sequences of genes involved in PGPH pathways, obtained from KEGG GENES Database (accessed 11 April 2022; https://www.genome.jp/kegg/genes.html), was used to query for homologs. PGPH synthesis genes from bacteria closely related to the 14 isolates examined here were collated for the IAA, GA and TZ pathway genes, and eukaryotic (plant) proxy genes were used as query for ABA, JA and BSN (Supplementary Table S2–S7). Reciprocal BLASTp and BLASTn searches for bacterial PGPH homologs were performed on the whole genome sequences of the 14 bacterial isolates and homolog PGPH genes as the query. BLASTp hits with an e-value $\geq 1 \times 10^{-10}$ or less was used as a cut-off [13].

Extraction protocol for PGPH quantification

All 14 bacterial strains were phenotypically screened for PGPH biosynthesis, whereby PGPH levels during the exponential growth phase for each of the isolates were measured. To prepare cultures for PGPH extraction, 50 ml of each bacterial isolate was grown to exponential phase in quadruplicate. A 200 µl aliquot was collected from each replicate, and bacterial enumeration was performed using a CytoFLEX LX flow cytometer (Beckman Coulter). Samples were stained with SYBR-Green I (final concentration 1:10,000; SYBR-Green I, Invitrogen) and incubated for 15 min at 4 °C. Bacterial cells were then identified according to side scatter (SSC) and green fluorescence (SYBR-Green) and enumerated [44]. Two different PGPH extraction protocols were carried out to quantify: (i) intracellular and (ii) exuded PGPHs. Each replicate was then transferred to a 50-mL Falcon tube (Eppendorf), and centrifuged at 2000 rpm for 30 min (Eppendorf). The supernatant was collected and transferred into a clean 50-mL Falcon tube. Supernatant and pellet samples were flash frozen in liquid nitrogen and stored at − 80 °C until PGPH extraction.

Intracellular PGPH extractions

To quantify intracellular PGPH concentrations within the bacterial cell pellets, each sample was separated into two pellets, and two separate extraction protocols were implemented, (i) for the quantification of Auxins, Gibberellins, Cytokines and Zeatins, and (ii) for the quantification of Steroids. For the extraction of (i), 2 mL of a 14:4:1 solution of 99% methanol (Sigma-Aldrich), ultrapure water, and 99% formic acid (Sigma-Aldrich) was added to each tube, which was then sonicated for 2 min to lyse the cells. The cellular debris was subsequently centrifuged down at 2000 rpm, and quadruplicate samples of supernatant extract were freeze-dried at − 80 °C. The sample residues were then reconstituted into 1 mL of 1 M formic acid, and further purified on Evolute express CX columns (Biotage) that had been pre-conditioned with methanol and 1 M formic acid. The samples were first eluted in 3 mL of 99% methanol, then 3 mL of 0.35 M ammonium hydroxide (Sigma-Aldrich) and finally 3 mL of 0.35 M in ammonium hydroxide in 60% methanol. All samples were then freeze-dried at − 80 °C and reconstituted with 99% ultrapure high-pressure liquid chromatography (UHPLC) grade methanol (Sigma-Aldrich). For the extraction of (ii), the pellet was freeze dried at − 80 °C, then reconstituted in 1 mL of 60% Acetonitrile supplemented with 5-α-Cholestane (internal standard 1 mg/mL) (Sigma-Aldrich). Samples were then further lysed through bead beating using a mixture of ≤ 106 µm and 425–600 µm acid-washed glass beads (0.5 g of each) (Sigma-Aldrich) and sonication in an ultrasonic cleaner (Unisonic PTY.LTD, Australia) for 3 min at 4 °C. Samples were then centrifuged and the supernatant was collected for LC–MS/MS analysis [45, 46].

Extracellular PGPH extraction

To quantify the PGPH concentrations exuded by bacterial cells, the supernatant of the bacterial cultures was first filtered through a 0.22-µm filter (Sartorius) to remove all bacteria. A sixteen-valve SUPELCO manifold and 6 cc 200 mg Oasis HBL cartridge were used to extract PGPH from the samples. The cartridges were pre-conditioned with 20 mL of ultrapure water and then 20 mL of 99% methanol (Sigma-Aldrich). The bacterial supernatant was then loaded into the column 10 mL at a time. The samples were eluted with 3 mL of 20% methanol, then 3 mL of 70% methanol (Sigma-Aldrich). All samples

were then freeze dried at − 80 °C and reconstituted with 99% UHPLC grade methanol (Sigma-Aldrich) [45, 46]. Extracellular quantification of BSN concentration was not conducted as extraction protocols to detect released concentrations of BSN have not yet been developed.

Chemical characterisation using LC MS–MS analysis

Known standards of IAA, Indole-3 butyric acid (IBA), Abscisic acid (ABA), Gibberellic acid (GA), Jasmonic acid (JA), Trans-zeatin (TZ) and Brassinolide (BSN) were used to calibrate the LC–MS/MS to mobile phase, run time and energy requirements for phytohormone fragmentation. A 10-point standard curve spanning 50, 25, 12.5, 6.25, 3.12, 2.5, 1.25, 0.624, 0.312 and 0.03 ng/mL was prepared for each PGPH. Quantification of PGPH was performed using a Shimadzu LCMS-8060 (Shimadzu, Kyoto, Japan) instrument with a dual ion source (DUIS) interfaced to Shimadzu Nexera X2 liquid chromatography system. The separation was performed on an Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 150 mm) using a binary gradient of MilliQ water (A) and methanol (B) for 15 min, at a flow rate of 0.18 mL/min. The linear gradient programme was run as follows: 0–10 min, 20–95% B; 10–11 min, 95–20% B; 11–15 min, 20% B. Column temperature was maintained at 30 °C. Samples were analysed in the multiple reaction monitoring mode. The following transitions were monitored with a fragmentor voltage of 160 V. Collision energy for each hormone were as follows: IAA − 6 eV m/z 174 → 130, d_5- IAA m/z 179 → 135, IBA − 10 eV, m/z 202 → 116, ABA − 9 eV, m/z 263 → 219, GA − 21, m/z 345 → 221, JA − 25 eV, m/z 209 → 59, TZ, − 10 eV, m/z 220 → 136, BSN − 14 eV, m/z 481 → 445. The resulting peaks were then quantified using the 10-point calibration curves [45]. Statistical differences in hormone concentrations between the bacteria isolates were assessed using a one-way ANOVA.

PGPH and cell density index correlation analysis

We next aimed to determine whether the concentrations of bacterial-produced PGPHs measured here could lead to phytoplankton growth enhancement. Specifically, we correlated levels of PGPH production with the growth-promoting effect of 12 out of the 14 bacteria isolates when co-grown with *Actinocyclus*. For each bacterial strain tested, we used Pearson's correlation to investigate the relationship between the extracellular concentration of each PGPH they release and the effect the strain has on *Actinocyclus* growth (using the previously reported cell density index, a metric that integrates the growth-promoting effect of a bacterial strain in co-culture with *Actinocyclus* over time). As reported in Le Reun et al. (2023), to assess the growth-promoting effect of each bacterial strain on the diatom over time, the co-cultures were divided into three time periods: days 0–6, days 8–12 and days 14–16. For each strain and time period, a cell density index (CD) was calculated. This index was determined by calculating the area under the curve (AUC) for each co-culture (using the AUC() function from the DescTools R package) and then dividing it by the AUC of the axenic culture [29].

Measuring the effect of PGPH on diatom growth

To determine the effect of each PGPH on the growth of *Actinocyclus* sp., each hormone identified in the bacterial isolates was added individually to axenic *Actinocyclus* sp. cultures. Axenic cultures of *Actinocyclus* sp. were grown to exponential phase, diluted to 5000 cells/mL and aliquoted into a 50 mL volume in f/2 medium. Stock solutions of the PGPHs were prepared to concentrations matching those measured in the extracellular production by bacterial isolates. An initial screening of the growth effects was conducted at three different concentrations, including one magnitude above and below the extracellular concentrations measured in our experiment, in order to determine the optimal concentration of PGPH required for growth enhancement. The optimal concentrations were found to be 1×10^{-8} µg/mL for IAA, 1×10^{-9} µg/mL for BA, 1×10^{-10} µg/mL for TZ, GA and BSN, and 1×10^{-11} µg/mL for IBA, ABA and JA (Sigma-Aldrich). All hormones were dissolved in f/2 medium with the exception of IAA and ABA, which were diluted in ethanol and dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Individual hormones were added at early exponential (day 2) and early stationary (day 6) of *Actinocyclus* sp. growth phases to mimic a supply by associated bacteria.

The axenic cultures of *Actinocyclus* sp. were generated in Le Reun et al. (2023) and also used in this study. Following antibiotic treatment, cultures were subjected to a range of tests to ensure axenicity: (1) no bacterial growth was identified when 1 mL of algal culture was inoculated into 100% Marine Broth for 7 days; (2) drop-plating of algal culture onto 100% Marine Agar resulted in no bacterial growth; (3) no bacterial population was present in algal cultures stained with SYBR-Green (Sigma-Aldrich S9430) and enumerated with flow cytometry; (4) no bacterial 16S rRNA gene sequences were identified following amplicon sequencing of the V1-V3 region of these cultures ($n = 3$) with all reads identified as chloroplast, confirming the *Actinocyclus* sp. cultures were indeed axenic [29].

Axenic *Actinocyclus* sp. cultures were spiked with each hormone in 70 mL sterile tissue culture flasks ($n = 4$ flasks for each pair). Each tissue culture flask contained 50 mL of *Actinocyclus* sp. at 5000 cells/mL and 5 µL of

PGPH diluted to the aforementioned final concentrations. Additionally, quadruplicate controls were maintained alongside the treatments, which consisted of axenic phytoplankton without any hormones added. All flasks were maintained in the same incubation conditions as described above. The cultures were monitored over time using flow cytometry to enumerate diatom cell numbers and to confirm the culture remained axenic. Samples were taken every second day at the same time, whereby 200 µL samples were collected in 1.5 mL microcentrifuge snap-seal Eppendorf tubes and fixed with 2% glutaraldehyde. Cells were enumerated using a CytoFLEX LX flow cytometer (Beckman Coulter). *Actinocyclus* cells were discriminated according to forward scatter (FSC), side scatter (SSC) and red fluorescence (chlorophyll), and bacterial samples were enumerated as previously described. To confirm the absence of attached bacteria, axenic *Actinocyclus* cultures were DAPI stained and checked for bacteria under epifluorescence microscopy after the experiment. There was no evidence of bacteria attached to the diatom or in the surrounding suspension [47].

All data was analysed using repeated-measures ANOVA to identify significant changes in growth between control and treatment cultures. All data was assessed for normality and homogeneity of variance using Shapiro and Levene's test before performing pairwise comparison to assess the overall effect of the PGPH treatments. Differences in cell numbers between the hormone treatment and axenic control at each time point were assessed using a two-way repeated-measure ANOVA (Simple Main Effect test) [48].

Results

Genomic survey of PGPH biosynthesis pathway genes in bacterial isolates

Here, we examined PGPH biosynthesis capacity in the genomes of 14 unique bacterial strains (Supplementary Table S1) that have previously been demonstrated to enhance the growth of the diatom *Actinocyclus* sp. [29]. Our analysis revealed that all 14 of the tested bacteria have the genomic potential to synthesise at least one of the PGPHs investigated.

Indole-3 acetic acid biosynthesis pathway

Five different IAA biosynthesis pathways have previously been identified, including the Indole-3-acetamide, Indole-3-pyruvate, Indole-3 acetonitrile, Tryptophan side chain oxidase and Tryptamine pathways [13, 41]. All 14 bacterial isolates possessed clear orthologues of all the enzymes required for one or more pathways for IAA production (Fig. 1, Supplementary Table S2), with genes involved in the tryptamine and indole-3-acetonitrile pathways the most common. Of the 14 bacteria screened, 92% displayed the genomic capacity to synthesise *Tryptophan decarboxylase* (*TDC*), which is a precursor to the Tryptamine dependent pathway (Fig. 1; green). In addition, 85% of the bacterial genomes harboured *Amine oxidase* (*AO*), a gene required to synthesise Indole-3-acetaldehyde. The capacity to synthesise IAA via the Indole-3-acetonitrile pathway was present in 64% of the bacteria (Fig. 1; red), whereby 85% possessed the *Nitrate hydrolase* (*NH*) gene, and 85% had the *Indole-acetaldoxime dehydratase* (*NIT*) gene, which is responsible for the last step of the IAA synthesis pathway.

Gibberellic acid biosynthesis pathway

GA synthesis is mediated by a single linear pathway categorised into three distinct stages. The first stage involves catalysis by soluble enzymes to form molecules such as ent-kaurene, while the second stage involves oxidation to form precursor GA molecules such as GA12-aldehyde. The final stage involves further catalysis via *mono-oxygenases* to form four bioactive GA molecules (Fig. 2A) [23]. Only 70% of the targeted bacteria (mainly from the Rhodobacteraceae and Vibrionaceae clades) possessed genes responsible for the second and third stages of the pathway (Fig. 2B, Supplementary Table S4). Interestingly, none of the genomes of the tested bacteria contained the *Copalyl diphosphate synthase* (*CPS*) or *Kaurene synthase* (*KS*).

Trans-zeatin, Abscisic acid and Jasmonic acid brassinosteroid pathways

Analysis of homologs for TZ pathway-specific genes revealed that all 14 bacteria examined possessed the putative bacterial pathways via one or more biosynthesis routes (Supplementary Fig. S1 and Table S6). The TZ synthesis pathway comprises two distinct routes, comprising known and unknown TZ biosynthesis genes. With the exception of *Isopentenyl-diphosphate delta-isomerase* (*IPP isomerase*), 100% of the screened bacteria harboured all other known genes necessary for synthesising TZ (Supplementary Fig. S1 and Table S6). Putative pathways for ABA, JA and BSN biosynthesis were constructed based on eukaryotic (plant) organisms. Our analysis revealed that the genomes of the screened bacteria include limited gene orthologues for pathways of plant origin, with none of these bacteria having a complete set of known genes necessary for hormone synthesis (Fig. 5, Supplementary Fig. S1, S3 and Table S3, S5, S7).

Quantification of PGPH production by bacterial isolates

Intracellular PGPH concentrations

Strikingly, with the exception of IBA, all 14 bacterial strains produced each of the PGPHs tested (Fig. 3). This

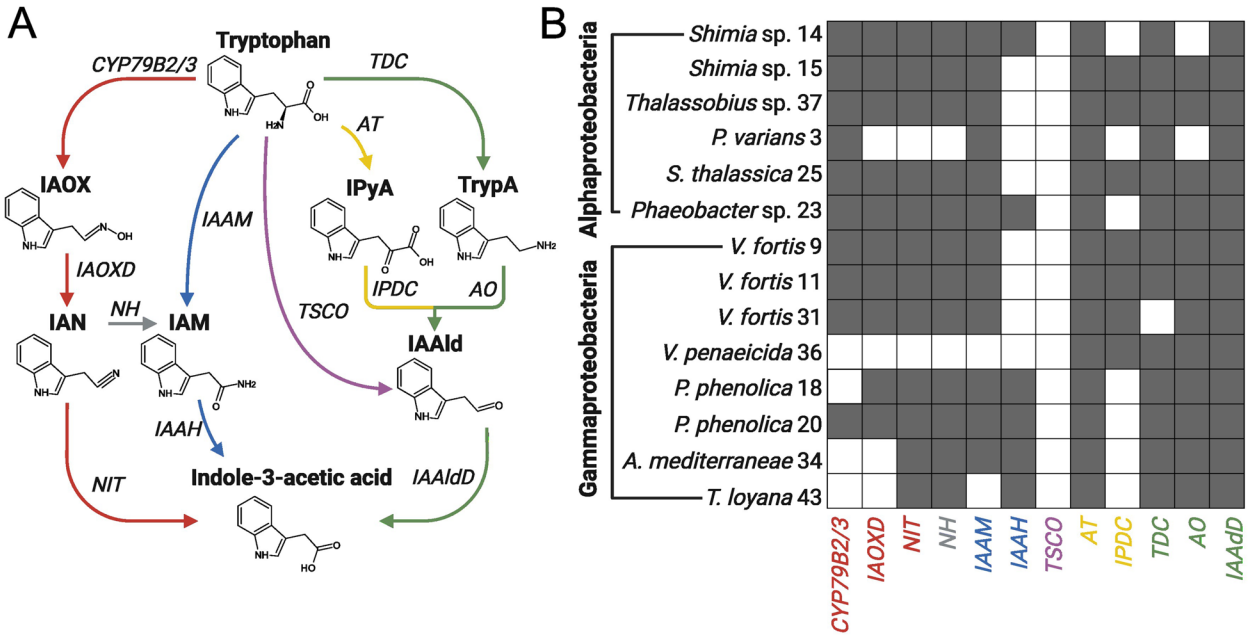

Fig. 1 Indole-3-acetic acid biosynthesis pathway and production potential in the 14 bacterial isolates. **A** Tryptophan-dependent IAA pathways in bacteria. Five main IAA biosynthesis pathways, including the Indole-3-acetonitrile (red), Indole-3-acetamide (blue), Tryptophan side chain oxidase (purple), Indole-3-pyruvate (yellow) and Tryptamine (green) pathways are depicted. Intermediate molecules are depicted with black bold text. IAOx, Indole-3-acetaldoxime; IAN, Indole-3-acetonitrile; IAM, indole-3-acetamide; IPyA, Indole-3-pyruvate; TrypA, Tryptamine; IAAld, indole-3-acetaldehyde. Enzymes are depicted in un-bold black text and lines. CYP79B2/3, cytochrome P450; IAOxD, Indole-acetaldoxime dehydratase; NIT, Nitrilase; NH, Nitrile hydrolase; IaaM, Tryptophan-2-monooxygenase; IaaH, Indole-3-acetamide hydrolase; TSO, Tryptophan side chain oxidase; IPDC, TDC, Tryptophan decarboxylase; Indole-3-pyruvate decarboxylase; AO, Amine oxidase; IAAldD, indole-3-acetaldehyde dehydrogenase. **B** Heatmap of the presence of orthologues for IAA biosynthesis in the 14 bacterial strains tested. Grey squares depict the detection of orthologues of IAA synthesis genes (bottom) in the bacteria (left), as indicated by a BLASTp search (e-value < 10^{-10}, Supplementary Table S2). Strain numbers, as defined in Le Reun et al. (2023), are reported next to the scientific name of each isolate

result was particularly notable given that the capacity of most strains to produce a wide diversity of PGPHs was not expected based on their genome sequences. On average, the concentrations of BSN were 100 times higher than the other hormones tested (one-way ANOVA, $p = 0.000271$, Supplementary Table S14). This was followed by IAA and ABA (one-way ANOVA, $p = 0.000397$ and $p = 1.1 \times 10^{-13}$, Supplementary Table S8 and S10, respectively), which exceeded that of IBA, JA, GA and TZ by 10 to 100 times (Supplementary Fig. S5). Out of the 14 bacterial strains, *Phaeobacter* sp. 23 produced significantly higher concentrations of IBA, ABA, TZ and BSN, which exceeded all other tested bacteria tested by 10 to 1000 times (one-way ANOVA, $p < 0.05$, Supplementary Table S9, S10, S13 and S14).

Extracellular PGPH concentrations

Out of the 14 bacterial strains tested, 6 (42%) released all six of the tested PGPHs into the surrounding medium, while 10 (71%) released at least five of these compounds (Fig. 4). However, there was considerable variation in the concentrations and diversity of PGPHs released among the strains. Of the seven targeted PGPH, only IAA was produced by 13 of the 14 tested bacteria. Production of the six other PGPHs — GA, JA IBA, ABA, TZ and BSN — was heterogeneously distributed across the screened strains (Fig. 4). On average, out of the seven hormones tested, IAA was exuded at significantly higher concentrations (one-way ANOVA, $p < 0.05$, Supplementary Table S8), closely followed by TZ (one-way ANOVA, $p < 0.05$, Supplementary Table S13), with exuded concentration of IAA exceeding IBA, JA, GA and ABA by 10 to 1000 times (Fig. 4). *T. loyana* 43 released the highest concentrations of IAA ($2.7 \pm 1 \times 10^{-8}$ µg/cell; one-way ANOVA $p < 0.05$, Supplementary Table S8), while *Phaeobacter* sp. 23 released the highest concentrations of IBA, ABA, GA and JA (one-way ANOVA, $p < 0.05$, Supplementary Table S9–S12). Notably, *P. varians* 3 did not exude detectable amounts of any of the tested PGPHs, despite producing all 7 of the hormones.

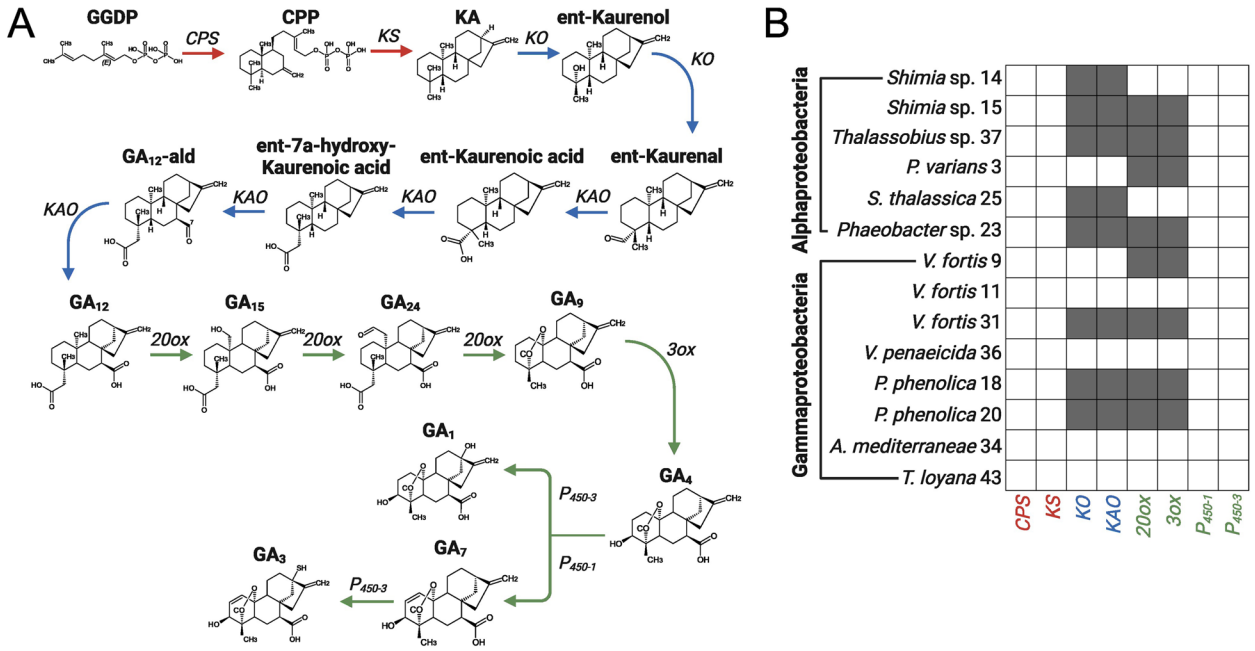

Fig. 2 Gibberellic acid biosynthesis pathway and production potential in the 14 bacteria isolates. **A** GA pathway in bacteria. Each stage of the pathway is categorised by red (stage 1), blue (stage 2) and green (stage 3) coloured arrows. GA biosynthesis pathway intermediate molecules are depicted with black bold text. GGDP, Geranylgeranyl diphosphate; CCP, Copalyl diphosphate; KA, Ent-kaurene; GA_{12}-ald, Gibberellic acid-12-aldehyde; GA, Gibberellic acid. Genes and enzymes are depicted with un-bold black text. CPS, Copalyl diphosphate synthase; KS, Kaurene synthase; KO, Kaurene oxidase KAO, Kaurenoic acid oxidase 20ox, 20-oxoglutarate-dependent dioxygenase; 3ox, 3-oxidase. **B** The presence of orthologues for GA biosynthesis in the 14 bacterial strains tested. Grey squares depict the detection of orthologues of GA synthesis genes (bottom) in the bacteria (left), as indicated by a BLASTn and BLASTp search (e-value < 10^{-10}, Supplementary Table S4). Strain numbers, as defined in Le Reun et al. (2023), are reported next to the scientific name of each isolate

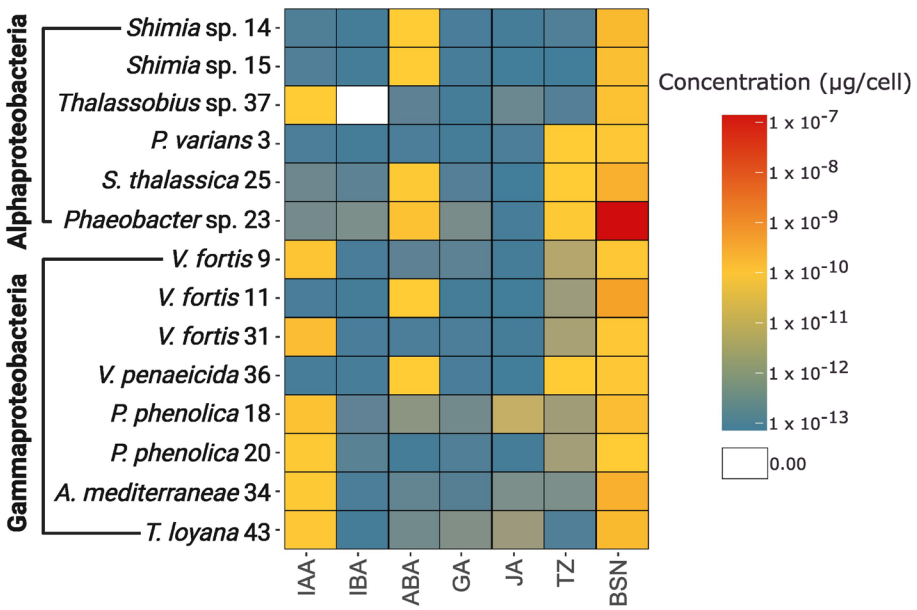

Fig. 3 Quantification of intracellular concentrations of plant growth-promoting hormones (µg/cell) in the 14 bacterial strains tested. A heat map illustrating the intracellular concentration (coloured squares) of the plant growth-promoting hormones Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Abscisic acid (ABA), Gibberellic acid (GA), Jasmonic acid (JA), Trans-zeatin (TZ) and Brassinolide (BSN) of the 14 bacteria tested

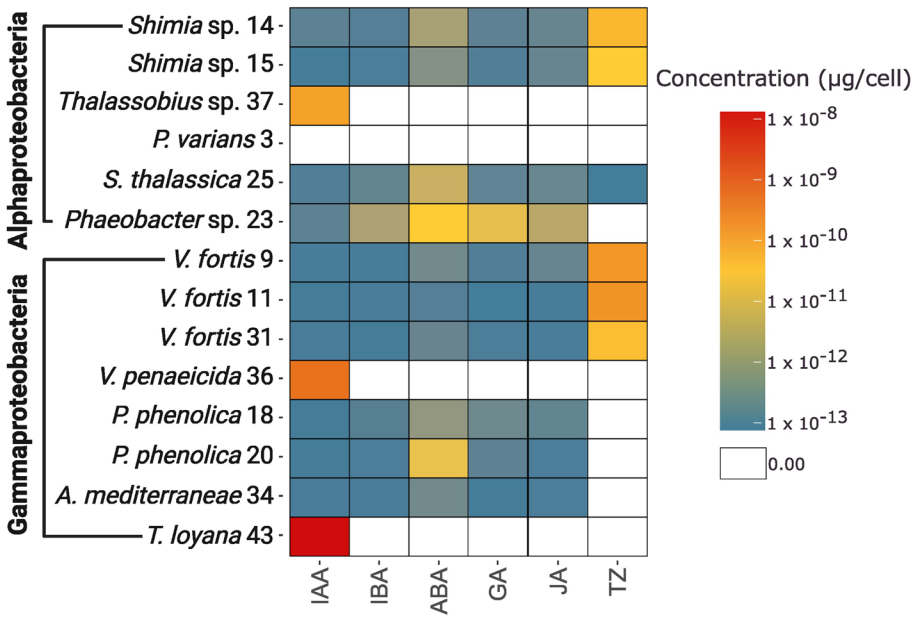

Fig. 4 Quantification of plant growth-promoting hormones (µg/cell) exuded by the 14 bacterial strains tested. A heat map illustrating the concentration (coloured squares) of the plant growth-promoting hormones Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Abscisic acid (ABA), Gibberellic acid (GA), Jasmonic acid (JA) and Trans-zeatin (TZ) released into the surrounding media from the 14 bacteria tested. Quantification of released BSN was not conducted as extraction protocols to detect extracellular concentrations of BSN have not yet been developed

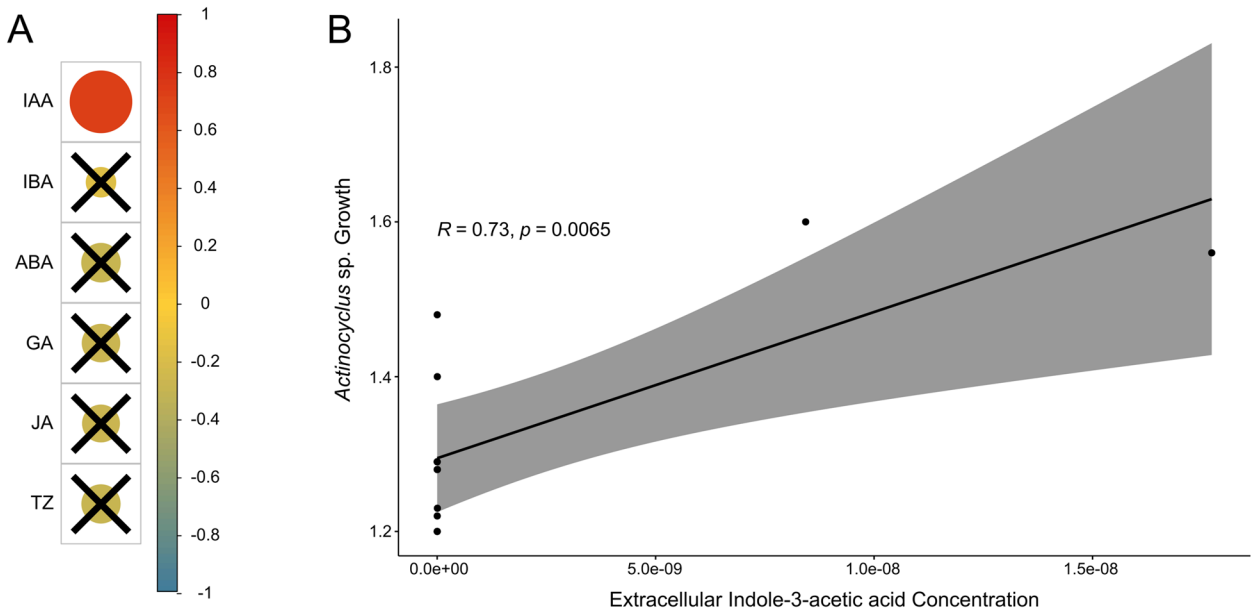

Fig. 5 Correlation analysis between extracellularly released PGPHs and maximum *Actinocyclus* growth enhancement in co-culture experiments. *Actinocyclus* growth enhancement (cell density index) was calculated as reported in Le Reun et al. 2023, whereby the bacteria co-cultures were divided into three time periods: days 0–6, days 8–12 and days 14–16. For each strain and time period, the cell density index was determined by calculating the area under the curve (AUC) for each co-culture (using the AUC() function from the DescTools R package) and then dividing it by the AUC of the axenic culture [29]. **A** Correlogram for each PGPH tested (the size and colour of each bubble are proportional to the strength of the correlation). Only statistically significant correlations are not crossed (Pearson's correlation, $p < 0.05$). **B** Linear regression of the only statistically supported correlation. The *x*-axis represents released IAA concentrations measured for each bacterial isolate (µg/cell), while the *y*-axis represents the maximum growth enhancement of *Actinocyclus* cells for 12 out of the 14 bacteria isolates when co-grown with *Actinocyclus* [29]. R-square and *p*-value (Pearson's correlation) are displayed in italics

Relationship between phytoplankton growth and PGPH concentrations

To determine if the production of specific PGPHs may be linked to bacterial augmentation of *Actinocyclus* growth, we used results derived from a previous experiment that quantified the growth promotion (cell density index) of each of the bacterial isolates during co-culture experiments with *Actinocyclus* [29]. Our results revealed that out of the 7 hormones tested, IAA was the only hormone for which extracellular concentrations were positively and significantly correlated to increases in *Actinocyclus* cell density (Pearson's correlation, $R = 0.73$; Fig. 5).

Measuring the effect of PGPH on *Actinocyclus* sp. growth

To measure whether the seven detected bacterial PGPHs enhanced the growth of *Actinocyclus*, axenic diatom cultures were inoculated with each hormone at concentrations one order of magnitude higher and lower than the highest extracellular PGPH concentrations released by the bacteria. Provision of IBA, ABA, JA, TZ and BSN did not result in any significant increase in *Actinocyclus* sp. cell numbers (Fig. 6 and Supplementary Table S15). However, the addition of IAA and GA led to significantly higher *Actinocyclus* cell numbers compared to the axenic control (repeated-measures ANOVA, p-value < 0.05; Fig. 6, Supplementary Table S15). All three tested IAA concentrations significantly increased *Actinocyclus* cell numbers compared to the control (repeated-measures ANOVA, p-value < 0.05; Fig. 6A, Supplementary Table S15). This effect started after 4 days and lasted until the end of the experiment. The highest IAA concentration tested (10^{-7} µg/mL) caused a 2.5 times increase in cell numbers compared to the control after 7 days (repeated-measures ANOVA, p-value < 0.05, Supplementary Table S15). For GA, only the lowest concentration (10^{-11} µg/mL) significantly increased the number of *Actinocyclus* cells compared to the control (repeated-measures ANOVA, p-value < 0.05; Fig. 6B, Supplementary Table S15). This effect started on day 6 and lasted until the end of the experiment with the largest effect of GA on *Actinocyclus* cell numbers which was recorded on day 10, when a 30% increase in cell abundance occurred (repeated-measures ANOVA, p-value < 0.05; Fig. 6B, Supplementary Table S15).

Discussion

Diverse chemical currencies are involved in the ecological interactions between phytoplankton and bacteria [4–6, 28, 49]. Among exchanged metabolites, the bacterial production of PGPHs may be a critical regulator of phytoplankton growth [50, 51], yet the production and effects of PGPHs remain understudied in the marine environment. Here, our goal was to deliver a comprehensive analysis of the PGPH-producing capabilities of a suite of marine bacteria that have previously been demonstrated to enhance the growth of the diatom *Actinocyclus* [29], and quantify the impacts of these hormones on diatom growth. Both analysis of the genomic potential for PGPH synthesis and direct chemical quantification revealed that the diverse range of marine bacteria tested can produce a wide range of PGPHs and that some of these PGPHs enhance the growth of *Actinocyclus*.

Widespread production of bacterial-produced PGPHs

PGPH biosynthesis was widespread across the tested bacteria, with each of the 14 strains producing at least six different hormones. Surprisingly, 85% of the strains synthesised all seven PGPHs tested, including Brassinolide, which had never been reported in the marine environment before. This result is striking because the simultaneous biosynthesis of more than three PGPHs has not previously been reported in marine bacteria [52]. In addition, the capacity of most strains to produce a wide diversity of PGPHs was not expected based on their genome sequences. Indeed, despite the absence of genes thought to be required for JA, ABA and BSN biosynthesis and the partial presence of genes required for GA biosynthesis,

(See figure on next page.)

Fig. 6 Growth of *Actinocyclus* inoculated with IAA, GA, IBA, ABA, JA, TZ, BSN and the average PGPH concentration released by the 14 bacteria. **A** *Actinocyclus* cell numbers per millilitre in response to three different IAA concentrations (chosen based on quantifications data, see methods). **B** *Actinocyclus* cell numbers per millilitre in response to three different GA concentrations (chosen based on quantification data, see methods). Error bars are standard error of the mean (SEM), $n = 4$. Red arrows depict inoculation times for IAA and GA treatments (days 2 and 6 for both). Number of *Actinocyclus* sp. cells per millilitre (*y*-axis) when treated with **C** IBA, **D** ABA, **E** JA, **F** TZ and **G** BSN at three different concentrations. The blue line, yellow line and red line each represent concentrations of 1×10^{-12}, 1×10^{-11} and 1×10^{-10} µg/mL for IBA, ABA, JA and BSN, 1×10^{-10}, 1×10^{-9} and 1×10^{-8} for BA and 1×10^{-9}, 1×10^{-8} and $1 \times 10.^{-7}$ µg/mL for TZ. *Actinocyclus* sp. axenic control grown solely in F/2 is represented by the black line with filled circles over the course of 11 days (*y*-axis). The data displays a typical phytoplankton growth curve pattern with a lag, exponential and stationary phases. Error bars are displayed as black vertical lines on each data point (± SEM; $n = 4$). A significant difference between a given concentration and the control is depicted as an empty circle (Simple Main Effect Test, $p < 0.05$, Supplementary Table S15). **H** Average extracellular PGPHs released by the 14 bacteria. Error bars are SEM, $n = 56$

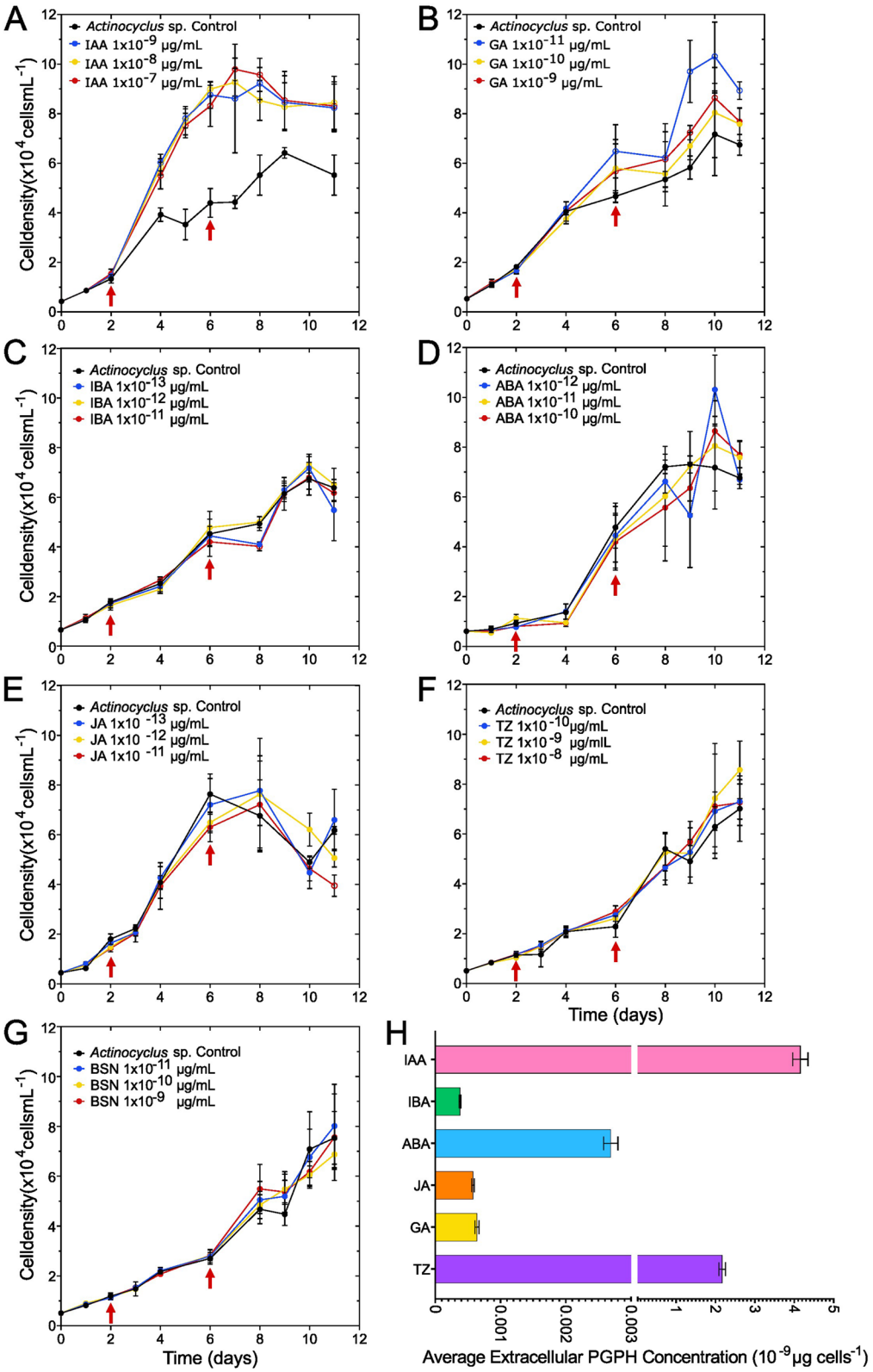

Fig. 6 (See legend on previous page.)

the 14 screened strains still produced quantifiable amounts of each of these PGPHs, suggesting that uncharacterised pathways may mediate the biosynthesis of these hormones in the bacteria tested.

The concentrations of PGPHs produced varied between the different bacterial strains, with the Alphaproteobacterium *Phaeobacter* sp. 23 often producing the highest concentration of PGPHs. This strain produced the highest intracellular concentrations of IBA, ABA, TZ and BSN, and released the highest extracellular concentrations of IBA, ABA, GA and JA. Further genomic analysis of this strain's PGPH biosynthesis capacity also revealed that *Phaeobacter* sp. 23 harbours three different pathways to synthesise IAA (Indole-3 acetonitrile, Indole-3-acetamide and Tryptamine), the majority of genes within the GA pathway and all genes necessary for TZ biosynthesis, a pathway of eukaryotic origin. The ability of *Phaeobacter* sp. 23 to produce large concentrations of PGPHs may contribute to the abundance of this genus in phytoplankton-associated bacterial communities and its ability to modulate phytoplankton growth [53–55].

Although the production of IAA and GA by phytoplankton-associated bacteria had been reported, none of the other five hormones were previously identified in phytoplankton-bacteria studies. However, some hormones, such as IBA, ABA, and JA, are known to enhance phytoplankton biomass in biotechnological settings [17–19, 56–58]. Indeed, only rhizobacteria within the Pseudomonadaceae family were previously known to produce both TZ and BSN, while only members of the Bacillus and Enterobacteriaceae produced BSN [59, 60]. Furthermore, BSN intracellular concentrations in our marine bacteria were the highest among the seven PGPHs quantified here, while TZ concentrations surpassed those of more well-known PGPHs, such as GA. These results clearly demonstrate that BSN and TZ production is not limited to specific rhizobacteria clades and may be widespread in phytoplankton-associated bacteria.

Effect of bacterial-produced PGPH on *marine diatoms*

The concentrations of IAA exuded by the 14 bacterial strains were significantly and positively correlated with the ability of these strains to enhance *Actinocyclus* growth [29]. This correlation suggests that IAA provision by the different bacterial isolates may be a key determinant of the *Actinocyclus* growth promotion previously reported [29]. This finding was further confirmed when we examined *Actinocyclus* growth responses to direct PGPH additions. Indeed, only IAA and GA additions elicited a growth-enhancing effect on *Actinocyclus* cells. The provision of IAA to *Actinocyclus* cells resulted in a nearly two-fold increase in cell numbers. This is consistent with observations in other phytoplankton species, where IAA additions resulted in significant growth enhancement [61, 62]. All three concentrations of IAA tested here elicited a significant growth-promoting effect, but studies in other species have found that an inhibitory effect can occur at high concentrations [63, 64], suggesting that the concentration of hormones received by the phytoplankton is very important to induce a growth benefit. Importantly, the concentrations of IAA that caused a growth-promoting effect on *Actinocyclus* here are the same order of magnitude as the extracellular concentrations we quantified in our bacterial strains, further confirming that the exuded concentrations can affect the growth of phytoplankton.

In contrast, the addition of GA only impacted *Actinocyclus* growth at low (10^{-9} µg/mL) and high (10^{-7} µg/mL) concentrations, perhaps suggesting a narrower range of effective phytoplankton growth enhancement compared to IAA. Significant changes in the growth of *Actinocyclus* were only noted beyond the late exponential phase (days 6–11; repeated-measures ANOVA, p-value < 0.05; Fig. 6, Supplementary Table S15). This suggests GA may only affect *Actinocyclus* cell proliferation after cells have reached late exponential stages of growth. It is indeed plausible that the growth-promoting effects of PGPHs may be influenced by a range of factors, including the concentration of the hormone and the physiological state of the cells.

Despite the 14 bacteria producing quantifiable amounts of IBA, ABA, TZ, JA and BSN, supplementation of *Actinocyclus* cultures with these PGPHs had negligible effects on the diatom's growth. This indicates that *Actinocyclus* sp. may only respond to certain PGPHs (i.e. IAA and GA), despite the 14 tested bacteria also producing other PGPHs. This is in contrast to other studies that have shown that some of these hormones can have beneficial properties for phytoplankton. These include changes in cell growth in response to JA supplementation experiments [17–19], the stimulation of heterocyst formation and chlorophyll *a* accumulation in response to IBA [57], or increases in carotenogenesis (algal defence system) when treated with ABA [56]. Given that most of these health-related phenotypes were not measured during our experiments and may be independent of cell density, it is possible that these five PGPHs may still positively affect *Actinocyclus* health. It is also possible that *Actinocyclus* sp. require a combination of PGPHs to elicit a growth response. Previous studies in terrestrial systems have demonstrated that a combination of IAA and Benzyl adenine enhanced morphological parameters (shoot length, leaves numbers and branching) [65]. Given that all isolates tested here exhibit a growth-promoting

effect on *Actinocyclus* and, on average, produce 6 out of the 7 PGPHs, it is possible these PGPHs could affect phytoplankton growth and remains an intriguing area for future study. The diverse and abundant microbiome of *Actinocyclus* [29] may therefore have a crucial role in enabling phytoplankton to access a suitable mixture of PGPHs.

Conclusion

In planktonic systems, bacterial-produced metabolites play important roles in regulating the biological functions of phytoplankton [66]. Emerging evidence suggests that PGPHs could play a pivotal role as metabolites in mutualistic partnerships between phytoplankton and bacteria. However, there is limited knowledge regarding the range of PGPHs produced by marine bacteria and their precise impact on the growth of phytoplankton. Here, we provide evidence that production and exudation of PGPHs is widespread among marine bacteria and that some of these PGPHs enhance the growth of the marine diatom *Actinocyclus*. Strikingly, all 14 bacteria tested here produced a wide suite of PGPHs. We found that several bacteria have the capacity to simultaneously produce and extracellularly release significant quantities of a suite of PGPHs, pointing towards a potentially significant ecological importance of these chemicals. Our demonstration that some of these PGPHs, in particular IAA, enhance phytoplankton growth provide clear evidence for their role in bacteria-phytoplankton interactions. Yet, on the other hand, the limited impact of several of the bacterial-produced PGPHs on phytoplankton growth indicates a more complex dynamic, whereby a suite of PGPHs may be required for phytoplankton growth promotion. Previously, the simultaneous biosynthesis of several PGPHs by bacteria was only believed to occur in plant root systems, but our observations have confirmed the presence of numerous PGPHs within a phytoplankton-bacteria association. Cumulatively, these observations indicate that the production of PGPHs may be widespread in marine bacteria and the release of these chemicals may affect primary producers the base of the marine food web.

Supplementary Information

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Additional file 1: Figs. S1–S5 and Tables S1–S15.

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Authors' contributions
A.K., J.S., J-B.R., and A.B. designed all the experiments, wrote the main text and edited the supplementary material. N.R. isolated the 14 bacteria and completed preliminary experiments for the correlation analysis (Fig. 5). N.W. prepared Figs. 3 and 4 and A.B. isolated the diatom. A.K. performed all the experiments and processed all data. All authors edited the manuscript and agree to the final submitted version.

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Data availability
Sequence data that support the findings of this study (bacteria whole genome sequences) have been deposited in the National Center for Biotechnology Information (NCBI) with the bioproject number PRJNA1089792.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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