

# Production of a Dibrominated Aromatic Secondary Metabolite by a Planctomycete Implies Complex Interaction with a Macroalgal Host

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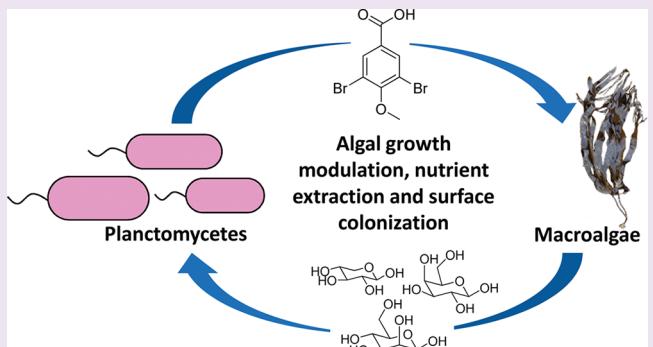
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## S Supporting Information

**ABSTRACT:** The roles of the majority of bacterial secondary metabolites, especially those from uncommon sources, are still elusive even though many of these compounds show striking biological activities. To further investigate the secondary metabolite repertoire of underexploited bacterial families, we chose to analyze a novel representative of the yet untapped bacterial phylum *Planctomycetes* for the production of secondary metabolites under laboratory culture conditions. Development of a planctomycetal high density cultivation technique in combination with high resolution mass spectrometric analysis revealed *Planctomycetales* strain 10988 to produce the plant toxin 3,5-dibromo-*p*-anisic acid. This molecule represents the first secondary metabolite reported from any planctomycete. Genome mining revealed the biosynthetic origin of this doubly brominated secondary metabolite, and a biosynthesis model for the compound was devised. Comparison of the biosynthetic route to biosynthetic gene clusters responsible for formation of polybrominated small aromatic compounds reveals evidence of an evolutionary link, while the compound's herbicidal activity points toward a complex interaction of planctomycetes with their macroalgal host.



Bacterial secondary metabolism has long been a source of chemically diverse and biologically active natural products.<sup>1,2</sup> Large numbers of biologically active entities have been isolated from extensively screened phyla such as actinobacteria, firmicutes, and proteobacteria.<sup>3–5</sup> To establish alternative sources, natural products research is increasingly focusing on taxa that have been less exploited to date but show potential for production of secondary metabolites according to the presence of secondary metabolite biosynthesis gene clusters (BGCs) in their genomes.<sup>2</sup> This strategic shift toward new producers increases chances for the discovery of novel bioactive secondary metabolite scaffolds that are chemically distinct from the scaffolds found in previously screened bacteria. While it has long been stated that phylogenetically distant species have a more distinct secondary metabolism, recent comprehensive secondary metabolome studies were able to validate this claim.<sup>6,7</sup> Accordingly, it is now widely recognized that there is an urgent need to scrutinize novel bacterial taxa, alongside the use of sensitive mass spectrometry and varied cultivation conditions to unearth novel natural products from bacterial secondary metabolomes.<sup>8</sup> Planctomycetes represent an underexploited phylum of bacteria in terms

of their secondary metabolite potential.<sup>9</sup> However, no secondary metabolite of planctomycetal origin has been reported, although planctomycetes were already discovered in 1924.<sup>10</sup> This bleak picture is in clear contrast to previous *in silico* genome analysis that suggested planctomycetes to contain a significant number of secondary metabolite BGCs.<sup>11,9</sup> In this work, we describe the first secondary metabolite from any planctomycete, including its structural characterization and biosynthesis. The biological activity of the discovered compound leads us to postulate an ecological role for it within the planctomycete's natural habitat.<sup>9</sup>

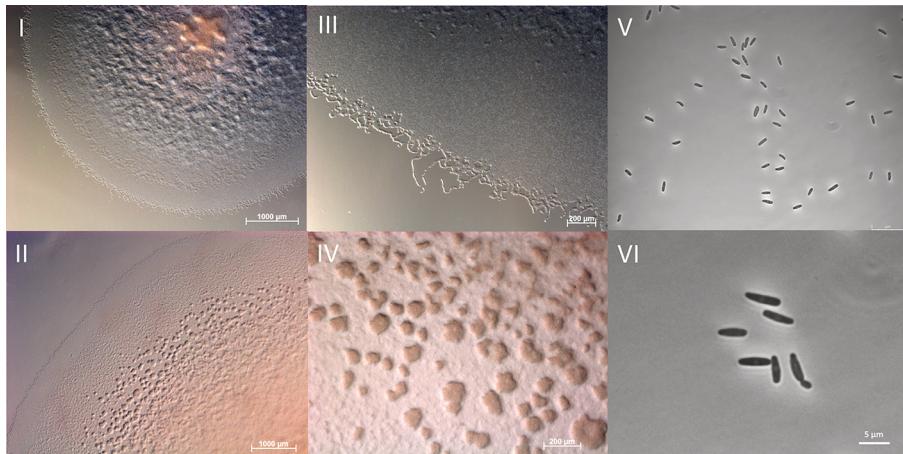
## RESULTS AND DISCUSSION

**Cultivation of *Planctomycetales* Strain 10988.** In order to investigate the biosynthetic capacity of uncommon and underexploited bacteria, we set out to isolate new strains from marine sediment samples. Our efforts revealed a swarming,

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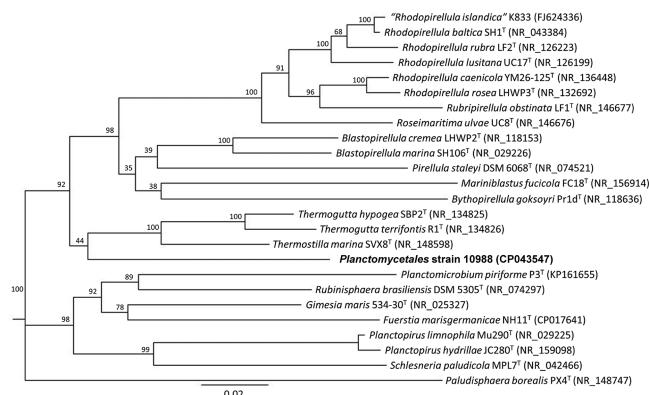
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**Figure 1.** Growth characteristics of *Planctomycetales* strain 10988 on solid medium displaying swarming (I and III), “fruiting body-like” aggregate formation (II–IV), and phase contrast microscopy images of single cells from swarm (V) and from “fruiting body-like” aggregates (VI).

rose colored bacterial isolate that was designated as strain 10988 (see Figure 1).

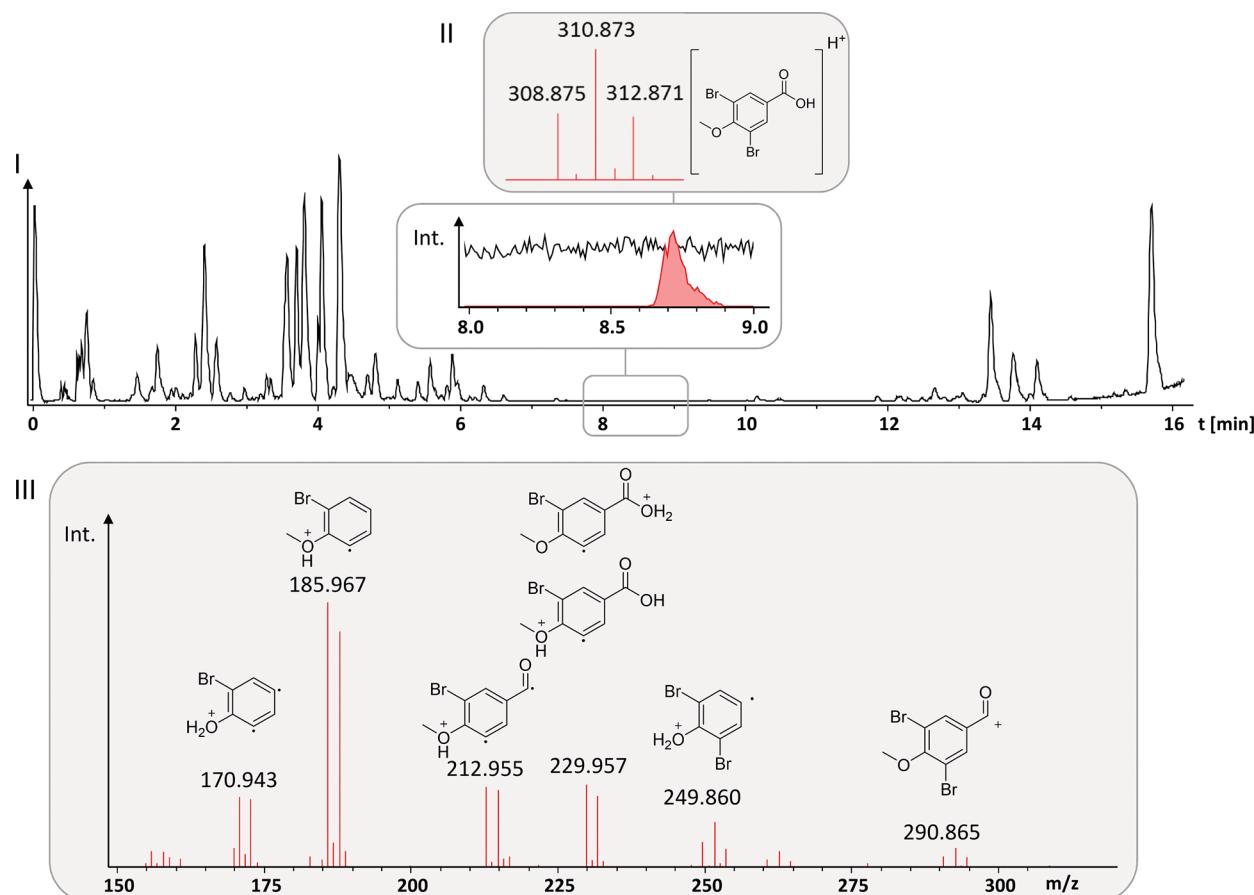
16S rRNA-gene based phylogenetic analyses revealed the bacterium to belong to the order *Planctomycetales* while being genetically distant from previously characterized planctomycetal genera such as *Thermogutta*, *Thermostilla*, *Pirellula*, *Rhodopirellula*, and *Blastopirellula* (see Figure 2).<sup>9,12–14</sup>



**Figure 2.** Phylogenetic tree inferred from 16S rRNA gene sequence similarity showing *Planctomycetales* strain 10988 among its nearest neighbors in the family *Planctomycetaceae*. GenBank accession numbers are indicated in parentheses. *Paludisphaera borealis* strain PX4<sup>T</sup> was used as an outgroup to root the tree. The scale bar indicates nucleotide substitutions per site.

The nearest neighbors of strain 10988 among the *Planctomycetales* are the genera *Thermogutta* and *Thermostilla*, both of which are thermophilic as well as anaerobic or microaerophilic. However, strain 10988 showed very different characteristics. It not only displays optimum growth at 24–37 °C, but it also grows aerobically. These findings agree with our 16S rDNA gene classification attempt, which classified strain 10988 as only distantly related to all characterized *Planctomycetes*. We thus sought to evaluate its secondary metabolome as a potential source for new natural products. Although *Planctomycetes* have been continuously studied since the late 1980s and *Planctomycetes* have been shown to possess cellular features completely distinct from other prokaryotes, little has been done to investigate the planctomycetal secondary metabolome.<sup>15,16</sup> One of the key limiting factors

that needs to be overcome in order to uncover planctomycetal secondary metabolism is the requirement to develop suitable cultivation techniques first. While there has been some success with cultivation of freshwater *Planctomycetes*, cultivation of marine species such as strain 10988 turned out to be challenging.<sup>17</sup> Cultivation of strain 10988 showed that it is an obligate halophile, as it did not grow in the absence of sea salts. The halophilicity of the strain is underpinned by an ectoine biosynthesis gene cluster present in the genome of strain 10988 that serves as a means to counterbalance the osmotic stress exerted by seawater brine on the cell. Furthermore, the strain depends on surface adsorption for efficient growth which is exemplified by its enhanced growth in early stages if cellulose powders are added to the medium. As these fine cellulose filter particles turn to rose color before the medium in the shake flask contains a significant number of suspended cells, the bacterium seems to preferentially colonize surfaces before dispersing into the suspension in a shake flask (**Supporting Information**). This finding is well in line with planctomycetal growth in nature that occurs in parts fixed to the surfaces of macroalgae.<sup>9</sup> The slow growth of this isolate in combination with initially low secondary metabolite production rates—as judged by LC-MS analysis—led us to devise a fermenter based cultivation to obtain increased secondary metabolite yields that could not be achieved in shake flask cultivations. As a means to stimulate productivity of the planctomycetal strain for secondary metabolite isolation, we added adsorber resin to shake flask cultures. This should circumvent productivity limitations arising from feedback inhibition mechanisms.<sup>18</sup> However, addition of adsorber resin led to complete suppression of planctomycetal growth unless the culture was inoculated with a high concentration of actively growing cells. When strain 10988 was grown in the absence of adsorber resin, inoculation of liquid cultures with a very low concentration of live cells was sufficient to stimulate planctomycetal growth. The most probable explanation for this phenomenon is that the presence of adsorber resin in low density cultures masks certain quorum sensing signals by binding them, inhibiting cooperative growth of planctomycetes. While quorum sensing has been linked to different effects such as the inhibition of biofilm formation or virulence, a quorum sensing signal that increases or stalls cell division speed has not been described yet.<sup>19</sup> As a result, in order to avoid lack of growth or unnecessarily lengthy lag phases in



**Figure 3.** (I) LC-MS base peak chromatogram of *Planctomycetales* strain 10988, magnification of the extracted ion chromatogram for 3,5-dibromo-p-anisic acid (red) and the base peak chromatogram in that area (1). (II) Magnification of the corresponding MS spectrum and structure formula of 1. (III) MS<sup>2</sup> spectrum of 1 and putative product ions formed in MS<sup>2</sup> fragmentation experiments.

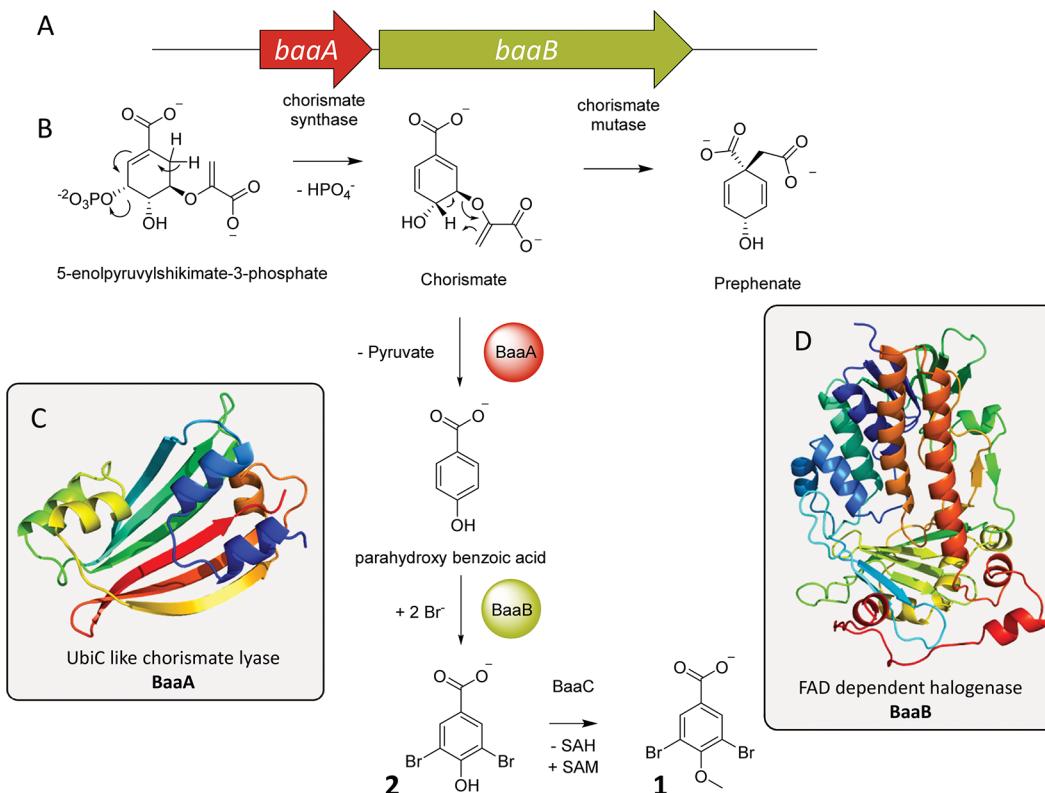
planctomycetal fermentations in larger scale production cultures, adsorber resin addition was performed several days post inoculation of the respective fermenter. In analytical scale shake flask cultivations, the effect of adding resin directly could be mitigated by inoculation of the cultures with a higher concentration of live cells.

**Discovery of 3,5-Dibromo-p-anisic Acid.** To assess the secondary metabolome of planctomycetal strain 10988, methanolic extracts of the strain's culture supplemented with adsorber resin were prepared and compared to methanolic extracts of the corresponding medium ("blank" sample) to obtain an overview about its secondary metabolome. The bacterial extract and the blank were subjected to high-resolution LC-MS analysis using a reverse-phase UPLC-coupled qTOF setup (Supporting Information). This analysis revealed an intriguing signal presenting a monoisotopic mass of 308.873 Da [ $\text{M} + \text{H}]^+$  ( $\text{C}_8\text{H}_6\text{Br}_2\text{O}_3$ ,  $\Delta m/z = 6$  ppm) and an isotope pattern that pointed toward double bromination.<sup>20</sup> Maximum cell density as well as the production rate of this doubly brominated compound remained limiting for material supply in shake flask cultures even after media optimization (Supporting Information). We therefore developed a method to grow strain 10988 in a fermenter which allowed the purification of the candidate compound by semipreparative HPLC (Supporting Information).

According to molecular formula calculation, the molecule possesses six hydrogen atoms. As the <sup>1</sup>H NMR spectrum contained only two singlet signals, the molecule had to be

highly symmetrical. One of the signals representing two protons had a strong downfield shift of 8.11 ppm, indicating a heavily electron deficient symmetrical aromatic system, while the other singlet signal representing three protons was characteristic for an oxygen-linked methyl group. The <sup>13</sup>C shift of the corresponding methyl group indicated its connection to the phenolic oxygen of the molecule and not to the carboxylic acid (Supporting Information). This was further supported by the fact that the tandem MS spectra show a strong water loss, as expected from free carboxylic acid moieties, while we did not observe neutral loss of methanol in tandem MS experiments (Figure 3). This neutral loss would be expected if the molecule contained a methyl ester. The double brominated compound produced by 10988 was therefore determined to be 3,5-dibromo-p-anisic acid (1), which could be later confirmed using synthetic standard material.

**Biosynthesis of 3,5-Dibromo-p-anisic Acid.** In order to identify the biosynthetic origin of 1, we determined the complete genome of *Planctomycetales* strain 10988 using PacBio long read sequencing technology (Supporting Information). Genome assembly resulted in a single circular bacterial chromosome of 6.6 Mbp with a total GC content of 50.4% (GenBank accession number CP043547). AntiSMASH analysis of the bacterial genome annotated three terpene BGCs, an ectoine BGC, a cluster for lasso peptide biosynthesis, and a type III polyketide synthase BGC.<sup>21</sup> Contrary to earlier genome mining results from planctomycetes, the genome of strain 10988 does not encode any multimodular secondary



**Figure 4.** (A) Gene cluster for 3,5-dibromo-*p*-anisic acid (**1**) biosynthesis. (B) Proposed model for biosynthesis of **1** including the nonmethylated precursor **2**. (C) Phyre2 based homology model for the chorismate lyase BaaA. (D) Phyre2 based homology model for the brominase BaaB.

metabolite pathways in its genome.<sup>11,22</sup> Since our newly elucidated secondary metabolite is likely not produced by a multimodular megasynthase, the biosynthesis gene cluster predictors run by antiSMASH are in this case unsuitable to annotate the corresponding biosynthesis pathway, which possibly consists of a set of “stand-alone” enzymes.<sup>21</sup> We therefore searched the obtained genome for flavin dependent halogenase enzymes, as most regioselective bromination or chlorination reactions on aromatic systems are catalyzed by this protein family in nature.<sup>23,24</sup> The flavoprotein showing the highest homology to halogenating enzymes was named BaaB. It was found encoded adjacent to—and putatively on the same mRNA strand as—a chorismate lyase-like protein termed BaaA. This protein could plausibly deliver the precursor *p*-hydroxybenzoic acid from the cellular chorismic acid pool (Figure 4).<sup>25</sup> Homology modeling of the two proteins on the protein fold recognition server Phyre2 supports this finding, as both proteins involved in biosynthesis of **1** are correctly mapped onto the expected protein families.<sup>26</sup> Unfortunately, and despite serious efforts, we were unable to develop methods to genetically manipulate the planctomycetal strain and we were thus unable to further validate our hypothesis by an inactivation mutant of the *baaA* and *baaB* locus. The mechanism of UbiC-like chorismate lyases such as BaaA is less studied in comparison to the mechanism of chorismate mutases. Chorismate mutase reactions consist of an electrocyclic six-electron rearrangement reaction that leads to prephenate formation (Figure 4).<sup>25</sup> Chorismate lyase enzymes like BaaA use a closely related electrocyclic six-electron rearrangement reaction that removes pyruvate from chorismate to form *p*-hydroxybenzoic acid.<sup>27</sup> At this point, we could not differentiate whether *p*-hydroxybenzoic acid is methylated to *p*-

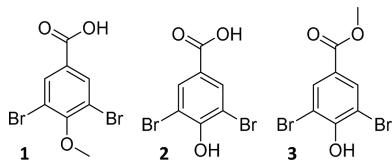
anisic acid first or if methyl transfer occurs after double bromination of *p*-hydroxybenzoic acid by the brominase enzyme BaaB. In order to complete the biosynthesis of **1** after the action of BaaA and BaaB, an SAM dependent O-methyl transferase (BaaC) is needed that transforms **2** into **1**. When analyzing the genetic locus encoding BaaA and BaaB, we did not find such an enzyme, meaning BaaC is encoded in a different genetic locus. The fact that the *baaA* and *baaB* genes are encoded adjacently in the genome while the corresponding methyl transferase *baaC* is encoded in a different location may indicate that dibromo *p*-hydroxybenzoic acid is produced first and subsequently methylated. It is worth noting that we could not identify either of the possible intermediates via LC-MS from the 10988 strain extracts. As BaaB is the only halogenase enzyme encoded in the *baa* BGC, it is certainly responsible for 3,5 dibromination of the aromatic moiety. As both positions that are brominated are chemically equivalent, it is not surprising that both halogenations are performed by the same enzyme. Furthermore, we observe strict specificity of BaaB for bromine, as no chlorinated or mixed brominated and chlorinated anisic acid derivatives could be identified in the fermentation broth. Thus, BaaB is either unable to bind chloride anions instead of bromide anions due to a difference in binding cavity size, or the redox potential of BaaB is not sufficient to oxidize chloride anions but is sufficient to oxidize bromide ions to an activated species. Still, BaaB is not the only such enzyme unable to process chlorine, as the brominase BmpS from *Pseudoalteromonas* strains involved in biosynthesis of polybrominated phenols is also specific for bromine over chlorine.<sup>28</sup> The architectures of the responsible loci producing polybrominated biphenyllic secondary metabolites show remarkable similarity to the Baa operon even though the

host organisms are phylogenetically very distant. While Bmp5-like proteins from *P. luteoviolacea* 2ta16 and *P. phenolica* O-BC30 are very similar as they share 96% homology, their similarity to BaaB remains around 44%. This finding is readily explained as *Pseudoalteromonads* and *Planctomycetes* are phylogenetically far apart and BaaB only catalyzes meta-position bromination, while Bmp5 also catalyzes ipso substitution of CO<sub>2</sub> at the aromatic core. This reaction removes the carboxylic acid, and the phenols are not methylated afterward.<sup>29,30</sup> Furthermore, in biosynthesis of polybrominated biphenyl ethers in *Pseudoalteromonads*, an additional enzyme called Bmp7 uses phenolic coupling reactions to form biphenyl structures that do not exist in our planctomycetes strain, as the baa gene cluster in strain 10988 does not possess the corresponding CYP P450 enzymes.<sup>30</sup> The absence of CYP enzymes in the planctomycetal BGC explains why the planctomycete only synthesizes monocyclic polybrominated aromatic compounds, as it lacks the CYP enzyme required to perform phenol couplings, leading to the formation of biphenylic compounds.<sup>30,31</sup>

As shown in Figure 4, in order to finish biosynthesis of **1**, the formerly mentioned O-methyl transferase BaaC is required that transfers a methyl group and thus forms the methoxy group in **1**. The corresponding oxygen-methyl transferase responsible for methyl transfer to the phenolic oxygen of the precursor compound is not clustered with the genes responsible for *p*-anisic acid production. Although there are candidate sequences for the BaaC protein, it is currently not possible to pinpoint the specific methyl transferase in the 10988 genome responsible for methylation of **2** due to our inability to perform directed mutagenesis with the strain. Blast analyses of the 10988 genome for homologues to the StiK protein from stigmatellin biosynthesis in *S. aurantiaca* (NCBI protein acc. Nr. CAD19094.1) and UbiG from *Escherichia coli* K12 (NCBI protein acc. Nr. BAA16049.1) were performed.<sup>32,33</sup> We obtained eight sequences encoding proteins that putatively catalyze methyl transfer to an aromatic hydroxyl group, the enzymatic functionality of BaaC. These proteins showing similarity to both aforementioned enzymes are thus considered plausible candidates to catalyze the transformation of **2** to **1**.

**Bioactivity Evaluation of **1** and Its Analogues.** Due to the double bromination of **1**, its biosynthesis is costly to the strain in terms of energy and resources. Therefore, **1** can be plausibly expected to confer a competitive advantage to strain 10988 in its environment. To evaluate this biological role of **1**, we set out to profile its bioactivity as well as the bioactivity of its biological precursor **2** and its isomer methyl-3,5-*p*-hydroxybenzoic acid (**3**). The compounds **2** and **3** that cannot be obtained from the planctomycetal culture broth are commercially available.

**Scheme 1.** The Natural Product 3,5-Dibromo-*p*-anisic Acid (**1**), Its Putative Biological Precursor 3,5-Dibromo-*p*-hydroxybenzoic Acid, and the Natural Product Analogue Methyl-3,5-dibromo-*p*-hydroxybenzoate (**3**)



The planctomycetal natural product **1**, its precursor **2**, and its analogue **3** were tested in an antibiotics screening against the bacterial pathogens *Citrobacter freundii*, *Acinetobacter baumanii*, *Escherichia coli*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Micrococcus luteus*, the yeasts *Candida albicans* and *Pichia anomala*, as well as the filamentous fungus *Mucor hiemalis*. The compounds did not display any inhibition of these microbial indicator strains at concentrations up to 64 µg mL<sup>-1</sup>. To evaluate cytotoxicity, we tested **1**, **2**, and **3** in a cell line cytotoxicity assay. This assay revealed both methylated compounds (**1** and **3**) to display moderate cytotoxicity to the human cervical carcinoma cell line KB3.1. While **1** and **3** showed IC<sub>50</sub> values of 60 µg mL<sup>-1</sup>, no cytotoxicity was found for the free acid **2** up to 300 µg mL<sup>-1</sup>. To assay the herbicidal activity of *p*-methoxy-dibromobenzoic acid as well as its precursor and isomer, we tested the compound's activity on the germination of *Agrostis stolonifera* penncross. IC<sub>50</sub> values for seed germination inhibition were determined to be 32 µg mL<sup>-1</sup> for **1**, 64 µg mL<sup>-1</sup> for **2**, and 16 µg mL<sup>-1</sup> for **3**. The fully decorated methoxy derivative **1** shows higher biological activity than its precursor **2**, while the non-natural methyl ester derivative **3** shows the best anti-germination activity in the *A. stolonifera* germination assay (Supporting Information). As compound **1** shows no significant antibacterial activity or mammalian cell cytotoxicity but displays moderate phytotoxicity and as **1** is likely released to the marine environment, we assume its role as a putative algal toxin (Supporting Information).

**On the Potential Biological Role of **1**.** Many planctomycetes live in close association with macroalgae which they cover almost completely.<sup>34</sup> Given that the strain 10988 was isolated from a marine sediment, the strain is likely associated with marine algae in its natural habitat. The exact mode of interaction between the planctomycetes and the macroalgae has yet to be determined. Still, the high abundance of planctomycetes on algal species, which can reach up to 50% of the algal microbiome, indicates that these bacteria are significant interaction partners for the algae.<sup>34</sup> One hypothesis considers the algae as a food source for planctomycetes, since they are able to utilize uncommon sugars such as rhamnose and fucose contained in algal biomass. In our case, strain 10988—like other planctomycetes—was able to grow on uncommon sugars such as galactose, mannose, lactose, sucrose, maltose, raffinose, xylose, and rhamnose (Supporting Information, Figure S4), indicating that this nutritional option could apply to strain 10988.<sup>11</sup> On the other hand, the planctomycetes bacterium possesses the ability to produce the plant toxin 3,5-dibromo-*p*-anisic acid, whose production seems to be tightly regulated, as judged by the low production titers under laboratory conditions. We therefore reason that an ambivalent interaction model might take place between planctomycetes and their plant hosts. The planctomycetes strains may live on the algal surface and modulate the local microbial community until they sense the algal species they live on is weakening. This might trigger expression of said plant toxin to kill and decay this part of the algae, and the bacterium would subsequently move on to colonize different algae. Similar “Jekyll and Hyde” behavior, meaning the ability to switch between commensalism or symbiosis, comparable to planctomycetal colonization of algae, and a virulent state that is hostile to its host organism, has been described for *P. gallaeciensis* in its interaction with algae as well as for the human pathogen *C.*

*albicans*.<sup>35,36</sup> The ability of strain 10988 to produce a plant toxin as a bacterium associated with macroscopic plants can be seen as a strong hint that the bacterium adopts such a strategy.

**Conclusion.** In this work, we describe the cultivation of a new marine planctomycete that is genetically distant from all planctomycetes known to date and reveal *Planctomycetales* strain 10988 as a producer of a dibrominated secondary metabolite. Isolation of this secondary metabolite required a bioreactor setting and optimized medium and culture conditions. Subsequent structure elucidation of **1** by NMR revealed an intriguing structure and thus sparked interest in the biosynthetic origin and ecological role of the compound. We were able to pinpoint the core biosynthesis genes *baaA* and *baaB* that can accomplish the core structure of **1**. Investigation of the bioactivity of **1** as well as the bioactivity of its isomer **3** and putative precursor **2** showed that this compound class displays herbicidal activity in *A. stolonifera* penncross germination assays. We thus hypothesize on a biological role of this compound in the life cycle of the algal symbiont *Planctomycetales* strain 10988. In conclusion, we contribute to the understanding of the biogenesis of small polyhalogenated compounds in marine bacteria, whereas it remains astonishing to what extent such polybrominated aromatic substances are apparently released into the ecosystem from biological instead of anthropogenic sources.

This study also identifies planctomycetes as an under-exploited source of biologically active secondary metabolites, as **1** to the best of our knowledge is the first natural product described from this bacterial taxon. However, we would like to point out that previous studies may have overestimated the genome encoded secondary metabolite diversity of planctomycetes as a group, since strain 10988 under study here did not show the presence of any multimodular megasynthetase.<sup>11,22</sup> Even though strain 10988 shows some BGCs, especially BGCs linked to terpene biosynthesis, BGCs containing megasynthases are often considered as benchmark indicators for secondary metabolite production capability.<sup>2</sup> On the other hand, the example presented here shows how important it is to evaluate new taxa on the metabolomics stage, since metabolites of the type described here are easily missed by genome mining. Thus, the overall potential of planctomycetes awaits further investigation. While growing these bacteria under laboratory conditions may be tedious and nontrivial, devising methods for their cultivation is a valuable tool to tap into the planctomycetal secondary metabolite space. The discovery of polybrominated compounds in strain 10988 is well in line with both the observation that this bacterium is an obligate halophile and reminiscent of the strain's marine origin. The isolated and characterized natural product 3,5-dibromo-*p*-anisic acid shows that Nature, especially the marine microbial community, is able to biosynthesize polyhalogenated small aromatic compounds that look like anthropogenic products of chemical synthesis.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscchembio.9b00641](https://doi.org/10.1021/acscchembio.9b00641).

An in detail description of the planctomyces strain, all utilized fermentation protocols, *in silico* analyses on gene

and protein level, as well as all relevant NMR data for structure elucidation ([PDF](#))

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### Notes

The authors declare no competing financial interest.

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