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The planctomycete *Stieleria maiorica* Mal15^T employs stielериacines to alter the species composition in marine biofilms

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Bacterial strains of the phylum Planctomycetes occur ubiquitously, but are often found on surfaces of aquatic phototrophs, e.g. alga. Despite slower growth, planctomycetes are not outcompeted by faster-growing bacteria in biofilms on such surfaces; however, strategies allowing them to compensate for slower growth have not yet been investigated. Here, we identified stielериacines, a class of *N*-acylated tyrosines produced by the novel planctomycete *Stieleria maiorica* Mal15^T, and analysed their effects on growth of the producing strain and bacterial species likely co-occurring with strain Mal15^T. Stielериacines reduced the lag phase of Mal15^T and either stimulated or inhibited biofilm formation of two bacterial competitors, indicating that Mal15^T employs stielериacines to specifically alter microbial biofilm composition. The genetic organisation of the putative stielериacine biosynthetic cluster in strain Mal15^T points towards a functional link of stielериacine biosynthesis to exopolysaccharide-associated protein sorting and biofilm formation.

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From a heterotrophic bacterial perspective, the vastness of the sea represents a hostile oligotrophic ‘desert’. In contrast, surfaces of marine macroscopic phototrophs are nutrient-rich ‘oases’, densely packed with all sorts of alluring organic compounds, which can serve as nutrient sources. In aquatic environments, such biotic surfaces represent desirable ecological niches and are thus rapidly occupied by bacterial biofilms^{1,2}. Members of the ‘*Roseobacter* group’ are particularly successful in such habitats³. For example, *Phaeobacter inhibens* employs *N*-acyl homoserine lactones (AHLs) as quorum sensing signals to initiate biofilm formation⁴. In *P. inhibens*, AHLs trigger the expression of the gene encoding AHL synthase along with other genes that promote biofilm formation. This positive feedback loop is often associated with the production of bioactive small molecules, e.g., the antibiotic tropodithietic acid (TDA) by *P. inhibens*^{5,6}.

While *P. inhibens* and other proteobacteria are well known to dominate biotic surfaces, it was found that members of the phylum Planctomycetes can sometimes also be the dominating taxon⁷. Species belonging to the family Pirellulaceae, including the marine model planctomycete *Rhodopirellula baltica*, switch between different lifestyles, in which they either live as motile free-swimming cells or attach to surfaces⁸. Such a complex life-cycle requires control at additional stages beyond canonical transcription factor-based regulation of gene expression. Members of the family Pirellulaceae might thus rely on similar regulatory mechanisms of secreting signalling molecules for enabling cell-to-cell communication as the *Roseobacter* clade and many other microorganisms⁹. Large planctomycetal genomes of up to 12.4 Mb and high numbers of predicted clusters involved in small molecule production are in line with the assumed portfolio of bioactive compounds with potential regulatory activities, in addition to two component systems and extracytoplasmic function sigma factors¹⁰. Although planctomycetes grow rather slowly compared to competing microorganisms occupying the same ecological niche, they are not outcompeted by their natural competitors. On the contrary, planctomycetes can even account for up to 70% of the bacterial community in certain habitats⁷. The deficit in growth rate is suggested to be compensated by the production of small molecules with antimicrobial properties, while the chemical nature of such molecules remains elusive¹¹. The current knowledge gap mainly results from the insufficient number of planctomycetes available as axenic cultures⁷. Recently, we developed an isolation pipeline to obtain novel planctomycetal strains in axenic culture. Basis for the isolation of 79 novel strains was an optimised formulation of cultivation media, supplemented with a blend of carefully titrated antibiotics and fungicides¹⁰. In Mal15^T, one of the strains isolated by this strategy, we detected production of novel compounds belonging to the class of *N*-acylated tyrosine derivatives, which might have regulatory activities in this strain. In order to validate our working hypothesis, we performed assays with purified stieleriaceine for studying its role on intraspecies and interspecies communication. Additionally, we inferred a putative biosynthetic pathway based on the genome sequence of strain Mal15^T obtained during a detailed characterisation of the novel strain.

Results

Description of *Stieleria* gen. nov. *Stieleria* (Stie.le’ri.a’ N.L. fem. n. *Stieleria* named in honor of Anja Heuer, née Stieler, an extraordinary skilled German technician at the Leibniz Institute DSMZ, who played a key role in the cultivation of literally hundreds of novel planctomycetal strains). The round to pear-shaped cells with a smooth cell surface form rosettes or short chains. Cells reproduce by polar budding. In liquid culture, they

produce an extracellular matrix compound which interconnects cells in aggregates. Daughter cells are motile, while mother cells are non-motile. The lifestyle is heterotrophic, obligatory aerobic and mesophilic. The genus belongs to the phylum Planctomycetes, class Planctomycetia, order Pirellulales, family Pirellulaceae. The type species is *Stieleria maiorica*.

Description of *Stieleria maiorica* sp. nov. *Stieleria maiorica* (ma’i.ori.ca’ N.L. fem. adj. *maiorica*, pertaining to the island Mallorca, Spain, on which the type strain was isolated). In addition to the features described above the species exhibits the following properties. Colonies are pink-coloured on solid medium. Cells are $1.9 \pm 0.2 \times 1.4 \pm 0.2 \mu\text{m}$ in size. Motile daughter cells originate through budding from sessile mother cells. Gram staining delivers no clear results. The oxidase assay was negative while the catalase assay was positive. The organism can degrade a wide range of carbon sources. In particular, strong signals were observed for *N*-acetyl-d-galactosamine, *N*-acetyl-d-glucosamine, l-arabinose, d-cellobiose, l-fucose, d-fructose, d-galactose, gentiobiose, α -d-glucose, d-gluconic acid, glucuronamide, d-glucuronic acid, α -d-lactose, lactulose, d-mannose, d-melibiose, β -methyl-d-glucoside, d-raffinose, l-rhamnose, sucrose, d-trehalose, turanose and d-psicose, while for the carbon sources acetic acid, dextrin, d-galactonic acid lactone, d-glucose-6-phosphate, α -ketoglutaric acid, maltose, d-mannitol only weak signals were detected. The enzyme repertoire includes alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase. Growth of the type strain occurs between pH 5.5 and 9.0 with an optimum at pH 7.5. The optimal growth temperature of the type strain was determined to be 35 °C, but cells could grow in a range from 11 °C up to at least 37 °C. The complete chromosome of the type strain comprises 9,894,293 bp, with a G + C content of 59.3%. The type strain is Mal15^T (DSM 100215^T = LMG 29790^T, synonym Malle15), which was isolated from seawater sediment on Mallorca island, Spain.

Identification of stieleriaceines in cultures of strain Mal15^T. Given our working hypothesis that planctomycetes are a promising source for novel bioactive small molecules, we continuously extend the current collection of axenic cultures from this phylum¹⁰. Here, we analysed the novel biofilm-forming strain Mal15^T (DSM 100215^T = LMG 29790^T) isolated from sediments on Mallorca island in the Mediterranean Sea. Our phylogenetic analysis suggests that strain Mal15^T belongs to a novel genus and species in the family Pirellulaceae. Hence, we propose the name *Stieleria maiorica* gen. nov., sp. nov. for the novel isolate. A detailed characterisation of the novel strain is presented in the Supplementary Information.

During metabolite analysis in culture supernatants of strain Mal15^T, we found that it produces a distinct class of small molecules belonging to the group of long-chain *N*-acyl tyrosine derivatives, which we named stieleriaceines (in accordance with the proposed genus name *Stieleria*). Stieleriaceines are composed of a lauric acid or *trans*-2-dodecenoic acid moiety as fatty acid residue, which is ligated to a dehydrotyrosine derivative additionally *C*-methylated in *meta*-position of its aromatic ring (Fig. 1). Stieleriaceines were found to be produced during laboratory-scale shaking flask cultivations of axenic Mal15^T cultures, indicating that their production does not require presence of other microorganisms as an external stimulus.

Analysis of in vitro and in vivo effects of stieleriaceine A₁. In previous studies, it has been shown that *N*-acyl amino acids can display antimicrobial activities against various bacteria^{12–17},

Fig. 1 Stieleriactins. Structures of stieleriactins A_1 , A_2 (upper panel), B_1 , B_2 (middle panel) and C (lower panel) are depicted.

while *N*-acylated tyrosine derivatives were shown to inhibit the enzyme tyrosinase¹⁸. Employing *in vivo* and *in vitro* assays with purified stieleriactin A_1 , the major stieleriactin produced by strain Mal15^T, we could show that these compounds exhibit only moderate antimicrobial activity against Gram-positive bacteria (Supplementary Table 1) and negligible inhibition of tyrosinase, indicating that neither of the two effects reflects the major function of stieleriactins. To get first hints on the natural function of stieleriactin A_1 , we thus applied time-lapse microscopy coupled to microfluidics as experimental strategy to allow observation of individual cells over time spans of several hours to days. Such unusual approaches were necessary since most of the canonical biofilm formation assays did not work for planctomycetes. Mal15^T cells were treated with 1.34 μ M stieleriactin A_1 , a ‘physiological’ concentration found to be produced in the mid-exponential phase during laboratory-scale cultivation of the strain (Fig. 2). The natural production of stieleriactins by strain Mal15^T can be neglected in this experimental setup as a constant medium flow ensures that compounds secreted by the strain are immediately flushed away. We found that presence of stieleriactin A_1 significantly reduced the duration of the lag phase (5.2 h) compared to untreated Mal15^T cells (6.3 h; $p = 0.0058$) (Fig. 3, Supplementary Table 2, Supplementary Movie 1 and 2). This observation points towards a bioactivity to the advantage of its producer, however, we did not exclude any additional inhibitory effect on competing microbes at this stage.

The artificial laboratory cultivation conditions and the tested concentration of stieleriactin A_1 probably only insufficiently reflect natural conditions in seawater, in which densities of planktonic Mal15^T cells are much lower. From an ecological perspective, stieleriactin production by individual swarming cells or smaller aggregates appears irrational as the secreted molecules are immediately diluted to inactive concentrations. We hypothesised that sufficient stieleriactin concentrations can only be

Fig. 2 Stieleriactin A_1 production in relation to growth of strain Mal15^T.

Triplicates of Mal15^T cultures were harvested every 12 h for five days, followed by harvesting every 16 h for two days. The optical density at 600 nm (OD_{600nm}), the biomass dry weight and the stieleriactin A_1 titre in the cell supernatant were quantified. Error bars indicate the standard deviation. Where error bars are not visible, they are shorter than the size of the symbols.

Fig. 3 Effect of stieleriactin A_1 on growth of strain Mal15^T. **a** Time-lapse series of untreated Mal15^T cells during the 6.25 h lag-phase (1–5) and after initialisation of cell division and entrance into the exponential phase (6–8). The division as such from budding to daughter cell release required 1.9 h. **b** The lag phase of Mal15^T cells was significantly shorter (1–5: 5.16 h) once treated with 1.34 μ M stieleriactin A_1 , while the division as such lasted 1.9 h as well (6–8); (untreated: 6.25 h (**a**), treated: 5.16 h (**b**), $p = 0.0058$). The scale bar is 1 μ m.

reached by larger numbers of cells inhabiting a local environment, such as a biofilm with a rather gel-like texture and that stieleriactins might thus play a more important role in such micro-environments. Following this argumentation, we were curious to test for a potential effect of stieleriactin A_1 on biofilm formation capabilities of natural competitors of strain Mal15^T. We chose two members of the ‘*Roseobacter* group’, *P. inhibens* DSM 17395 and *Sulfotobacter dubius* DSM 16472^T, which were shown to co-occur with species of the family Pirellulaceae^{19,20}. The biofilm assays were performed with isolated stieleriactin A_1 in absence of Mal15^T cells to exclude any additional effects, which might e.g. be caused by other natural compounds produced by strain Mal15^T. Unexpectedly, application of stieleriactin A_1 led to an increased biofilm formation of *P. inhibens* (+35%, $p < 0.001$), but reduced biofilm formation of *S. dubius* (–15%, $p < 0.001$) (Fig. 4a). This effect was observed when 134 μ M stieleriactin A_1 was used, a 100-fold higher concentration than in the microfluidics experiment performed with strain Mal15^T. The biofilm assay required empirical optimisation of the stieleriactin A_1 concentration; since we were aware that concentrations in liquid

Fig. 4 Box plot of biofilm formation capacity for cells treated with stielieriacine A₁. **a** Box plot of biofilm formation capacity of *P. inhibens* (P) and *S. dubius* (S). **b** Box plot of biofilm formation capacity of *P. inhibens* (P) compared to *P. inhibens* Δ*luxR* transposon mutant (M). Experimental setup: **P.C+**: *P. inhibens* in MB medium; **P.C++**: *P. inhibens* in MB with acetone; **P.A₁1**: *P. inhibens* in MB with stielieriacine A₁ 1.34 μM in acetone; **P.A₁2**: *P. inhibens* in MB with stielieriacine A₁ 134 μM in acetone; **S.C+**: *S. dubius* in MB medium; **S.C++**: *S. dubius* in MB medium with acetone; **S.A₁1**: *S. dubius* in MB with stielieriacine A₁ 1.34 μM in acetone; **S.A₁2**: *S. dubius* in MB with stielieriacine A₁ 134 μM in acetone; **M.C+**: Δ*luxR* transposon mutant in MB medium; **M.C++**: Δ*luxR* transposon mutant in MB with acetone; **M.A₁1**: Δ*luxR* transposon mutant in MB with stielieriacine A₁ 1.34 μM in acetone; **M.A₁2**: Δ*luxR* transposon mutant in MB with stielieriacine A₁ 134 μM in acetone; **C-**: MB medium. For each experiment, four biological replicates and two technical replicates were performed. The minimum and maximum value, sample median, and the first and third quartiles are shown (box-and-whisker plot).

cultures might not properly reflect local concentrations in biofilms, in which cells are in very close proximity and local metabolite concentrations are thus considerably higher. We decided to follow the empirical optimisation approach of the stielieriacine A₁ concentration to reconstruct the natural conditions in the biofilm as good as possible. Natural local concentrations of stielieriacines in biofilms are difficult to assess and largely dependent on size and microbial community composition of the biofilm. The observed stimulating effect of stielieriacine A₁ on *P. inhibens* was surprising and was thus analysed further. To this end, we tested for a potential interaction of stielieriacine A₁ with the AHL-dependent quorum sensing system in *P. inhibens*, in particular the AHL-responsive transcriptional regulator protein LuxR²¹. Mutant analysis with a *luxR*-deficient *P. inhibens* strain (Supplementary Fig. 1) demonstrated that the positive effect on biofilm formation is largely independent from LuxR, indicating that stielieriacine A₁ does not directly interfere with the quorum sensing system of *P. inhibens* (Fig. 4b). But, why should strain Mal15^T produce a molecule that stimulates biofilm formation of its natural competitor *P. inhibens*, while reducing the fitness of its other competitor *S. dubius*? The reason possibly relates to production of TDA²². Promoting the biofilm formation of *P. inhibens* would in return lead to increased production of AHLs and TDA^{23,24}. This benefits strain Mal15^T in two ways: (i) Mal15^T turned out to be resistant against TDA while other competitors are not. (ii) TDA production reduces the growth speed of *P. inhibens* approximately by 41% due to the increased metabolic burden²⁵ and may ensure that strain Mal15^T is not outcompeted. Thus, we suggest that strain Mal15^T ‘invites’ *P. inhibens* via stielieriacine production to join the biofilm and to produce TDA in order to challenge other faster growing competitors. In contrast, biofilm formation of *S. dubius* is reduced by stielieriacine A₁ as this bacterium does not produce TDA and is in that regard useless for strain Mal15^T. The hypothesis suggests that planctomycetes actively shape the biofilm community to gain advantages employing a novel type of natural products: stielieriacines. Our results thus point towards the capability of certain slow-growing bacteria to mediate colonisation of their surrounding by production of small molecules. The hypothesis fits to the observation that slow-

growing planctomycetes can dominate biofilms on competitive marine surfaces⁷.

Genome analysis identifies a putative stielieriacine biosynthesis pathway. Although microbial synthesis of *N*-acyl tyrosine molecules has been reported in several recent studies^{18,26,27}, the genetic basis for biosynthesis has not been elucidated in greater detail. The key enzyme for stielieriacine biosynthesis in strain Mal15^T is likely an *N*-acyl amino acid synthase (NAS) (Fig. 5). We sequenced the 9.9 Mb genome of strain Mal15^T and identified a cluster (locus tags Mal15_37240 to Mal15_37430, Fig. 6), which contains not only four putative NAS-encoding genes, but all genes required for a postulated stielieriacine biosynthesis pathway. The NAS-encoding genes (Mal15_37340, Mal15_37370, Mal15_37380 and Mal15_37430) were originally annotated as hypothetical proteins, but were identified as candidates by BLASTp and InterPro scan. The four enzymes might have different substrate spectra, which can explain the production of five slightly different stielieriacines in strain Mal15^T (Fig. 1). However, it should not be excluded that the strain is capable of producing other *N*-acyl amino acids. The C-methylation in *meta*-position of the aromatic ring and an uncommon double bond in the α,β-position of the tyrosine moiety were identified as characteristic molecular features separating stielieriacines from other *N*-acyl tyrosines identified so far^{18,26}. In the biosynthetic cluster, Mal15_37250 encodes a putative methyltransferase similar to the L-tyrosine C3-methyltransferase SfmM2 of *Streptomyces lavendulae*. This enzyme catalyses an early step in saframycin biosynthesis²⁸ identical to the reaction required during synthesis of stielieriacines (Fig. 5). The gene encoded immediately downstream, Mal15_37560, codes for a putative tRNA threonylcarbamoyladenine dehydratase. When only taking the putative protein annotation into consideration, involvement in stielieriacine biosynthesis appears rather unlikely. However, the substrate of the dehydratase (tRNA threonylcarbamoyladenine) strongly resembles the amide bond obtained after the *N*-acylation reaction in the postulated stielieriacine pathway. The catalysed lactonization and dehydration reaction would give rise to a cyclic pathway intermediate, in which the remaining double bond can be introduced by a coupled oxidation and hydrolysis reaction

induced by a keto-enol tautomeric rearrangement (Fig. 5). This reaction may be catalysed by the putative oxidoreductase encoded by Mal15_37310. Other proteins in the cluster code for a putative transcriptional regulator or putative transporters (Supplementary Table 3), which could have additional function for regulation of the pathway and product export, however, we had no clear indication for this based on published information. Mal15_37240, encoding a putative fatty acid desaturase, is likely involved in the

introduction of the double bond in the fatty acid part of the molecule (Fig. 5), however it remains to be elucidated whether this reaction takes place before or after the *N*-acylation.

Discussion

In this study, we isolated and characterised *Stieleria maiorica* Mal15^T, which represents a novel species and genus within the family Pirellulaceae, order Pirellulales, class Planctomycetia, phylum Planctomycetes. The strain was shown to produce *N*-acylated tyrosine derivatives, which we designated stieleriocines and which belong to the class of *N*-acylated amino acids. In previous studies, compounds of this class showed antimicrobial activities against various bacteria^{12–17} and probably act as signalling molecules mediating intraspecies and interspecies communication^{4,15,29}. *N*-acylated tyrosine derivatives isolated from the marine γ -proteobacterium *Thalassotalea* sp. PP2-459 (Alteromonadales) were also shown to inhibit the enzyme tyrosinase¹⁸. Recently, an *N*-acyl tyrosine congener harbouring an uncommon α -methyl group in the tyrosine moiety was identified in *Alteromonas* sp. RKMC-009 isolated from the surface of a marine sponge²⁶, while its natural function remains to be elucidated. Stieleriocine A₁ showed only moderate antimicrobial activity against Gram-positive bacteria and negligible tyrosinase inhibitory activity. At this early stage of research with the novel isolate, we can only speculate on potential quorum sensing activity of stieleriocines. Analysis of potential target genes of a respective regulator part of a putative quorum sensing system is challenging given that 42% (2897 out of 6920) proteins encoded in the genome of strain Mal15^T are of unknown function. Experiments with the strain itself instead of isolated stieleriocine are particularly interesting, however, these require a Mal15^T mutant incapable of stieleriocine biosynthesis as negative control. Such a mutant can also provide important information on the stieleriocine biosynthesis pathway, however, due to lacking experience in terms of the genetic accessibility of strain Mal15^T, this question needs to be addressed in follow-up studies.

Although some *N*-acyl amino acid compounds have been identified, the current knowledge on the underlying biosynthetic pathways is limited. Evidence for the presence of *N*-acylated derivatives of aromatic or basic amino acids was mainly based on a multitude of environmental DNA libraries. Characterisation of biosynthetic pathways for *N*-acyl amino acids is an important task in the future given that such compounds find potential biotechnological applications as antimicrobial surfactants and precursors for biodegradable polyesters in pharmaceutical and biomedical applications^{30,31}. In case of the surfactants, the C₁₂ chain as acyl residue (as present in stieleriocine A₁) proved to

Fig. 5 Proposed biosynthesis pathway of stieleriocines. The proposed stieleriocine biosynthesis pathway starting from L-tyrosine and lauryl-ACP is depicted. The locus tags of genes coding for candidate enzymes participating in the pathway are shown in boxes. ACP acyl carrier protein.

Fig. 6 The putative stieleriocine biosynthetic gene cluster in strain Mal15^T. Genes with a predicted role in the stieleriocine biosynthesis cluster are shown in different colours. For these genes the automated gene annotation and the postulated function are shown in the table. The complete list of automated gene annotations of the cluster can be found in Supplementary Table 3.

have the optimum antibacterial activity among the single chain surfactants tested³¹. In that regard, genes of strain Mal15^T are interesting target for expression in heterologous microbial hosts.

A report on screening of soil metagenomic libraries revealed that genes coding for NAS proteins are frequently found adjacent to genes involved in the PEP-CTERM/exosortase protein sorting system (an exopolysaccharide-associated protein targeting system), indicating a functional link between both pathways²⁹. Indeed, the same situation is also found in the genome of strain Mal15^T. A PEP-CTERM motif protein-encoding gene (Mal15_37300) was found within the proposed stieleriaceae biosynthetic gene cluster (Fig. 6), and many exopolysaccharide biosynthesis-related and secretion-related genes are encoded in the region upstream (Mal15_36520 to Mal17_37230, Supplementary Table 3). The conserved genetic organisation supports the notion of a functional link between stieleriaceae biosynthesis, exopolysaccharide assembly and biofilm formation, which requires additional attention in future studies.

In conclusion, the identified stieleriaceae stimulated growth initiation of Mal15^T cells and either promoted or inhibited biofilm formation of two different microorganisms, likely naturally competing with strain Mal15^T for biotic surfaces in marine environments. Although the two tested competitors might only be a small fraction of microorganisms dwelling in such environments, it allows first insights into strategies how slow-growing microorganisms compensate for lower growth rates and avoid being outcompeted. Stieleriaceae are likely only one of several strategies how the survival of planctomycetes in aquatic environments is ensured and their high abundance on several marine biotic surfaces can be explained.

Methods

Sample collection and preparation. Seawater sediment samples from 30 cm depth were collected from Spain, Mallorca island, El Arenal, between Balneario 4 and 5 (39° 30' 45.2" N, 2° 44' 49.1" E) on September 23th, 2014. Samples were collected in sterile 50 mL polypropylene tubes, transferred to the laboratory, homogenised and processed.

Culture and isolation conditions. For maintenance of strain Mal15^T M1 medium with HEPES (H) as buffering agent and additionally supplemented with *N*-acetylglucosamine (NAG) and artificial seawater (ASW) (M1H NAG ASW medium) was used and prepared as described before³². Solid medium was prepared with 12 g/L (w/v) agar, washed three times with double distilled H₂O and cooled to 55 °C prior to the addition of heat-sensitive solutions. For the initial strain isolation, solid M1H NAG ASW medium was supplemented with 100 µL of an ampicillin (50 mg/mL) and cycloheximide stock solution (20 mg/mL), dried for 30 min, inoculated with 100 µL homogenised sample material per plate and incubated at 20 °C in the dark until colony formation became visible. Single colonies were inoculated on fresh solid medium with the respective antibiotics. Axenic cultures were cryo-preserved in M1H NAG ASW medium supplemented with 50% glycerol or 5% DMSO and stored at −80 °C. Exponentially growing Mal15^T cells were used for analysis of carbon source utilisation (Supplementary Table 4) and enzymatic activities (Supplementary Table 5).

Strain characterisation. Electron microscopy and basic light microscopy were performed as previously described^{33,34}. For time-lapse microscopy, cells were observed using the CellASIC ONIX Microfluidic Platform (Merck Millipore). Physiological tests as well as phylogenetic and genome analysis were performed as previously described³⁵. The results are shown in Supplementary Figs. 2–6.

Statistics and reproducibility. All experiments were performed at least in biological triplicates. If not stated otherwise, data represent average and standard deviation. In all cases, detailed information is given in the respective figure captions. Significance of results is indicated by *p*-values given in the text.

Phylogenetic analysis. 16S rRNA gene sequence-based phylogeny was computed for strain Mal15^T (GenBank acc. no. MK554562), the type strains of all described planctomycetal species (assessed in May 2019) and all isolates recently published¹⁰ and described^{1,36,37}. Phylogenetic inference based on 16S rRNA gene sequences and multi-locus sequence analysis (MLSA) was performed as described³⁶. The 16S rRNA gene sequences were aligned with SINA³⁸ and the phylogenetic inference was calculated with RAXML³⁹ with a maximum likelihood approach with 1000

bootstraps, nucleotide substitution model GTR, gamma distributed rate variation and estimation of proportion of invariable sites (GTRGAMMAI option). Three 16S rRNA genes of bacterial strains from the PVC superphylum outside of the phylum Planctomycetes were used as outgroup. For MLSA the unique single-copy core genome of the analysed genomes (GenBank acc. no. for strain Mal15^T: CP036264.1) was determined with proteinortho5⁴⁰ with the 'selfblast' option enabled. The protein sequences of the resulting orthologous groups were aligned using MUSCLE v.3.8.31⁴¹. After clipping, partially aligned C-terminal and N-terminal regions and poorly aligned internal regions were filtered using Gblocks⁴². The final alignment was concatenated and clustered using the maximum likelihood method implemented by RAXML³⁹ with the 'rapid bootstrap' method and 500 bootstrap replicates. Four planctomycetal genomes from different families in the class Planctomycetia were used as outgroup. The average nucleotide identity (ANI) was calculated using OrthoANI⁴³. The average amino acid identity (AAI) was calculated using the aai.rb script of the enveomics collection⁴⁴ and the percentage of conserved proteins (POCP) was calculated as described⁴⁵. The *rpoB* nucleotide sequences were taken from publicly available planctomycetal genome annotations and the sequence identities were determined as described⁴⁶. Alignment and matrix calculation were done with Clustal Omega⁴⁷.

Isolation and purification of stieleriaceae from culture broth. For getting sufficient amounts of Mal15^T biomass, nutrient content of the culture broth was increased (1.0 g/L peptone, 1.0 g/L yeast extract, 40 mL/L 2.5% (w/v) glucose solution). Six hundred microlitre Mal15^T cultures were incubated for three days at 28 °C and 80 rpm in 2 L baffled flasks until 2% (w/v) of purified adsorbent resin XAD-16N (Rohm and Haas) were added. Cultures were further incubated for three days. In total, XAD-16N from 32 L culture was harvested and processed as previously described³². The combined crude extract was filtered with water/methanol (1:1) using a Strata-X 33 mm, Polymeric Reverse Phase Solid-Phase cartridge (Phenomenex). The solid phase was eluted with acetone and hexane yielding 308.25 mg crude product, which was fractionated using preparative RP-HPLC (PLC 2020, Gilson with Kromasil C₁₈ column 250 × 20 mm, 7 µm; MZ-Analyse-technik) with deionised water and 0.1% formic acid (solvent A), and acetonitrile with 0.1% formic acid (solvent B). The following elution gradient was used: 40% B for 10 min, increased to 50% B in 3 min, and a gradient from 50–70% B in 60 min, thereafter 100% B for 10 min. Ultraviolet (UV) detection was carried out at 215 and 310 nm. Fractions were collected yielding five pure compounds. Stieleriaceae A₁ (1) (6.3 mg) was obtained at a retention time (*t_R*) of 32.5 min, B₁ (2) (2.0 mg) at *t_R* = 34.0 min, C (3) (0.8 mg) at *t_R* = 36.0 min, A₂ (4) (4.9 mg) at *t_R* = 39.5 min, B₂ (5) (3.5 mg) at *t_R* = 41.1 min (Fig. 1, Supplementary Fig. 7).

Structure elucidation. HRESIMS mass spectra were measured with an Agilent 1200 series HPLC-UV system in combination with an ESI-TOF-MS (Maxis, Bruker) [column 2.1 × 50 mm, 1.7 µm, C₁₈ Acquity UPLC BEH (Waters), solvent A: water with 0.1% formic acid, solvent B: acetonitrile with 0.1% formic acid, gradient: 5% B for 0.5 min increasing to 100% B in 19.5 min, maintaining 100% B for another 5 min, *R_F* = 0.6 mL/min, UV detection 200–600 nm. Nuclear magnetic resonance spectra were recorded on a Bruker Avance III 500 MHz spectrometer with a BBFO(plus) SmartProbe (¹H 500 MHz, ¹³C 126 MHz), and a Bruker Avance III 700 MHz spectrometer with a 5 mm TCI cryoprobe (¹H 700 MHz, ¹³C 175 MHz, ¹⁵N 71 MHz). UV spectra were recorded using a Shimadzu UV-VIS spectrophotometer UV-2450. Optical rotation was determined using a PerkinElmer 241 polarimeter. Correlations are shown in Supplementary Fig. 8.

Antimicrobial activity assay. Determination of minimum inhibitory concentrations (MIC) was performed using serial dilution assays in 96-well microtiter plates. YMG medium (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose, pH 7.2) was used for yeasts and filamentous fungi, while EBS medium (5 g/L casein peptone, 1 g/L meat extract, 1 g/L yeast extract, 5 g/L glucose, 50 mM HEPES, pH 7.0) was used for bacteria. Tested species were inoculated to initial cell densities of 10⁵–10⁶ colony-forming units per mL in the respective growth medium. MIC were assessed after 16 and 48 h of cultivation at 37 °C (bacteria) and 28 °C (yeasts and moulds), respectively. The MIC values represent the lowest concentration of stieleriaceae at which no visible growth was observed.

Cytotoxicity assay. In vitro cytotoxicity (IC₅₀) was investigated using mouse fibroblast cell line L929 and HeLa KB3.1 cells as previously described⁴⁸. Briefly, cells were cultivated in EBM-2 supplemented with 10% foetal bovine serum under 10% CO₂ at 37 °C. Sixty microlitre of serial dilutions from an initial stock of 1 mg/mL stieleriaceae in acetone was added to 120 µL aliquots of a cell suspension (50,000 cells per mL) in 96-well microtiter plates. After 5 days of incubation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. The absorbance was measured at 590 nm using an ELISA plate reader (Victor). The concentration, at which the growth of cells was inhibited to 50% of the control (IC₅₀), was obtained from the dose–response curves. Acetone served as negative control.

Tyrosinase inhibition assay. The tyrosinase inhibition assay was performed as previously described¹⁸ with the following modifications: 80 µL of compound,

dissolved in PBS (0.1 M, pH 6.8) with 10% methanol at various concentrations, were added to 80 µL of PBS. PBS with 10% methanol served as a negative control. Eighty microlitre of mushroom-tyrosinase (100 units per mL) and 40 µL of L-tyrosine (2.5 mM) were added to each well. Phosphate buffer and tyrosinase served as a negative control. Reactions were incubated at 37 °C for 45 min and the absorbance was measured at 490 nm using the Infinite 200 Pro multimode reader (Tecan). The percentage of tyrosinase inhibition was calculated as follows: $[(\Delta A_{\text{control}} - \Delta A_{\text{sample}}) / \Delta A_{\text{control}}] \times 100$.

Time-lapse microscopy. To analyse the physiological effect of stieleriace A₁ with time-lapse microscopy, a preculture of Mal15^T cells was inoculated 1:10 in fresh M1H NAG ASW medium and incubated for 72 h (28 °C, 80 rpm). Before loading, the CellASIC® microfluidic chamber was washed with a constant flow of medium (2 × 5 psi, 5 min). Stieleriace A₁ was provided dissolved in 132 µL acetone at 1.34 µM, while medium supplemented with the same volume of acetone was used as negative control. Cells were monitored for up to 36 h at 28 °C at 2 × 1 psi flow rate. Phase contrast images were acquired and processed as previously described⁴⁹. In total, 1264 cells were analysed and all experiments were performed in three biological replicates.

Biofilm formation assay. Biofilm formation of *P. inhibens* and *S. dubius* was analysed by a crystal violet assay^{3,50}. *P. inhibens* and *S. dubius* were grown in 10 mL MB medium (MB, Carl Roth) at 28 °C with vigorous shaking to the early exponential phase (OD₆₀₀ of 0.4). Cultures were subsequently diluted to an OD₆₀₀ of 0.065. 100 µL of the diluted culture was transferred to a respective well of a sterile polystyrene 96-well assay plate (Corning, New York, NY, USA; Costar 3370). 1.34 µM or 134 µM of stieleriace A₁ solved in acetone or pure acetone were added to the wells prior to statically culturing of the plates for 24 h. After incubation, medium along with planktonic cells was removed and wells were washed twice with H₂O. Two hundred microlitre of 0.5% crystal violet (CV) was added to the wells, which were incubated at room temperature for 10 min. After staining, the CV solution was removed and each well was washed twice with H₂O to remove the residual dye and dried overnight. For scoring of cell attachment, the CV was extracted from the biofilm with 200 µL 95% (v/v) ethanol, of which 100 µL were transferred to a new 96-well plate before the absorbance was determined at 595 nm using the Tecan microplate reader (Infinite® 200 PRO). For each experiment, four biological replicates and two technical replicates were performed.

Analysis of the stieleriace production rate. To analyse the production rate of stieleriace A₁, precultures of strain Mal15^T were inoculated 1:10 in fresh M1H NAG ASW medium in triplicates and incubated for 7 days (152 h, 28 °C, 80 rpm). For the next five days 30 mL of each culture were harvested every 12 h, followed by 2 days harvesting after every 16 h. The harvested material was analysed for optical density (OD₆₀₀), biomass and stieleriace A₁ concentration.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The genome and 16S rRNA gene sequence of strain Mal15^T are available from GenBank under accession numbers CP036264.1 and MK554562.1, respectively. Other relevant data are available from the corresponding authors upon request. The putative stieleriace biosynthetic gene cluster was deposited at the MiBiG (Minimum Information about a Biosynthetic Gene cluster) database and can be found under accession number BGC0002080.

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References

- Kohn, T. et al. *Planctopirus ephydatiae*, a novel Planctomycete isolated from a freshwater sponge. *Syst. Appl. Microbiol.* **43**, 126022 (2019).
- Lage, O. M. & Bondoso, J. Planctomycetes and macroalgae, a striking association. *Front. Microbiol.* **5**, 267 (2014).
- Frank, O. et al. Plasmid curing and the loss of grip—the 65-kb replicon of *Phaeobacter inhibens* DSM 17395 is required for biofilm formation, motility and the colonization of marine algae. *Syst. Appl. Microbiol.* **38**, 120–127 (2014).
- Van Wagoner, R. M. & Clardy, J. FeeM, an N-acyl amino acid synthase from an uncultured soil microbe: structure, mechanism, and acyl carrier protein binding. *Structure* **14**, 1425–1435 (2006).
- Whiteley, M., Diggle, S. P. & Greenberg, E. P. Progress in and promise of bacterial quorum sensing research. *Nature* **551**, 313–320 (2017).
- Papenfort, K. & Bassler, B. L. Quorum sensing signal-response systems in Gram-negative bacteria. *Nat. Rev. Microbiol.* **14**, 576–588 (2016).
- Wiegand, S., Jogler, M. & Jogler, C. On the maverick planctomycetes. *FEMS Microbiol. Rev.* **42**, 739–760 (2018).
- Frank, C. S., Langhammer, P., Fuchs, B. M. & Harder, J. Ammonium and attachment of *Rhodopirellula baltica*. *Arch. Microbiol.* **193**, 365–372 (2011).
- Waters, C. M. & Bassler, B. L. Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* **21**, 319–346 (2005).
- Wiegand, S. et al. Cultivation and functional characterization of 79 planctomycetes uncovers their unique biology. *Nat. Microbiol.* **5**, 126–140 (2020).
- Jeske, O., Jogler, M., Petersen, J., Sikorski, J. & Jogler, C. From genome mining to phenotypic microarrays: planctomycetes as source for novel bioactive molecules. *Antonie Van. Leeuwenhoek* **104**, 551–567 (2013).
- Clardy, J. & Brady, S. F. Cyclic AMP directly activates NasP, an N-acyl amino acid antibiotic biosynthetic enzyme cloned from an uncultured beta-proteobacterium. *J. Bacteriol.* **189**, 6487–6489 (2007).
- Thies, S. et al. Metagenomic discovery of novel enzymes and biosurfactants in a slaughterhouse biofilm microbial community. *Sci. Rep.* **6**, 27035 (2016).
- Brady, S. F., Clardy, J. & Long-Chain N-Acyl amino acid antibiotics isolated from heterologously expressed environmental DNA. *J. Am. Chem. Soc.* **122**, 12903–12904 (2000).
- Brady, S. F., Chao, C. J., Clardy, J. & Long-chain N-acyltyrosine synthases from environmental DNA. *Appl. Environ. Microbiol.* **70**, 6865–6870 (2004).
- Brady, S. F. & Clardy, J. N-acyl derivatives of arginine and tryptophan isolated from environmental DNA expressed in *Escherichia coli*. *Org. Lett.* **7**, 3613–3616 (2005).
- Brady, S. F., Chao, C. J. & Clardy, J. New natural product families from an environmental DNA (eDNA) gene cluster. *J. Am. Chem. Soc.* **124**, 9968–9969 (2002).
- Deering, R. W. et al. N-acyl dehydrotyrosines, tyrosinase inhibitors from the marine bacterium *Thalassotalea* sp. PP2-459. *J. Nat. Prod.* **79**, 447–450 (2016).
- Morris, R., Longnecker, K. & Giovannoni, S. *Pirellula* and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. *Environ. Microbiol.* **8**, 1361–1370 (2006).
- Hengst, M. B., Andrade, S., González, B. & Correa, J. A. Changes in epiphytic bacterial communities of intertidal seaweeds modulated by host, temporality, and copper enrichment. *Microb. Ecol.* **60**, 282–290 (2010).
- Zan, J., Liu, Y., Fuqua, C. & Hill, R. T. Acyl-homoserine lactone quorum sensing in the *Roseobacter* clade. *Int. J. Mol. Sci.* **15**, 654–669 (2014).
- Wilson, M. Z., Wang, R., Gitai, Z. & Seyedsayamdost, M. R. Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl cycle as a proton sink. *Proc. Natl Acad. Sci. USA* **113**, 1630–1635 (2016).
- Berger, M., Neumann, A., Schulz, S., Simon, M. & Brinkhoff, T. Tropodithietic acid production in *Phaeobacter gallaeciensis* is regulated by N-acyl homoserine lactone-mediated quorum sensing. *J. Bacteriol.* **193**, 6576–6585 (2011).
- Beyersmann, P. G. et al. Dual function of tropodithietic acid as antibiotic and signaling molecule in global gene regulation of the probiotic bacterium *Phaeobacter inhibens*. *Sci. Rep.* **7**, 730 (2017).
- Will, S. E. et al. The limits to growth—energetic burden of the endogenous antibiotic tropodithietic acid in *Phaeobacter inhibens* DSM 17395. *PLoS ONE* **12**, e0177295 (2017).
- MacIntyre, L. W., Charles, M. J., Haltli, B. A., Marchbank, D. H. & Kerr, R. G. An Ichip-domesticated sponge bacterium produces an N-acyltyrosine bearing an α-methyl substituent. *Org. Lett.* **21**, 7768–7771 (2019).
- Lee, C.-M. et al. Characterization of a novel antibacterial N-acyl amino acid synthase from soil metagenome. *J. Biotechnol.* **294**, 19–25 (2019).
- Li, L. et al. Characterization of the saframycin A gene cluster from *Streptomyces lavendulae* NRRL 11002 revealing a nonribosomal peptide synthetase system for assembling the unusual tetrapeptidyl skeleton in an iterative manner. *J. Bacteriol.* **190**, 251–263 (2008).
- Craig, J. W., Cherry, M. A. & Brady, S. F. Long-chain N-acyl amino acid synthases are linked to the putative PEP-CTERM/exosortase protein-sorting system in Gram-negative bacteria. *J. Bacteriol.* **193**, 5707–5715 (2011).
- Taresco, V. et al. New N-acyl amino acid-functionalized biodegradable polyesters for pharmaceutical and biomedical applications. *RSC Adv.* **6**, 109401–109405 (2016).
- Joondan, N., Jhaumeer-Laulloo, S., Caumul, P. & Akerman, M. Synthesis, physicochemical, and biological activities of novel N-acyl tyrosine monomeric and gemini surfactants in single and SDS/CTAB-mixed micellar system. *J. Phys. Org. Chem.* **30**, e3675 (2017).
- Jeske, O. et al. Developing techniques for the utilization of planctomycetes as producers of bioactive molecules. *Front. Microbiol.* **7**, 1242 (2016).
- Kohn, T. et al. *Fuerstia marisgermanicae* gen. nov., sp. nov., an unusual member of the phylum planctomycetes from the German Wadden Sea. *Front. Microbiol.* **7**, 2079 (2016).

34. Kohn, T. et al. Corrigendum: *Fuerstia marisgermanicae* gen. nov., sp. nov., an unusual member of the phylum planctomycetes from the German Wadden Sea. *Front. Microbiol.* **10**, 1029 (2019).
35. Rast, P. et al. Three novel species with peptidoglycan cell walls form the new genus *Lacunisphaera* gen. nov. in the family *Opitutaceae* of the verrucomicrobial subdivision 4. *Front. Microbiol.* **8**, 202 (2017).
36. Kallscheuer, N. et al. Description of three bacterial strains belonging to the new genus *Novipirellula* gen. nov., reclassification of *Rhodopirellula rosea* and *Rhodopirellula caenicola* and readjustment of the genus threshold of the phylogenetic marker *rpoB* for *Planctomycetaceae*. *Antonie van Leeuwenhoek* <https://doi.org/10.1007/s10482-019-01374-51-17> (2019).
37. Boersma, A. et al. *Alienimonas californiensis* gen. nov. sp. nov., a novel planctomycete isolated from the kelp forest in Monterey Bay. *Antonie van Leeuwenhoek* <https://doi.org/10.1007/s10482-019-01367-4> (2019).
38. Pruesse, E., Peplies, J. & Glöckner, F. O. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* **28**, 1823–1829 (2012).
39. Stamatakis, A. RAXML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313 (2014).
40. Lechner, M. et al. Proteinortho: detection of (co-)orthologs in large-scale analysis. *BMC Bioinform.* **12**, 124 (2011).
41. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
42. Castresana, J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540–552 (2000).
43. Lee, I., Ouk Kim, Y., Park, S. C. & Chun, J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int. J. Syst. Evol. Microbiol.* **66**, 1100–1103 (2016).
44. Rodríguez-R, L. M. & Konstantinidis, K. T. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ Prepr.* **4**, e1900v1 (2016).
45. Qin, Q.-L. et al. A proposed genus boundary for the prokaryotes based on genomic insights. *J. Bacteriol.* **196**, 2210–2215 (2014).
46. Bondoso, J., Harder, J. & Lage, O. M. *rpoB* gene as a novel molecular marker to infer phylogeny in *Planctomycetales*. *Antonie Van. Leeuwenhoek* **104**, 477–488 (2013).
47. Sievers, F. et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
48. Surup, F. et al. Deconins A–E: cuparenic and mevalonic or propionic acid conjugates from the basidiomycete *Deconica* sp. 471. *J. Nat. Prod.* **78**, 934–938 (2015).
49. Boedeker, C. et al. Determining the bacterial cell biology of planctomycetes. *Nat. Commun.* **8**, 14853 (2017).
50. O'Toole, G. A. & Kolter, R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol. Microbiol.* **28**, 449–461 (1998).

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Author contributions

N.K. wrote the final version of the manuscript with help of C.J. based on an initial version written by O.J., B.S. and F.S. O.J. performed most biological experiments. N.K. analysed the genome and proposed the biosynthesis pathway with help from M.H.M. B.S. performed most of the chemical experiments with the help of F.S. C.B. performed time-lapse and light microscopy experiments. S.W. sequenced and characterised the genome and performed the phylogenetic analysis. P.B. performed *Roseobacter*-related experiments and constructed the mutants with help from J.P. M.J. developed the novel isolation strategy with help from C.J., isolated the strain and performed general microbiological experiments. M.R. took electron micrographs. J.P. designed the *Roseobacter*-related experiments and supervised P.B. M.H.M. supported N.K. and both analysed genes related to small molecule production. F.S. supervised B.S. and guided structure elucidation. C.J. and F.S. designed the concept of this study. C.J. supervised O.J. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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