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Research review paper

The bacterial phylum *Planctomycetes* as novel source for bioactive small molecules

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ABSTRACT

Extensive knowledge and methodological expertise on the bacterial cell biology have been accumulated over the last decades and bacterial cells have now become an integral part of several (bio-)technological processes. While it appears reasonable to focus on a relatively small number of fast-growing and genetically easily manipulable model bacteria as biotechnological workhorses, the for the most part untapped diversity of bacteria needs to be explored when it comes to bioprospecting for natural product discovery. Members of the underexplored and evolutionarily deep-branching phylum *Planctomycetes* have only recently gained increased attention with respect to the production of small molecules with biomedical activities, e.g. as a natural source of novel antibiotics. Next-generation sequencing and metagenomics can provide access to the genomes of uncultivated bacteria from sparsely studied phyla, this, however, should be regarded as an addition rather than a substitute for classical strain isolation approaches. Ten years ago, a large sampling campaign was initiated to isolate planctomycetes from their varied natural habitats and protocols were developed to address complications during cultivation of representative species in the laboratory. The characterisation of approximately 90 novel strains by several research groups in the recent years opened a detailed in silico look into the coding potential of individual members of this phylum. Here, we review the current state of planctomycetal research, focusing on diversity, small molecule production and potential future applications. Although the field developed promising, the time frame of 10 years illustrates that the study of additional promising bacterial phyla as sources for novel small molecules needs to start rather today than tomorrow.

1. Introduction

In the quest for novel small molecules with health-promoting properties, it became obvious that gene clusters responsible for their biosynthesis are not equally distributed among bacteria. There are hotspots known as groups or even phyla of so-called 'talented producers', e.g. *Streptomycetes*, *Myxobacteria* or *Cyanobacteria*, that share certain traits with respect to their capability to synthesize bioactive small molecules (Bader et al., 2020; Harir et al., 2018; Kultschar and Llewellyn, 2018). These traits include large genomes and complex lifestyles (Konstantinidis and Tiedje, 2004; O'Brien and Wright, 2011). However, the mentioned bacterial groups were subject of intense research in the past decades and continued screening for novel small molecules inevitably leads to high rediscovery rates. Thus, such bacterial groups do not meet the current need for novel bioactive lead compounds. This said, where should we look instead for producers of natural products, including novel antibiotics?

Thus far, less than 1% of the bacterial diversity is studied employing axenic cultures (van Teeseling and Jogler, 2021). Such pure cultures are key for purification of large amounts of small molecules required to elucidate structures and study their bioactivities. Furthermore, genetic modification and the determination of enzyme functions require axenic cultures as well. In the past, it was claimed that the remaining 99% of elusive microbes cannot be studied as they belong to the 'microbial dark matter' recalcitrant to any cultivation attempt (Jiao et al., 2021). The enormous number of microbes would make the untargeted cultivation, if possible at all, the search for a needle in a haystack as most bacteria are specialized to certain ecological niches and thus not necessarily a source for novel small molecules. About a decade ago, we asked the question if a targeted cultivation approach is possible and if such an approach is feasible to unearth both, novel elusive microbes and novel bioactive small molecules. We coined the term deep-cultivation for such a

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hypothesis-driven approach. The underlying main hypothesis was the quintessence of 'talented producers': large genomes and complex lifestyles correlate with the production of bioactive small molecules. If an understudied bacterial phylum comprising these traits is intensively studied, the likelihood for finding novel small molecules is high. We chose the phylum *Planctomycetes* as example for two reasons: i) the first genome obtained from the planctomycete *Rhodopirellula baltica* is large (7.1 Mb), and ii) planctomycetes show complex lifestyles: at this time, they were believed to incorporate large carbon substrates via endocytosis, divide by polar budding, a process often linked to a lifestyle switch between sessile mother cells and motile daughter cells (Boedeker et al., 2017; Glöckner et al., 2003; Wiegand et al., 2020b). Although key aspects of the planctomycetal cell biology were recently revised (Wiegand et al., 2018), members of this phylum are still outstanding bacteria in terms of their genome organization, their lifestyle and their cell biology. Furthermore, planctomycetes are of both, environmental and biotechnological importance (Wagner and Horn, 2006). The role of planctomycetes as plentiful source of novel bioactivities is strongly supported by complex allelopathic interactions with aquatic phototrophs and the need for compensation of lower growth rates compared to heterotrophic bacterial competitors that occupy the same ecological niches (Faria et al., 2018; Kallscheuer et al., 2020a; Lage and Bondoso, 2014; Martens et al., 2006). In this overview article, we focus on the described diversity of planctomycetes, their genomic potential for the production of novel small molecules, their biotechnological potential and further provide three examples of recently discovered small molecules produced by planctomycetes. We end discussing the future perspectives on secondary metabolite discovery from planctomycetes and beyond.

2. Results and discussion

2.1. Ecophysiology and cell biology of planctomycetes

Planctomycetes are ubiquitous bacteria that play an important role in the global carbon and nitrogen cycle (Kuenen, 2008; Wiegand et al., 2018). Phylogenetically, the phylum *Planctomycetes* is part of the medically and biotechnologically relevant PVC (*Planctomycetes-Verrucomicrobia-Chlamydiae*) superphylum (Rivas-Marín and Devos, 2018; Wagner and Horn, 2006). While planctomycetes are frequently isolated from various abiotic and biotic surfaces of aquatic habitats, a high abundance of members of this phylum was observed in association with macroscopic phototrophs (Bengtsson et al., 2012; Bondoso et al., 2014; Bondoso et al., 2017; Lage and Bondoso, 2014). The phylum has been shown to account for up to 70–85% of the surface-associated bacterial community, for example on the macroalga *Ecklonia radiata* or the Neptune seagrass *Posidonia oceanica* (Kohn et al., 2020; Wiegand et al., 2018).

During an investigation of the planctomycetal cell biology, exceptional traits such as a lack of peptidoglycan (König et al., 1984), a compartmentalized cell plan (Lindsay et al., 1997), a nucleus-like structure (Fuerst and Webb, 1991) and endocytosis (Lonhienne et al., 2010) were proposed, however, most of these traits have been refuted based on novel superresolution microscopic techniques and by employing genetic tools (Jogler et al., 2011; Jogler and Jogler, 2013; Rivas-Marín et al., 2016). The observed compartments turned out to be rather invaginations of the cytoplasmic membrane (Acehan et al., 2014; Boedeker et al., 2017; Santarella-Mellwig et al., 2013) and with some exceptions the cell envelope architecture of planctomycetes is now considered to be similar to that of Gram-negative bacteria (Devos, 2014a; Devos, 2014b). Despite this re-interpretation, the planctomycetal cell plan is still uncommon in many ways. The planctomycetal divisiome, for example, does not rely on the otherwise essential structural protein FtsZ (Jogler et al., 2012; Pilhofer et al., 2008; Wiegand et al., 2020b). The cell surface of several planctomycetal strains is covered by pili originating from unique crateriform structures (Boedeker et al., 2017). In combination with an enlarged periplasmic space, the observed pili may be part of a specialized system for the internalization and intracellular degradation of complex polysaccharides as it has been shown for the model substrate dextran (Boedeker et al., 2017). The uncommon cell morphology and physiology of the phylum is also reflected in 35–65% of genome-encoded proteins having an unknown function, values which are the highest among bacterial phyla investigated so far (Overmann et al., 2017).

A recent strain isolation and characterization campaign targeting the phylum *Planctomycetes* yielded 79 novel strains in axenic cultures that could be assigned to 1 known and 65 previously unknown species that in turn belong to 8 known and 31 previously unknown genera (Devos et al., 2020; Wiegand et al., 2020b). One of the key factors that enabled the isolation of novel members of the phylum was an optimized medium formulation that relied on the use of *N*-acetyl glucosamine as carbon source and exploited the natural resistance of planctomycetal strains to antibiotics. A combination of carbenicillin, ampicillin and streptomycin turned out to be suitable to selectively enrich planctomycetes from material sampled from biotic and abiotic surfaces on the European or American coastline or from several limnic habitats in Germany.

2.2. Currently described diversity within the phylum Planctomycetes

Hitherto, strains belonging to 125 species have been described that together constitute the current phylum *Planctomycetes* (as of July 2021). The current taxonomy follows the common notation for the ranks class (suffix *-ia* or *-ae*), order (suffix *-ales*) and family (suffix *-aceae*). Characterized species are distributed among the two classes *Planctomycetia* (108 species in 56 genera) and *Phycisphaerae* (8 species in 7 genera) and the proposed class *Candidatus* Brocadiae (20 proposed species in 5 proposed genera; holding the *Candidatus* status due to lack of axenic cultures) (Fig. 1). The class *Planctomycetia* was recently reorganized by subdivision into the orders *Pirellulales* (harboring the three families *Pirellulaceae*, *Lacipirellulaceae* and *Thermoguttaceae*), *Planctomycetales* (with the sole family *Planctomycetaceae*), *Gemmatales* (sole family *Gemmataceae*) and *Isosphaerales* (sole family *Isosphaeraceae*) (Dedysh et al., 2020). The class *Phycisphaerae* harbours two orders with a single family each (*Phycisphaerales* – *Phycisphaeraceae*; *Tepidisphaerales* – *Tepidisphaeraceae*) and one order with two families (*Sedimentisphaerales* – *Sedimentisphaeraceae* and *Anaerohalosphaeraceae*) (Fukunaga et al., 2009; Pradel et al., 2020; Spring, 2015). In the class *Candidatus* Brocadiae all strains are currently placed in the single family *Candidatus*

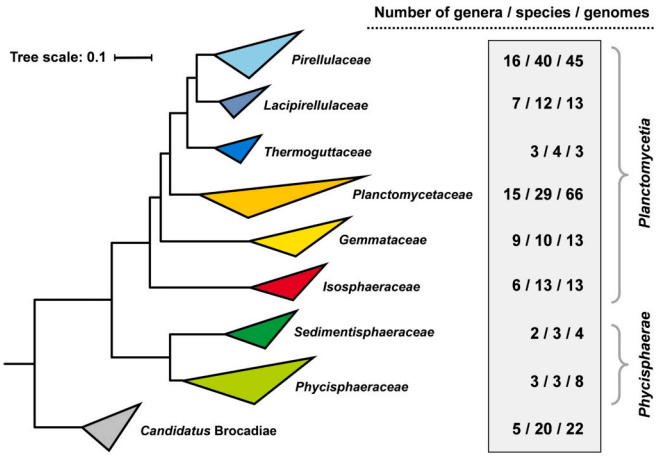

Fig. 1. Phylogenetic overview of the current phylum *Planctomycetes*. The phylogenetic tree highlights the current families of the phylum. Families that currently only harbour a single genus and species (*Anaerohalosphaeraceae* and *Tepidisphaeraceae*, both belonging to different orders within the class *Phycisphaerae*) are not shown. The number of available genomes is based on data of the taxonomy browser in the NCBI database. The taxonomic state of orders is not shown in the figure (please see text for details).

Brocadiaceae of the sole order *Candidatus* Brocadiales (Jetten et al., 2015). Members of this class are well-known for their ability to perform anaerobic ammonium oxidation (referred to as 'anammox' process) (Jetten et al., 2009). The classes *Planctomycetia* and *Phycisphaerae* can be delineated by differences in cell biological and genomic features. Members of the class *Planctomycetia* divide by budding, whereas strains belonging to the class *Phycisphaerae* divide by binary fission (Wiegand et al., 2020b). The latter have relatively small genomes of 3.0–4.3 Mb, whereas genomes in the current class *Planctomycetia* are considerably larger (4.8–12.4 Mb). When taking into account that larger genomes are typically enriched in genes related to secondary metabolite production and regulatory processes (Konstantinidis and Tiedje, 2004), such differences have direct implications in terms of the potential for discovery of novel small bioactive molecules (Wiegand et al., 2020b). Higher numbers of giant genes and suggested gene clusters coding for carbohydrate-active enzymes (CAZymes) involved in the breakdown of high molecular weight sugars probably also contribute to larger genomes of the class *Planctomycetia* (Andrade et al., 2017; Reva and Tümmler, 2008).

2.3. Analysis of biosynthetic gene clusters putatively involved in secondary metabolite production

Based on the characterized planctomycetal strains with an available genome sequence, we analyzed biosynthetic gene clusters (BGCs) potentially related to secondary metabolite production using antiSMASH (Blin et al., 2019; Supplementary Table 1 in [dataset] Kallscheuer and Jogler, 2021). The analysis yielded between 0 and 13 BGCs per genome and a clear correlation between the number of clusters and the genome size was observed ($r^2 = 0.41$, p-value <0.0001) (Fig. 2A). Only a single strain, *Limihaloglobus sulfuriphilus* SM-Chi-D1^T (family *Sedimentisphaeraceae*) (Pradel et al., 2020), lacks BGCs according to the antiSMASH analysis, while the highest number of 13 BGCs each was found in *Gemmata massiliana* IIL30^T (family *Gemmataceae*) and *Rhodopirellula pilleata* Pla100^T (family *Pirellulaceae*) (Aghnatios et al., 2015; Kallscheuer et al., 2020c). *Fimbriiglobus ruber* SP5^T, the planctomycete with the currently largest genome of 12.4 Mb (Kulichevskaya et al., 2017; Ravin et al., 2018), harbours only 9 BGCs, indicating that other functionalities, including potentially unknown BGCs, are encoded in the genome that make up the large size.

For a comparison of the numbers of BGCs obtained for planctomycetes, we also analyzed species from other bacterial phyla that have equally large genomes and are known to be potent sources of bioactive natural products. The bacterium with the currently largest genome in the NCBI database (with assembly level 'closed genome') is *Minicystis rosea* DSM 24000^T, a member of the order *Myxococcales* (class *Deltaproteobacteria*) with a genome size of 16.0 Mb (NCBI accession number CP016211.1) (Garcia et al., 2014). An antiSMASH analysis for this strain yielded altogether 47 BGCs. *Streptomyces rapamycinicus* NRRL 5491 (NCBI accession number CP006567.1), the streptomycete with the currently largest genome of 12.7 Mb in the NCBI database (assembly level: chromosome), has a comparable genome size as the planctomycete *F. ruber* SP5^T, but yielded 53 BGCs in the antiSMASH analysis. At first sight, such large numbers might suggest that planctomycetes are rather 'untalented' in terms of secondary metabolite production. However, a direct comparison of these values is heavily biased when taking into consideration that the antiSMASH algorithm is based on empirical data, which is in turn derived from identified compounds and their respective BGCs from known 'talented' producers including streptomycetes and myxobacteria. This impression of an 'unfair' comparison is further reinforced by a recent analysis showing that the majority of putative planctomycetal BGCs is not connected to known BGCs (Wiegand et al., 2020b) as well as the fact that antiSMASH failed to predict BGCs for the small number of already isolated secondary metabolites from planctomycetes (Kallscheuer et al., 2020a; Panter et al., 2019). This even holds true for well-characterized compounds (e.g. carotenoids), for which no candidate biosynthetic enzymes could be identified based on known protein sequences or domains (Kallscheuer et al., 2019b). Further details on secondary metabolites identified in planctomycetes are presented in separate chapters in the second part of this

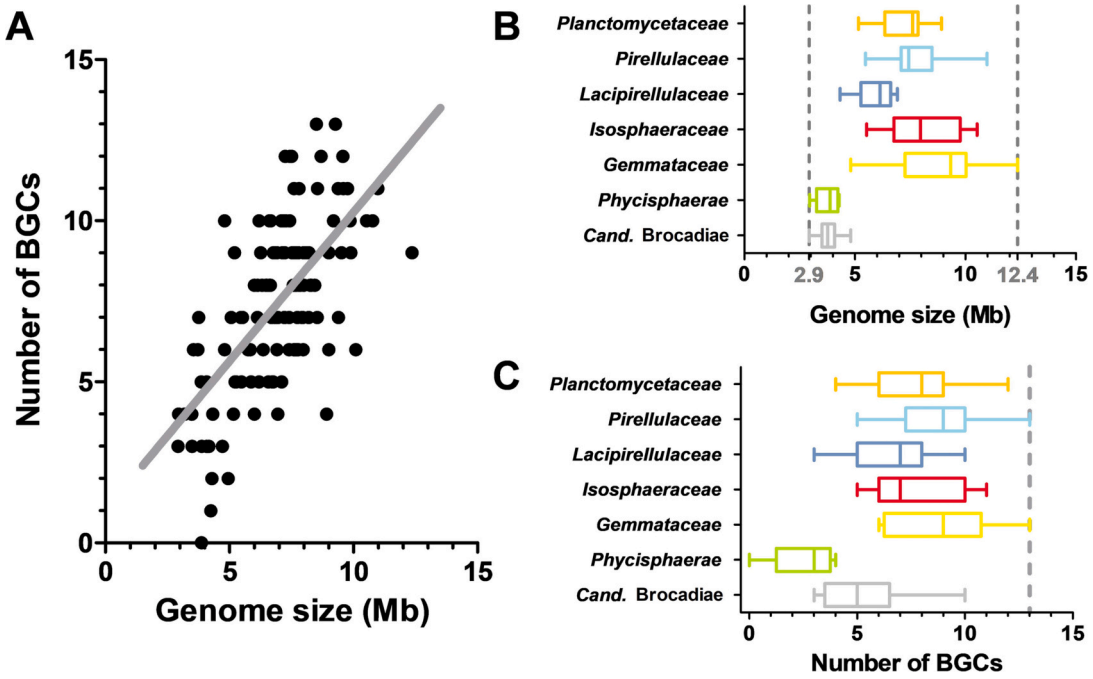

Fig. 2. Genome-based information and results of the antiSMASH analysis for the phylum *Planctomycetes*. (A) Correlation of the number of biosynthetic gene clusters (BGCs) predicted by antiSMASH and the genome size for characterized strains of the phylum. (B) Genome size ranges and (C) numbers of BGCs in the different families or classes of the phylum. Due to the low number of characterized strains in the classes *Phycisphaerae* and *Candidatus* Brocadiae, data is not resolved for the different orders and families within these two classes. The colours of the shown bars are the same as used for the collapsed clades in Fig. 1. Explicit data is presented in Supplementary Table 1 in [dataset] Kallscheuer and Jogler, 2021.

overview article.

2.4. Distribution of BGCs in different classes of the phylum

As expected from the observed differences in the genome size among planctomycetal classes, the distribution of the numbers of BGCs is quite heterogeneous. For some of the planctomycetal families the analysis is rather speculative in nature when taking into account that some of them harbour only a few described strains (e.g. within in the class *Phycisphaerae*). Hence, it should not be excluded that BGCs involved in the production of certain compound classes will be found in future isolates belonging to such families. The numbers of predicted BGCs turned out to nicely reflect the genome size ranges in the different planctomycetal families (Fig. 2B, C). With 6–13 BGCs per genome, members of the family *Gemmataceae* harbour the highest numbers of BGCs, closely followed by the family *Pirellulaceae* (5–13 BGCs) (Table 1). Although the family *Lacipirellulaceae* is currently the most closely related family to *Pirellulaceae*, its members have considerably smaller genomes (4.7–6.9 Mb, average: 6.0 Mb) and harbour only 3–10 BGCs per genome. The distribution of BGCs among the expected compound classes is quite similar for comparsion of the two families, however, the number of clusters per genome for individual compound classes is often lower for the family *Lacipirellulaceae*. It remains to be elucidated whether these differences have any phenotypic impact (e.g. regarding the number of different secondary metabolites produced). In terms of genome size and presence of BGCs, the family *Lacipirellulaceae* is currently the most striking outlier in the class *Planctomycetia*. The two described members of *Lacipirellula* (the type genus of the family *Lacipirellulaceae*) are e.g. currently the only members in the phylum that harbour three putative type III polyketide synthase (PKS) genes. The other species in the phylum either lack type III PKSs or harbour a single gene coding for such an enzyme.

Remarkably, a comparison of the phylum on class level showed that genome size ranges of the current classes *Phycisphaerae* (3.0–4.3 Mb) and *Planctomycetia* (4.7–12.4 Mb) do not overlap and the same is in principle also true for the numbers of BGCs (*Phycisphaerae*: 0–4 BGCs, *Planctomycetia*: 3–13 BGCs) (Table 1). When comparing the classes *Phycisphaerae* (0–4 BGCs) and *Candidatus* Brocadiae (3–10 BGCs), it turns out that the latter have a higher number of BGCs despite similar genome size ranges of the two classes. This, however, is related to 2–3 additional BGCs found in members of the class *Candidatus* Brocadiae that are required for the synthesis of ladderanes. Ladderanes are unusual lipids containing fused cyclobutane rings, that were shown to be components of anammoxosomes. These compartments are the site of the anaerobic ammonium oxidation ('anammox') process, an exclusive feature of members of the class *Candidatus* Brocadiae (Damsté et al., 2005; Rattray et al., 2008). Within this class, one species, *Candidatus* Scalindua japonica, clearly stands out. With 4.81 Mb (genome assembly level: 47 contigs, accession number GCA_002443295.1) it has a considerably larger genome than the other members of the proposed class (2.9–4.1 Mb) and harbours five putative type I PKS-BGCs not found in its close relatives. Of all the genomes analyzed here it is the only strain with more than 2 BGCs per Mb (Supplementary Table 1 in [dataset] Kallscheuer and Jogler, 2021).

In the entire dataset obtained for the phylum *Planctomycetes* and among all types of BGCs responsible for the production of different compound classes annotated in antiSMASH, mixed type I PKS-non-ribosomal peptide synthetase (NRPS)-related BGCs are the only clusters found in all families of the phylum. BGCs coding for type III PKSs or enzymes involved in terpenoid and bacteriocin biosynthesis are only absent in the family *Sedimentisphaeraceae* and *Phycisphaeraceae*, respectively (Table 1). Gene clusters putatively involved in the synthesis of *N*-acyl amino acids, resorcinols, lanthipeptides, ectoine and cyanobactins have only been found in the class *Planctomycetia* so far, but not necessarily in all families belonging to different orders within this class. For BGCs involved in the production of other compound classes listed in

Table 1
Numbers of BGCs related to the production of different compound classes for the families/classes in the phylum *Planctomycetes*.

Family	Genomesizerange(Mb)	Terpenoid	TypeIPKS	TypeIIPKS	TypeIIIPKS	NRPS	TypeIPKS – NRPS	N – acylaminoacid	Thiopeptide	Other	Bacteriocin	Betalactone	Resorcinol	Arylpolyene	N – Ac – Gln – Gln – amide	Lanthipeptide	Indole	Nucleoside	Cyanobactin	RiPP – like	Phenazine	Ectoine	Ladderane	ΣClusters
Candidatus Brocadiaceae	2.9–4.8	1	0–5	0	0–1	0	0–1	0	0	0	0–2	0–1	0	0	0	0	0	0	0	0–2	0	0	2–3	3–10
Phycisphaeraceae	3.9–4.3	1–2	0	0	0–1	0	0–1	0	0	0	0	0	0	0–1	0	0	0	0	0	0	0	0	0	2–3
Sedimentisphaeraceae	3.0–3.9	0	0	0	0–2	0–3	0–1	0–2	0	0	0–1	0	0	0	0	0	0	0	0	0–1	0	0	0	0–4
Gemmataceae	4.8–12.4	2–5	1–2	0	0–1	0–3	0–1	0	0	0	0–2	0	0–1	0	0	0–1	0	0	0	0–2	0	0	0	6–13
Isosphaeraceae	6.8–10.5	2–3	1–3	0	0–1	0–2	0–2	0–1	0	0	0–2	0	0	0	0	0–1	0–1	0	0–1	0–2	0–1	0	0	5–11
Lacipirellulaceae	4.7–6.9	0–2	0–2	0	0–3	1–3	0–1	0–1	0	0–1	0–1	0–3	0–1	0–1	0–1	0	0	0	0	0	0	0–1	0	3–10
Pirellulaceae	6.2–11.0	1–3	0–3	0	0–1	1–4	0–3	0–3	0	0–1	0–3	0–1	0–1	0	0	0	0	0–1	0–1	0	0	0–1	0	5–13
Planctomycetaceae	5.2–8.9	1–4	0–4	0	0–2	0–2	0–1	0–1	0–1	0–1	0–3	0	0–1	0–1	0	0–1	0	0	0–1	0	0	0–2	0	4–12

antiSMASH, no clear distribution pattern could be observed. A few BGCs (involved in the formation of thiopeptides, *N*-acetylglutaminylglutamine amides, indoles, nucleosides, or phenazines) are even single hits or restricted to strains belonging to the same species (Table 1 and Supplementary Table 1 in [dataset] Kallscheuer and Jogler, 2021). Type II PKSs were not found in the phylum according to the results of the antiSMASH analysis.

For comparison, a complementary search for putative PKSs in the UniProt database was carried out exemplarily. After curation of the list by removal of proteins from uncharacterized strains in the phylum, 265 hits remained (Supplementary Table 2 in [dataset] Kallscheuer and Jogler, 2021), of which 128 are putative type III PKSs (typical length of 274–461 aa) and 137 putative type I PKSs (length of 516–4670 aa). These numbers only roughly match the antiSMASH results, which is not surprising when taking into account that (a) BGCs can contain more than one PKS-encoding gene, (b) the algorithm for prediction of the protein function is probably different between UniProt and the antiSMASH pipeline and (c) type I PKSs and NRPSs are structurally closely related, thus, they are difficult to differentiate based on the protein sequence. The list of putative PKSs found in the phylum is a good starting point for more detailed in silico analyses of individual enzymes with regard to their modular architectures using computational tools, e.g. NP.searcher, ASMPK or several others (Li et al., 2009; Tae et al., 2007).

2.5. Giant genes in the phylum Planctomycetes

The automated gene annotation of all genomes from characterized members of the phylum *Planctomycetes* yielded a total number of 407 giant genes (with an open reading frame >15 kb, i.e. encoding proteins >5000 aa; list curated by removal of hits from uncharacterized strains, Supplementary Table 3 in [dataset] Kallscheuer and Jogler, 2021). The highest number of 14 giant genes each was found in *Stieleria neptunia* Enr13^T and *Rhodopirellula pilleata* Pla100^T (Kallscheuer et al., 2020c; Sandargo et al., 2020) (both family *Pirellulaceae*). About one third of the strains lack genes >15 kb; these include all current members of the classes *Phycisphaerae* and *Candidatus* Brocadiae and a few members of the class *Planctomycetia*. Members of the genera *Stieleria*, *Roseimaritima* and *Rubripirellula* (all belonging to the family *Pirellulaceae*) harbour 9 or more giant genes. These numbers reflect the differences in the genome size only to a certain extent. Their correlation to the genome size turned out to be less pronounced but still significant (r^2 = 0.27, *p*-value <0.0001) compared to the number of BGCs. Genome sizes of strains lacking giant genes cover a range of 2.9–9.4 Mb.

While several giant genes in bacteria were shown to encode multimodular megasynthases, typically either type I PKSs, NRPSs or mixed type I PKS-NRPSs (Ansari et al., 2004), giant genes are not necessarily or exclusively related to secondary metabolite biosynthesis. Instead, giant genes often also code for surface proteins that are involved in cell adhesion, biofilm formation, cell-cell communication or regulatory and transport processes (Reva and Tümmler, 2008). According to the annotation in the UniProt database none of the giant genes (with >15 kb) codes for a putative PKS or NRPS (Supplementary Table 3 in [dataset] Kallscheuer and Jogler, 2021). Around 20% of the proteins encoded by giant genes (87 out of 407) are annotated as uncharacterized proteins. For many of the other proteins with a putative function, the annotation and/or domain architecture points towards a role as surface protein involved in adhesion or extracellular matrix formation or modification (e.g. cadherin-related proteins, matrixins, etc.). The presence of such proteins is in line with the tendency of planctomycetes to attach to various biotic and abiotic surfaces or to form rosettes or larger aggregates in liquid culture. Some of the giant gene-encoded proteins show similarity to components required for the assembly of cellulosomes (e.g. dockerin). Cellulosomes are extracellular protein complexes involved in the degradation of plant cell wall-derived polysaccharides, in particular cellulose (Bayer et al., 1998; Doi and Kosugi, 2004). The presence of such protein complexes in planctomycetes is consistent with their suspected ability to utilize the biomass of aquatic phototrophs as nutrient source. This hypothesis is further supported by putative polysaccharide catabolic enzymes found to be encoded by some of the giant genes (pectate lyases, pectinesterases, etc.) (Supplementary Table 3 in [dataset] Kallscheuer and Jogler, 2021). An alternative role of these structures (previously proposed as 'planctosomes') for the degradation of polypeptides has been suggested (Andrei et al., 2019). Although particularly interesting, such functions involving giant genes (cell adhesion/biofilm formation and decomposition of plant-derived polysaccharides) have no direct relation to secondary metabolite formation and are thus not further discussed at this stage. Whether the 20% of uncharacterized proteins encoded by planctomycetal giant genes are involved in secondary metabolite formation in any form remains to be elucidated. Genes coding for putative megasynthases in hitherto characterized planctomycetal strains are smaller than 15 kb, which is in line with the results obtained from antiSMASH and from the UniProt database (largest putative PKS: 4670 aa = 14.0 kb, UniProt entry A0A286TTC6; largest putative NRPS: 4844 aa = 14.5 kb, UniProt entry L0DP05). The statement that planctomycetal PKSs or NRPSs are not encoded by giant genes is clearly a question of the definition of a 'giant gene'. Unfortunately, the term is quite vague and still lacks a clear definition. For consistency with previous publications, here we used the definition 'open reading frame length >15 kb' for a giant gene, while others use e.g. >20 kb (Reva and Tümmler, 2008). Experimental evidence for a PKS- or NRPS-derived secondary metabolite produced by a planctomycete has not yet been provided. The current list of secondary metabolites identified in the phylum is quite short. Its three entries include carotenoids (three individual compounds), *N*-acylated derivatives of the aromatic amino acid L-tyrosine (six individual compounds) and a dibrominated benzoic acid (Fig. 3). The current state of research for these compounds inlcuding potential applications is summarized in the following chapters.

2.6. Carotenoid biosynthesis in planctomycetes

The biosynthesis of carotenoids as pigmenting compounds is not an isolated but also not a highly conserved feature in the phylum *Planctomycetes*. Of the total number of 125 strains described and available as axenic culture, around 50% are unpigmented, 45% show a pink to red pigmentation and 5% have a rare yellow to orange colour. No clear distribution pattern regarding strain pigmentation can be observed when taking phylogenetic relationship in the phylum into consideration. In some cases, colony colours are even inconsistent among strains belonging to the same genus. For example, *Rhodopirellula solitaria* CA85^T is currently the only unpigmented member of the genus *Rhodopirellula*, in which all other described strains have a pink to red pigmentation (Kallscheuer et al., 2019c). *Rubinisphaera brasiliensis* DSM 5305^T

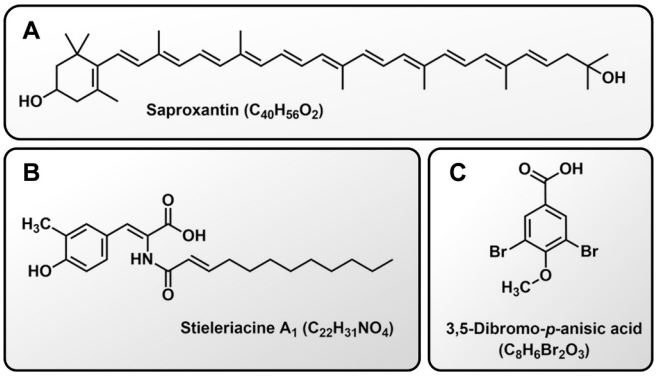

Fig. 3. Secondary metabolites identified in strains belonging to the phylum *Planctomycetes*. The structural and sum formulas for characteristic molecules in the three identified compound sets (A-C) are presented (see text for details).

(originally described as '*Plancomyces brasiliensis*') has a yellow to orange pigmentation (Schlesner, 1989), whereas its current closest relative *Rubinisphaera italica* Pan54^T lacks pigmentation (Kallscheuer et al., 2020b). Similarly, two orange strains representing the recently described species *Gimesia aquarii* differ in their pigmentation from all other described and unpigmented members of the genus *Gimesia* (Wiegand et al., 2020a). Carotenoid compounds were exemplarily analyzed in cell extracts of the pink-pigmented strain *Rubripirellula rubra* LF2^T and the orange strain *Rubinisphaera brasiliensis* Gr7, which led to the identification of two C_{40} and one C_{45} saproxanthin-type carotenoids (Kallscheuer et al., 2019b) (Fig. 3). All three compounds harbour a hydroxylated β-carotene-like cyclohexene ring at one end of the molecule, while the other end group results from simple hydration of the precursor backbone. Based on these modifications, a biosynthetic pathway from the common precursor geranylgeranyl pyrophosphate was postulated, however, gene candidates coding for the required enzymes could only be identified for the early steps up to the stage of lycopene (Kallscheuer et al., 2019b). Although the study gave a first insight into the carotenoid profile of planctomycetes, the limited number of investigated strains and identified compounds did not allow for conclusions on the metabolic basis for the different colours of the strains and also the underlying biosynthetic pathway responsible for carotenoid production remains enigmatic.

Bioinformatic analyses of planctomycetal genomes in the above-mentioned and other studies revealed that members of this phylum use the non-mevalonate pathway (also referred to as 2-*C*-methyl-D-erythritol-4-phosphate/1-deoxy-D-xylulose-5-phosphate pathway) for the production of isoprenoid units (Kallscheuer et al., 2019b; Kaushik et al., 2020), which then serve as precursors for the biosynthesis of carotenoids and other terpenoids, such as sterols and hopanoids (Rivas-Marin et al., 2019). In addition to genes coding for enzymes of the isoprenoid-forming non-mevalonate pathway, available genomes were re-analyzed for the presence of *crt* genes putatively involved in the biosynthesis of carotenoids in the course of preparing this overview article. Homologs of *crtB* encoding phytoene synthase, catalyzing the first committed step towards carotenoid biosynthesis, are present in the genomes of most planctomycetes, including those that are unpigmented. However, these results should be interpreted with caution given that phytoene synthases show a high degree of sequence similarity to squalene synthases, which catalyze a related reaction yielding the triterpene squalene ($C_{30}H_{50}$) instead of the tetraterpene phytoene ($C_{40}H_{64}$) (Summers et al., 1993). Other putative *crt* genes frequently observed in planctomycetal genomes include *crtI* (phytoene desaturase)/*carB* (*zeta*-carotene desaturase) (both involved in the conversion of phytoene to lycopene), *crtF* (demethylspheroidene *O*-methyltransferase), *crtP* (diapolycopene oxygenase) and *crtQ* (4,4′-diaponeurosporenoate glycosyltransferase). Lycopene, formed from phytoene by the activity of CrtI or CarB proteins, is an intermediate of the postulated pathway leading to the three identified carotenoids mentioned above (Kallscheuer et al., 2019b), however, all three lack chemical 'decorations' in the form of *O*-methyl or glycosyl groups. This indicates that the identified compounds are either further modified or that planctomycetes form additional carotenoids, for which CrtF, CrtP and CrtQ proteins are required. While these additional activities could not yet be attributed to metabolic pathways, the enzymes actually required for the conversion of lycopene to the identified carotenoids could not yet be identified using state-of-the-art bioinformatical methods. The involved enzymes might structurally differ from 'canonical' enzymes and thus escape the in silico analysis. In this context, the filamentous fungus *Phycomyces blakesleeanus* was for example shown to harbour the bifunctional protein CarRA, in which separate protein domains are responsible for phytoene synthase and lycopene cyclase activity (Arrach et al., 2001). Based on this observation, we analyzed planctomycetal phytoene synthase candidates (CrtB proteins) for a similar domain architecture, but found out that these protein are much smaller than CarRA (planctomycetal CrtB proteins: 290–340 aa; CarRA: 602 aa) and lack the additional lycopene cyclase domain. Hence, it appears unlikely that planctomycetes use bifunctional enzymes for catalysis of these two steps during carotenoid biosynthesis.

Recently, the genetic basis for carotenoid biosynthesis has been experimentally studied in the limnic model planctomycete *Planctopirus limnophila*. An individual transposon insertion-based inactivation of the genes Plim_2742 (NCBI accession number WP_013110996.1, annotated as phytoene desaturase) and Plim_3204 (NCBI accession number WP_052301613.1, annotated as FAD-dependent oxidoreductase) abolished the red pigmentation of the strain (Santana-Molina et al., 2020). This indicates that both proteins are involved in the first two steps of carotenoid biosynthesis, namely conversion of two molecules of geranylgeranyl pyrophosphate to phytoene and subsequent conversion of phytoene to lycopene. The relevance for these early steps is highly likely given that a catalytic role at later stages of the biosynthesis pathway would still allow for the formation of the bright red lycopene. The additional information that the putative phytoene synthase in *P. limnophila* is encoded by Plim_2244 suggests a role of both inactivated genes in the second reaction step, the conversion of phytoene to lycopene. This assumption is in line with the putative annotation of the two genes as well as the fact that the overall reaction consists of four subsequent oxidation reactions that can by catalyzed by separate enzymes in different organisms (Iniesta et al., 2007; Moise et al., 2014).

Closely related planctomycete strains differing in their pigmentation as well as the obtained carotenoid biosynthesis-deficient transposon insertion mutants of *P. limnophila* are a promising starting point to analyze the natural function of carotenoids in planctomycetes, in particular when considering that they have been shown to be non-essential for survival under laboratory-scale cultivation conditions. In addition to the protection against UV radiation and oxidative stress, carotenoids can for example also play a role in membrane stabilization and maintenance of membrane fluidity (Britton, 1995; Rizk et al., 2020; Santana-Molina et al., 2020; Stahl and Sies, 2002).

2.7. Stieleriacines, novel compounds in the class of N-acyl amino acids

Novel compounds belonging to the class of *N*-acyl amino acids have been identified in the planctomycete *Stieleria maiorica* Mal15^T (family *Pirellulaceae*), which were named stieleriacines based on the name of the genus to which the producer strain belongs (Kallscheuer et al., 2020a). Stieleriacine A_1, the major stieleriacine in *S. maiorica* Mal15^T, is composed of a *meta*-*C*-methylated 2,3-dehydrotyrosine residue representing the amino acid part, which is ligated to the mono-unsatured C_{12} fatty acid *trans*-2-dodecenoic acid (Fig. 3). Ligation of the carboxy group of the fatty acid to the amino group of the tyrosine derivative yields a carboxamide function placing the compound in the group of *N*-acylated amino acids. Closely related stieleriacines differing in the saturation state of the fatty acid and/or the tyrosine derivative as well as corresponding *Z*/*E*-stereoisomers were also identified in cell extracts of *S. maiorica* Mal15^T and were named stieleriacines A_2, B_1, B_2 and C (Kallscheuer et al., 2020a). The molecular structure of stieleriacines suggested that they are produced by dedicated long-chain *N*-acyl amino acid synthases (often designated NasY proteins). Biosynthetic gene clusters for *N*-acyl amino acids were implemented into the antiSMASH database only recently and were not available at the time of publication of the original study in 2020. For this reason, the putative biosynthetic gene cluster in *S. maiorica* escaped the automated prediction and was identified by manual analysis of the genome sequence and protein annotation. The suspected biosynthetic gene cluster harbours a gene coding for a putative tyrosine *C*-methyltransferase and altogether four putative *nasY* genes. The exact function of the individual NasY proteins still remains to be elucidated and they may well be involved in the production of additional *N*-acyl amino acids in *S. maiorica*. A biosynthetic pathway for stieleriacines was then postulated based on the genes encoded in the cluster (Kallscheuer et al., 2020a). *Stieleria neptunia* Enr13^T, a close relative of *S. maiorica*, was shown to produce two related

compounds designated stieleriacines D and E (Sandargo et al., 2020). These harbour an *O*-methylation in *para*-position of the aromatic ring in the tyrosine moiety instead of a *meta*-stable *C*-methylation and either stearic acid (octadecanoic acid) or oleic acid (*cis*-9-octadecenoic acid) as the acyl residue. In the genome of *S. neptunia* Enr13^T three putative *nasY* genes were found to be encoded in separate clusters. One of these clusters resembles the putative stieleriacine cluster in *S. maiorica* and is thus the best candidate for relevance during biosynthesis of stieleriacines D and E in *S. neptunia* (Sandargo et al., 2020). Similar to *S. maiorica*, the presence of additional *nasY* genes indicates the synthesis of other *N*-acyl amino acids also in *S. neptunia*. Astonishingly, the NasY proteins encoded in the identified clusters in *S. maiorica* and *S. neptunia* only have a maximal sequence identity of 20% on the protein level, which might also reflect the structural differences between the stieleriacines found in the two species. *Stieleria varia* Pla52n^T, the third described species of the genus (Surup et al., 2020), appears to lack *nasY* genes according to the antiSMASH results and BLASTp against the putative NasY proteins in the closest relatives. This indicates that the formation of stieleriacines or other *N*-acyl amino acids is not a conserved feature of the genus. Results of the antiSMASH analysis for the entire phylum showed that the majority of *N*-acyl amino acid BGCs is present in the family *Pirellulaceae*, in particular in the genus *Stieleria* and the currently closest related genera *Rubripirellula*, *Novipirellula* and *Rhodopirellula* (Supplementary Table 1 in [dataset] Kallscheuer and Jogler, 2021).

In cultivation experiments with *S. maiorica*, supplementation of stieleriacine A_1 led to shortening of the lag phase compared to a negative control condition with absence of stieleriacine A_1, while this compound had no influence on the duration of the division phase (Kallscheuer et al., 2020a). In biofilm formation assays with bacterial competitors probably naturally co-occurring with *S. maiorica*, different effects were observed for two tested bacterial species of the '*Roseobacter* group' (Kallscheuer et al., 2020a). Stieleriacine A_1 improved the biofilm formation capacity of *Phaeobacter inhibens*, while it reduced the biofilm formation of *Sulfitobacter dubius*. The observed stimulating effect in case of *P. inhibens* was largely independent of its quorum sensing system. The fact that *P. inhibens* is a natural producer of the antibiotic tropodithietic acid (TDA) led to the working hypothesis that *S. maiorica* might benefit from formation of stieleriacines and the resulting increased biofilm formation of *P. inhibens* in multiple ways: (a) stieleriacine formation shortens the lag phase, which can be decisive for invasion of existing biofilms or in the case that nutrients suddenly become available; (b) *S. maiorica* turned out to be naturally resistant against TDA, while several other bacterial competitors are not; and (c) increased formation of TDA by *P. inhibens* increases the metabolic burden, in turn reducing the growth speed of this competitor. At this stage, it should be taken into account that *S. maiorica* divides much slower than *P. inhibens*; under laboratory-scale cultivation conditions *S. maiorica* reaches a maximal growth rate of 0.093 h^{-1} (generation time of 7.5 h), while *P. inhibens* reaches 0.43 h^{-1} (generation time of 1.6 h) (Kallscheuer et al., 2020a; Martens et al., 2006). Such values do not necessarily reflect the natural conditions, under which nutrient levels are typically limiting and carbon needs to be obtained from polymeric compounds that are more difficult to degrade. Still, stieleriacines are likely one of the strategies followed to compensate for slower growth, they might even modulate community compositions of marine biofilms inhabited by *S. maiorica* or other planctomycetes.

More detailed studies are required to verify the postulated stieleriacine biosynthetic pathway and the potential mode of action of stieleriacines. Such information is also rare for *N*-acyl amino acids structurally related to stieleriacines identified in other microorganisms (Deering et al., 2016; MacIntyre et al., 2019). BGCs for long-chain *N*-acyl amino acids are often found adjacent to genes related to the PEP/CTERM exosortase system proposed to be relevant for sorting of secreted cell surface-associated proteins involved in adhesion processes or in the formation of exopolysaccharides or other extracellular polymeric substances (EPS) (Craig et al., 2011; Haft et al., 2006). A similar functional link also appears to exist in *S. maiorica* and *S. neptunia* given that several PEP/CTERM domain-containing proteins and EPS biosynthesis-related proteins (EpsD, EpsH, EpsI, etc.) are encoded in close proximity to the stieleriacine biosynthetic clusters in both genomes (Kallscheuer et al., 2020a; Sandargo et al., 2020). The potential link of stieleriacines to EPS synthesis is in line with the observed effects during initiation of (attached) growth and biofilm formation, definitely requiring further attention in the future.

2.8. The aryl halide 3,5-dibromo-p-anisic-acid

The strain "Planctomycetales bacterium 10988", a yet undescribed planctomycete likely belonging to a novel family in the order *Pirellulales* (and not *Planctomycetales* as the current name suggests), was shown to produce the aryl halide compound 3,5-dibromo-*p*-anisic acid (Panter et al., 2019). Chemically, this compound is a 4-hydroxybenzoic acid derivative brominated in both *meta*-positions of the aromatic ring (relative to the carboxyl group) and additionally *O*-methylated at the phenolic *para*-hydroxy group. Based on these modification steps, a putative biosynthetic pathway was proposed and the genome was analyzed for genes coding for enzymes potentially involved in the biosynthesis (Panter et al., 2019). The analysis yielded a gene encoding a putative flavin-dependent halogenase (BaaB, locus PBC10988_38600, UniProt entry A0A5Q3LFM1) as the best candidate for catalyzing the bromination reactions. The gene immediately downstream (PBC10988_38610, UniProt entry A0A5Q3LCP4) codes for a putative chorismate-pyruvate lyase (BaaA), which catalyzes the removal of pyruvate from the shikimate pathway intermediate chorismate to yield 4-hydroxybenzoate (Panter et al., 2019). The respective enzyme UbiC from *Escherichia coli* was shown to catalyze the first step in the ubiquinone biosynthesis pathway (Nichols and Green, 1992; Siebert et al., 1994). Although similar in length (180 vs. 165 aa), both proteins only share a sequence identity of 16% (similarity of 45%). A putative *O*-methyltransferase-encoding gene required for the last reaction step is not present in close proximity to the two identified genes. Nevertheless, the assumption that the two above-mentioned genes are relevant for 3,5-dibromo-*p*-anisic acid biosynthesis is justified when additionally taking into account that (a) no other genes related to ubiquinone biosynthesis are encoded in proximity to the two genes in the genome of strain 10988, (b) most planctomycetes produce menaquinones as the major respiratory quinones instead of ubiquinones (Ward, 2010), (c) menaquinone-biosynthesis related genes (*men* and *mqn* genes) are present in strain 10988 and localized elsewhere in the genome, (d) the brominated product was formed in an axenic culture, hence requiring de novo 4-hydroxybenzoic acid biosynthesis, and (e) a monohydroxylated and not otherwise substituted benzoic acid is very unlikely be produced by a polyketide synthase.

Cytotoxicity assays with 3,5-dibromo-*p*-anisic acid isolated from strain 10988 showed that this compound has no considerable antimicrobial activity and only low cytotoxic activity against a human carcinoma cell line. In contrast, moderate herbicidal activity was observed, indicating that it might naturally function as an algicide. This function is in line with the observed abundance of planctomycetes on macroalgae and their assumed role in the degradation of algal biomass for the sake of utilizing released compounds as a nutrient source (Erbilgin et al., 2014; Lage and Bondoso, 2014; Wiegand et al., 2018). 3,5-Dibromo-*p*-anisic acid (synonym: 3,5-dibromo-4-methoxybenzoic acid) was already isolated previously from the sponges *Amphimedon* sp. and *Psammaplysilla purpurea* (Campos et al., 2017; Venkateswarlu and Chavakula, 1995). The compound was also reported in the context of transgenic crops, in which resistance against the herbicide bromoxynitril (3,5-dibromo-4-hydroxybenzonitrile) was achieved by the heterologous expression of a nitrilase-encoding gene from *Klebsiella pneumoniae* subsp. *ozaenae* (Duke and Cerdeira, 2005). Nitrilase activity led to the conversion of bromoxynitril to 3,5-dibromo-4-methoxybenzoic acid. Astonishingly, the

latter is described as non-phytotoxic benzoic acid derivate, which somehow contradicts the results obtained in strain 10988, however, the statement might refer to a comparsion with the strong herbicide bromoxynitril.

Unfortunately, the two studies focusing on the sponge-derived metabolites focused only on the structure elucidation and present no information related to the natural function or biosynthesis of 3,5-dibromo-4-methoxybenzoic acid. At this stage, the postulated pathway and the putative gene cluster in strain 10988 are a promising basis to elucidate the role of this compound in more detail in future studies. It should also not be excluded that 3,5-dibromo-*p*-anisic acid is an early precursor of a more complex compound or that structurally related compounds are produced. Both theories might also provide an explanation why the gene coding for the *O*-methyltransferase is not part of the putative BGC.

2.9. Biotechnological potential of planctomycetes directly or indirectly related to natural product formation

Based on the complex lifestyle and specialized metabolic traits of planctomycetes, strains belonging to the phylum and/or natural compounds already identified to be produced by its members are directly linked to technical or biotechnological applications. As already mentioned earlier, members of the class *Candidatus* Brocadiae are capable of performing anaerobic ammonium oxidation ('anammox') (Kuenen, 2008). The 'anammox' process comprises the oxidation of ammonium (NH_4^+) to dinitrogen gas (N_2) using nitrite (NO_2^-) as electron acceptor and takes place in intracytoplasmic compartments ('anammoxosomes'), which are surrounded by ladderane lipid-containing membranes (Fernández et al., 2020; van Niftrik et al., 2004). It has been estimated that the 'anammox' reaction is responsible for 50% of the emitted dinitrogen gas from the oceans (Lam and Kuypers, 2011). This said, strains of the class *Candidatus* Brocadiae largely contribute to the global nitrogen cycle (Kuypers et al., 2003). The 'anammox' process is technically exploited for *N*-elimination during wastewater treatment (Peeters and van Niftrik, 2019). While the 'anammox' reaction is restricted to members of the class *Candidatus* Brocadiae (which harbour the ladderane-producing BGCs), other planctomycetes are suggested to rather contribute to the global carbon cycle. A striking association between planctomycetes and marine and limnic phototrophs has been observed (Bondoso et al., 2014; Kallscheuer et al., 2021; Lage and Bondoso, 2014). Planctomycetes can even account for 70–85% of the bacterial community on such biotic surfaces, e.g. of kelp or seagrasses (Kohn et al., 2020; Wiegand et al., 2018). The observed phototroph-planctomycete allelopathic interaction is likely orchestrated involving secondary metabolites and includes the ability of planctomycetes to degrade high-molecular weight polysaccharides released by the phototroph. This assumption is supported by (a) high numbers of carbohydrate-active enzymes encoded in planctomycetal genomes (Dabin et al., 2008; Wegner et al., 2013), (b) a postulated specialized machinery for the internalization of entire polysaccharide molecules (Boedeker et al., 2017), (c) the confirmed polysaccharide utilization pattern of model strains isolated from sea- and freshwater (Jeske et al., 2013), (d) the formation of potential plant toxins by planctomycetes (Panter et al., 2019) and (e) presence of genes coding for enzymes involved in the formation of cellulosome-like complexes (see section on giant genes). Bacterial strains capable of using plant-derived polysaccharides as carbon and energy source are of primary biotechnological interest as hosts for a sustainable production of chemical building blocks. Such bacteria receive significant attention, in particular in the current transition phase from 1st generation feedstocks (e.g. processed monosaccharides from wheat, corn, sugar cane, etc.) to 2nd generation feedstocks (e.g. cellulose and lignocellulose from wood waste, maize straw or bagasse) (Arevalo-Gallegos et al., 2017; Ayodele et al., 2020). The current shift towards the preferential use of 2nd generation feedstocks is driven by the rising world population and the resulting need to use 1st generation feedstocks in the food and feed industry instead of using them for the biotechnological production of chemical building blocks (Chen et al., 2017). Planctomycetes are potential candidates for establishing such production pipelines, in particular in the light that aquatic weeds are also included in the list of such next generation feedstocks (Kaur et al., 2018). As an alternative approach, respective genes associated with such 'catabolic functionalities' in planctomycetes can be transferred to well-established industrial production hosts such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Corynebacterium glutamicum* or others. This strategy opens up the possibility of using aquatic plant-derived polysaccharides (e.g. laminarin, alginates, carageenans, ulvan, fucoidan, etc.) as substrates for production purposes (Hehemann et al., 2014).

The antiSMASH analysis of planctomycetal genomes yielded several BGCs related to compound classes with potential antibiotic activities. Such activities are e.g. well-known for natural products synthesized by PKSs or NRPSs (Miyanaga, 2017; Schwarzer et al., 2003). The research on secondary metabolite production in planctomycetes is still at a very early stage and so far, no polyketide- or non-ribosomal peptide-derived molecule has been identified in a planctomycete. A previous analysis of planctomycetal BGCs obtained in a genome mining approach could show that these BGCs do not cluster with most of the BGCs belonging to known molecules listed in the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database (Wiegand et al., 2020b). This is a strong hint that planctomycetes represent a promising and yet for the most part untapped source of novel polyketides, non-ribosomal peptides or other molecules with potential health-promoting activities (Graça et al., 2016; Jeske et al., 2013). Such activities have already been observed in planctomycetal extracts (Calisto et al., 2019), however, have not been traced back to individual compounds yet.

Until today, three (sets of) compounds, namely C_{40} and C_{45} carotenoids, stieleriacines and the aryl halide 3,5-dibromo-*p*-anisic acid, have been identified in planctomycetes (Fig. 3). All three were published in the last two years (Kallscheuer et al., 2020a; Kallscheuer et al., 2019b; Panter et al., 2019; Sandargo et al., 2020) and have potential technical applications. Several planctomycetes show a strong pink to red pigmentation, for which deletion studies could confirm that it is caused by formation of carotenoids (Santana-Molina et al., 2020). Carotenoids are used as colorants in food applications or as feed additives, e.g. in aquaculture (Kallscheuer, 2018; Pasarin and Rovinaru, 2018; Sigurdson et al., 2017). Further characterization of the natural function of these pigments in planctomycetes requires an identification of the enzymes involved in their biosynthesis. State-of-the-art bioinformatic methods were used for comparison with enzymes known to catalyze the required reaction steps, however no hits were obtained for the proteomes of pigmented planctomycetes so far (Kallscheuer et al., 2019b). The genetic basis for carotenoid biosynthesis is the starting point for conducting further gene deletion and complementation studies and also provides the possibility to transfer the genes to heterologous bacteria that were already engineered for carotenoid overproduction, e.g. *C. glutamicum* (Henke et al., 2016). In *C. glutamicum*, expression of the genes coding for carotenoid biosynthetic enzymes was shown to be induced in a light-dependent manner (Sumi et al., 2019), whereas for planctomycetes no changes in the pigmentation were observed no matter if they were cultivated in the light or in the dark. This might point towards an additional function of the non-essential carotenoids in planctomycetes in addition or alternatively to the common photoprotective function of these pigments. The pink pigmented planctomycete *Rhodopirellula rubra* was tested as a supplementary food source for the water flea *Daphnia magna* (da Conceição Marinho et al., 2019). After uptake of the bacterial cells, the planctomycetal carotenoid pigments were retained and passed on to the next *D. magna* generation. It could be shown that *R. rubra*, when used as a supplementary food source, significantly improved life history parameters of *D. magna*, however it is not yet clear if the pigments played a role for the observed positive effect (da Conceição Marinho et al., 2019).

Stieleriacines, novel compounds in the class of *N*-acylated tyrosines,

were identified in two species of the genus *Stieleria* (Kallscheuer et al., 2020a; Sandargo et al., 2020). The biosynthesis pathway was postulated based on the presence of genes coding for the key enzyme *N*-acyl amino acid synthase (NAS); with the cluster in *S. maiorica* Mal15^T harboring four of such NAS-encoding genes (Kallscheuer et al., 2020a). The clusters in both species escaped the antiSMASH prediction at the time of the initial analysis of the genomes, however, *N*-acyl amino acid biosynthesis-related BGCs were implemented into antiSMASH recently (Blin et al., 2019). Stieleriacines might have regulatory functions related to biofilm formation. A quorum-sensing-like activity could not yet be investigated due to the lack of possible target genes as well as of a deletion mutant incapable of stieleriacine biosynthesis functioning as negative control (Kallscheuer et al., 2020a). *N*-acyl tyrosines can be technically applied as natural surfactants replacing their synthetic counterparts produced from petroleum-based chemicals (Joondan et al., 2017). *N*-acyl amino acid surfactants have a lower toxicity, are hypoallergenic and show a higher biodegradability compared to synthetic compounds. Remarkably, of the different fatty acid chain lengths tested, C_{12}, as present in stieleriacines A-C, showed the optimal antibacterial activity. Similarly, the oleoyl-derivative ($C_{18:1}\omega 9cis$), as present in stieleriacine D, showed a higher antibacterial activity than the saturated derivative (Joondan et al., 2017). In view of the amphiphilic properties of *N*-acyl amino acids, the similarity of the technical application as a surfactant and the natural function cannot be a coincidence. In biofilm formation assays, stieleriacine A_1 reduced the biofilm formation capacity of one of two tested competitors of the producer strain, while it increased biofilm formation of the other competitor (Kallscheuer et al., 2020a). These results substantiate the assumed natural function of stieleriacines as surface-active compounds and biophysical modulators of surfaces and biofilms. When taking into account that stieleriacines are postulated to be produced starting from the ubiquitous precursor molecules L-tyrosine and an activated fatty acid, the respective BGC is well-suited for heterologous expression experiments in a well-established microbial host.

The aryl halide 3,5-dibromo-*p*-anisic acid potentially acts as a plant toxin and was postulated to be produced by a three-step pathway starting from the shikimate pathway intermediate chorismate (Panter et al., 2019). Two adjacent genes coding for a putative 4-hydroxybenzoate-forming chorismate pyruvate lyase and a putative flavin-dependent halogenase are in line with the identified molecule structure, while the required *O*-methyltransferase-encoding gene is probably encoded elsewhere in the genome of the producer strain 10988. The distribution of the biosynthesis genes might suggest that either the *O*-methylation is a common and rather unspecific mechanism of strain 10988 to improve resistance of compounds against degradation or to improve hydrophobicity or that the identified compound is a pathway intermediate during synthesis of a more complex compound. Unexpectedly, the plant toxin was produced in absence of an external stimulus, e.g. presence of an alga or a compound secreted by algae. De novo production of the brominated compound was confirmed based on the finding that production occurred in an axenic culture of strain 10,988. In constrast, *P. inhibens*, a member of the '*Roseobacter* group' was e.g. shown to produce the plant toxin roseochelin B from the phenylpropanoid precursor sinapic acid that is released from algae (Wang and Seyedsayamdost, 2017). This strategy ensures that the plant toxin is only produced when the algal counterpart is actually physically present. In case of strain 10988, the trigger that induces production of 3,5-dibromo-*p*-anisic acid, if any, as well as the mode of action of the compound, remain to be elucidated. A reconstruction of the suspected biosynthetic pathway in a heterologous host appears feasible. Depending on the substrate specificity of the brominase, the *O*-methylation reaction can possibly be circumvented by supplementing *p*-anisic acid as a precursor. Potential technical applications of 3,5-dibromo-*p*-anisic acid are difficult to determine without additional information on the mode of action of this compounds. In a proof of concept study, five structurally related compounds were tested as potential algicides for waterborne paints (de Saravia et al., 2018). Suitable compounds should be able to prevent formation of algal biofilms on painted walls without the need for traditional and potentially toxic biocides. The tested compounds anisol, guaiacol, eugenol and thymol are plant-derived hydroxylated aromatic (= phenolic) compounds additionally harboring *C*- oder *O*-methyl groups. Walls painted with samples containing 2% guaiacol, eugenol or thymol were not colonized by algae (de Saravia et al., 2018). Except for the lack of bromination, all four tested compounds have a certain structural relationship to 3,5-dibromo-*p*-anisic acid. In an unrelated study, the *O*-methylated phenylpropanoid *trans*-ferulic acid specifically inhibited *Oscillatoria perornata* down to a concentration of 1 μM (Schrader, 2003). In case that 3,5-dibromo-*p*-anisic acid will be available in larger amounts in the future, either by chemical total synthesis of via biosynthesis in an engineered bacterial host, a related application of this compound could be conceivable.

2.10. The current 'knowledge gap' and suitable strategies to identify novel secondary metabolites from planctomycetes and beyond

The antiSMASH analysis yielded up to 13 BGCs associated to secondary metabolite production in the genomes of so far characterized planctomycete strains, whereas more than 50 BGCs are typically obtained e.g. for *Streptomycetes* or *Myxobacteria*. This gap cannot be explained by differences in the genome sizes given that at least member of the class *Planctomycetia* have comparably large genomes as strains harboring up to four times higher numbers of BGCs. Several 'hidden' planctomycetal BGCs are probably beyond what can be predicted using computational tools or protein sequence-based comparison. This notion is not only supported by the finding that planctomycetal BGCs do not cluster together with well-investigated BGCs (Wiegand et al., 2020b), but also by the fact that postulated pathways for identified compounds in planctomycetes cannot be traced back to BGCs. While BGCs involved in *N*-acyl amino acid biosynthesis were recently implemented into antiSMASH (Blin et al., 2019), genes putatively related to carotenoid and aryl halide production escaped the prediction algorithm and were either proposed by extensive manual analysis or still need to be discovered. This is particularly surprising for carotenoids, as these antioxidant compounds are common in nature and well-studied, both in terms of gene clusters and biosynthetic pathways.

In the light of the current state of research with only three identified compound sets, two experimental strategies appear to be purposeful to reveal the expected and yet for the most part untapped pool of secondary metabolites in planctomycetes. The most obvious strategy includes cultivation of different strains, followed by solvent-based extraction of cultures and mass spectrometrical analysis of obtained concentrated extracts combined with fractionation, isolation and structure elucidation. This strategy was followed in case of all three identified sets of compounds (Kallscheuer et al., 2020a; Kallscheuer et al., 2019b; Panter et al., 2019), however, it is limited to compounds that are produced in sufficient amounts during laboratory-scale cultivation in optimized media. Hence, this strategy appears unsuitable for the identification of compounds, for which production either requires an external stimulus (e.g. a specific metabolite acting as inducer of gene expression in a BGC that is otherwise silent) or that is based on a foreign compound acting as precursor of the biosynthetic pathway. Co-cultures of planctomycete strains and suspected bacterial competitors triggering the activation of planctomycetal defense mechanisms are often challenging in the light that planctomycetes grow slowly, reach low optical densities and require a special medium formulation, while they typically fail to grow in established complex or defined media (such as LB, BHI (brain heart infusion), M9 or YNB (yeast nitrogen base) medium). The decisive advantage of the metabolite extraction-based strategy is that it can yield novel compounds that either escaped a genome-based prediction and are thus not accessible for heterologous gene expression experiments or that require specific precursor molecules that are not present in commonly used heterologous microbial hosts. The same is also true for biosynthetic

pathways involving enzymes that (a) require posttranslational activation by dedicated auxiliary enzymes, (b) harbour uncommon cofactors or use uncommon cosubtrates or (c) that catalyze redox reaction with separate enzymes acting as electron donors or acceptors.

Single or multiple genes being part of identified BGCs are promising targets for the functional introduction of planctomycetal pathways or for the characterization of individual reaction steps in heterologous microbial host strains. Until today, planctomycetes can for the most part not be used for this purpose since genetic tools have only been developed for a very small number of model strains (e.g. the limnic strain *P. limnophila*) (Jeske et al., 2016; Jogler et al., 2011; Rivas-Marín et al., 2016). Currently, there is also no replicative plasmid available for planctomycetes, hence, native or heterologous genes need to be stably integrated into the genome. Further limitations result from the little information on activity of constitutive and inducible promoters and architecture of polycistronic operons in planctomycetes, while also the range of selection markers is restricted when considering that planctomycetes are naturally resistant to several of the commonly used antibiotics (Cayrou et al., 2010; Godinho et al., 2019). A molecular toolbox allowing the introduction of genetic modifications or an engineered overexpression of native genes in members of the phylum is an important prerequisite towards establishing planctomycetes as small molecule producers. Depending on the complexity of the involved pathway or in case that specialized precursors are required, planctomycetes themselves are required as production platform instead of well-established heterologous hosts. An investigation of the natural function of identified small molecules requires access to respective gene inactivation mutants, in which individual small molecule biosynthesis pathways are inactivated. Research projects focusing on the construction of molecular biological tools will benefit from the large number of novel strains that have been characterized in the recent years. In particular members of the family *Isosphaeraceae* are an excellent basis for the construction of expression plasmids given that strains in this family typically harbour multiple extrachromosomal elements. A maximum of five plasmids has, for example, been observed in *Tautonia plasticadhaerens* ElP^{T} (Jogler et al., 2020). As soon as an expression plasmid is established, different promoter/regulator (and inducer) combinations of commonly used expression systems can be tested, for example *lacI*/$P_{lac/tac}$ (IPTG), *tetR*/P_{tet} (anhydrotetracycline), *araC*/P_{araBAD} (arabinose), etc. The introduction of genomic modifications currently relies on recombination by double crossing-over events and can be used for the inactivation of genes or operons as well as for gene introduction (both with the simultaneous insertion of a kanamycin resistance gene for selection purposes). Other systems that include recycling of the selection marker by a two-step recombination protocol (i.e. insertion and subsequent excision of the resistance maker cassette) and CRISPR/Cas-based methods might be applicable for members of the phylum as well, however, respective protocols have not yet been developed and optimized for planctomycetes.

In the light of the current lack of such a molecular toolbox for planctomycetes, for the time being well-characterized microbial hosts appear to be more suitable for the expression of planctomycetal genes. Since many of the suspected secondary metabolites in planctomycetes have potential antibacterial activities, the list of hosts should include Gram-negative and Gram-positive bacteria (e.g. *E. coli* and *C. glutamicum*, respectively), but also eukaryotic hosts such as yeasts (e.g. *S. cerevisiae*). The functional expression of genes coding for putative type I PKSs or NRPSs requires specific host strains that harbour broad range phosphopantetheinyl transferases (PPTases). These auxiliary enzymes are crucial for the posttranslational activation of *apo*-PKSs or *apo*-NRPSs to the respective *holo* form (Beld et al., 2014). Specific hosts applicable for an expression of PKS- or NRPS-encoding genes include e.g. actinobacterial species such as *Streptomyces coelicolor* or *C. glutamicum* (Gomez-Escribano and Bibb, 2011; Kallscheuer et al., 2019a). The latter was additionally engineered for an increased supply of malonyl-CoA, for which a low natural metabolite pool is a well-known bottleneck during (engineered) polyketide production (Milke et al., 2019; Milke and Marienhagen, 2020). Both species also naturally produce methylmalonyl-CoA, which is used as an alternative chain elongation unit by some PKSs (Botella et al., 2009; Wu et al., 2005) and which is not naturally synthesized by *E. coli* (Dayem et al., 2002). A functional introduction of large multimodular type I PKS or NRPS enzymes often requires additional optimization in order to improve protein folding and solubility, e.g. by fine-tuning of the expression rate, translational coupling to other proteins as fusion partner (e.g. the maltose-binding protein) or co-expression of chaperone-encoding genes (Kallscheuer et al., 2019a; Skiba et al., 2018).

For further investigating the diversity of secondary metabolites in the phylum *Planctomycetes* and in view of the complementary character, both discussed strategies should urgently be followed in parallel. The isolated information on the structure of novel compounds found in culture extracts does not necessarily allow for conclusions regarding the underlying gene cluster responsible for production, in particular when taking into account that planctomycetes might also follow non-canonical pathways for secondary metabolite production (at this stage, it should also be remembered that even genes for well-characterized compounds could not be identified using state-of-the-art methods). On the other hand, heterologous expression approaches can currently only cover BGCs that were predicted using bioinformatic tools and genome mining algorithms such as antiSMASH. Relying only on this strategy would imply to entirely ignore compounds produced by pathways that are not yet implemented in prediction tools and can thus not be captured by the currently used algorithms.

When taking into account that between 35 and 65% of the genes encoded in planctomycetal genomes are of unknown function, it is reasonable to assume that several of them are involved in biosynthetic pathways for novel natural products. These complete the currently suspected portfolio of natural compounds produced by more frequent BGCs found in more than three families (related to production of terpenoids, polyketides, non-ribosomal peptides, bacteriocins and resorcinols) and by rare BGCs in the phylum *Planctomycetes* (related to synthesis of thiopetides, betalactones, arylpolyenes, lanthipeptides, phenazines, etc.).

3. Conclusions

Given the trillions of years that bacteria on earth struggle for life by committing chemical warfare against each other, it is no surprise that several compounds with health-promoting activities including about two third of clinically relevant antibiotics are actually produced by bacteria. However, they all originate from less than 1% of the known bacterial species out there. Thus, circumventing the high rediscovery rates by exploring the unresearched 99% of species sounds trivial. However, many of these species were thought to be impossible to cultivate – the 'microbial dark matter'. The endeavor to make little studied phyla accessible for the identification of bioactive compounds might already take 5–10 years. Establishing a huge and diverse library of natural small molecules might be the best way to find such urgently needed bioactivities. The identification of the first small molecules in the phylum *Planctomycetes* in the last two years together with observed bioactivities in whole-cell extracts (Calisto et al., 2019) emphasize the role of the phylum as promising source of bioactive natural products. Similar to other well-explored phyla, *Planctomycetes* can function as a blueprint, motivating to unearth novel 'talented' producers in under-explored bacterial phyla.

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I) All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

II) This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

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