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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

# Specificities and commonalities of the Planctomycetes plasmidome

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## Abstract

Plasmids, despite their critical role in antibiotic resistance and modern biotechnology, are understood in only a few bacterial groups in terms of their natural ecological dynamics. The bacterial phylum Planctomycetes, known for its unique molecular and cellular biology, has a largely unexplored plasmidome. This study offers a thorough exploration of the diversity of natural plasmids within Planctomycetes, which could serve as a foundation for developing various genetic research tools for this phylum. Planctomycetes plasmids encode a broad range of biological functions and appear to have coevolved significantly with their host chromosomes, sharing many homologues. Recent transfer events of insertion sequences between cohabiting chromosomes and plasmids were also observed. Interestingly, 64% of plasmid genes are distantly related to either chromosomally encoded genes or have homologues in plasmids from other bacterial groups. The planctomycetal plasmidome is composed of 36% exclusive proteins. Most planctomycetal plasmids encode a replication initiation protein from the Replication Protein A family near a putative iteron-containing replication origin, as well as active type I partition systems. The identification of one conjugative and three mobilizable plasmids suggests the occurrence of horizontal gene transfer via conjugation within this phylum. This comprehensive description enhances our understanding of the plasmidome of Planctomycetes and its potential implications in antibiotic resistance and biotechnology.

## INTRODUCTION

Most of our current knowledge of the bacterial world is largely based on model organisms from a restricted subset of phyla, mostly Proteobacteria and Firmicutes. Within this paradigm, notable exceptions have emerged, showcasing a rich tapestry of evolutionary adaptations. The phylum Planctomycetes stands out as one of the most enigmatic branches within the bacterial evolutionary tree. Planctomycetes exhibit distinctive cellular biology replete with attributes seldom encountered in the bacterial domain, thereby piquing scientific interest due to their potential and controversial relevance to eukaryogenesis (Devos, 2021; Forterre, 2011). Intriguingly, they manifest an atypical cellular configuration characterized by extensive invaginations of the cytoplasmic membrane, forming a complex endomembrane system (Acehan et al., 2013; Devos, 2014; Forterre, 2011; Santarella-Mellwig et al., 2013), previously mistaken as cell compartmentalization (Lindsay et al., 2001). Some of the cells display a phenotype similar to the typically eukaryotic characteristics of endo- and phagocytosis (Lonhienne et al., 2010; Shiratori et al., 2019). Furthermore, they encode homologues to several eukaryotic proteins (Arcas et al., 2013; Jenkins et al., 2002; Makarova & Koonin, 2010; Pearson et al., 2003; Santana-Molina et al., 2020; Santarella-Mellwig et al., 2010; Shiratori

et al., 2019). But beyond their distinctive cell attributes, Planctomycetes are recognized as pivotal contributors to global carbon and nitrogen cycles, owing to their metabolic proficiency in the degradation of complex carbon substrates (Jeske et al., 2013; Wiegand et al., 2018), and their unique ability to combine ammonium and nitrite or nitrate to form nitrogen gas (Kuenen, 2008; Strous et al., 1999). Moreover, their genomic repertoire encompasses genes associated with secondary metabolite pathways, which are implicated in the production of bioactive compounds, including antimicrobial and algicidal agents (Graça et al., 2016; Jeske et al., 2013, 2016; Vitorino et al., 2024).

Metagenomic data indicated the ubiquity of Planctomycetes in diverse ecosystems (reviewed by Wiegand et al., 2018), encompassing soil (Buckley et al., 2006; Ivanova et al., 2016; Stackebrandt et al., 1993; Wang et al., 2002), plant rhizosphere (Lei et al., 2023; Ravinath et al., 2022), seawater (Schlesner, 1994; Wiegand et al., 2020), freshwater (Dedysh et al., 2020; Schlesner, 1994; Wang et al., 2002), chlorinated water (Aghnatios & Drancourt, 2015), the human gastrointestinal tract (Cayrou et al., 2013), and very frequently in association with several algae (Bengtsson & Øvreås, 2010; Lage & Bondoso, 2011, 2012; Wiegand et al., 2020). However, the cultivation of Planctomycetes in pure axenic cultures has proven to be challenging, primarily attributed to their recalcitrance to grow on synthetic media and their inherently slow growth kinetics (Kaboré et al., 2020). Only a minute fraction, approximately 0.6%, of known planctomycetal operational taxonomic units are successfully isolated (Wiegand et al., 2018). Besides, only a few examples of genetic modifications have been carried out in this phylum, mostly in *Planctopirus limnophila* (formerly known as *Planctomyces limnophilus*), and to a lesser extent in *Gemmata obscuriglobus*, *Gimesia maris*, and *Blastopirellula marina* (Rivas-Marín et al., 2016). Foreign DNA incorporation has been accomplished through electroporation (Jogler et al., 2011) and conjugation (Rivas-Marín et al., 2016), while the generation of mutants was carried out by homologous recombination (Erbilgin et al., 2014; Rivas-Marín et al., 2016) or transposon mutagenesis, employing the EZ-Tn*5* system (Jogler et al., 2011; Rivas-Marin et al., 2023; Schreier et al., 2012).

In 2020, a concerted effort was made to selectively isolate axenic cultures from all major planctomycetal clades, subsequently culminating in the sequencing of 79 complete genomes (Wiegand et al., 2020). The current accessibility of this genome dataset facilitates a thorough genomic analysis of this phylum. Notably, one of the significant research gaps in this domain pertains to the comprehensive exploration of plasmids within the Planctomycetes on a global scale. Plasmids are nearly ubiquitous in bacteria, and Planctomycetes are no exception to this rule (Guo et al., 2014; Ivanova et al., 2017; Jogler et al., 2020; Kulichevskaya et al., 2020). These genetic elements play a pivotal role in mediating genetic exchange in bacteria (Halary et al., 2010). Besides, the absence of replicative vectors remains a notable limitation to genetically manipulating Planctomycetes. Plasmids are a natural source to construct replicative vectors, which, in turn, are essential to enhancing the construction of deletion mutants and the development of expression systems.

The idea that the presumed compartmentalization of Planctomycetes might serve as a barrier against horizontal gene transfer (HGT) has been under consideration (Pinos et al., 2016). However, this notion was dismissed, as no discernible differences were identified in terms of HGT quantity, proportion, or transfer partners when compared to other bacterial phyla (Pinos et al., 2016). Planctomycetes are not impermeable to HGT. Conjugative transfer to Planctomycetes from outside of this phylum has been documented. Plasmid pBF1, which was exogenously isolated from a marine microbial community, was successfully transferred from *Pseudomonas putida* to *Planctomyces maris* (Dahlberg et al., 1998). Shuttle vectors were mobilized by the same plasmid pBF1 from *E. coli* to *Gemmata obscuriglobus*, *Blastopirellula marina*, *Gimesia maris* (formerly *Planctomyces maris*), and *Planctopirus limnophila*, where the transfer was detected by recombination (Rivas-Marín et al., 2016). Other transfer attempts using the conjugative plasmid RP4 in matings from *E. coli* or *P. putida* to *Planctopirus limnophila* have failed (Fuerst, 2013). Nevertheless, bacterial conjugation between Planctomycetes has not been reported, nor have conjugative systems been investigated.

In the present study, we delve into the diversity within the planctomycetal plasmidome, elucidating its unique gene families, as well as those that are shared with plasmids originating from taxonomic groups outside this phylum or the planctomycetal host chromosomes. Moreover, we scrutinize the main characteristics of the planctomycetal plasmid modules, serving as an initial step for the prospective design of replicative vectors.

## EXPERIMENTAL PROCEDURES

### Planctomycetes dataset

The whole set of complete genome sequences of Planctomycetes was obtained from NCBI (August 2022). For the 83 complete genome assemblies (83 chromosomes and 21 plasmids; Supplementary Table S1), the genomic and protein fasta files were retrieved. Protein fasta files of bacterial plasmids (43,874 sequences) were obtained from the NCBI RefSeq212 database. To remove sequences

incorrectly tagged as plasmids, plasmid records were filtered by their description, using the regular expression "*contig|\sgene(?!tic|ral|rat|ric)|integron|transposon|scaffold|insertion sequence|insertion element|phage|operon|partial sequence|partial plasmid|region|fragment|locus|complete (?!sequence|genome|plasmid|\|,)|(?<!complete sequence,)whole genome shotgun|artificial|synthetic|vector*." The curated plasmid dataset comprised 43,217 sequences.

## Genome phylogenetic analysis

PhyloPhlAn v3.0.67 (Asnicar et al., 2020) was used for detecting the 400 most universal prokaryote markers in the set of 83 planctomycetal chromosomes and producing a concatenated multiple sequence protein alignment, using the parameters *-d phylophlan –accurate – diversity high*. The resulting alignment was in turn trimmed with Trimal v1.2 (Capella-Gutiérrez et al., 2009), using option *-automated1*. A total of 8253 aligned amino acid positions were used to build a Maximum-Likelihood (ML) tree with IQ-TREE v1.6.12 (Nguyen et al., 2015) with the best-fit amino acid substitution model LG + F + R5 (Kalyaanamoorthy et al., 2017) and 1000 ultrafast bootstrap replicates (Hoang et al., 2018). *Chlamydia pecorum* E58 chromosome (GenBank Acc. No. NC_015408.1) was included in the analysis as an outgroup. The phylogenetic tree was visualized with the iTOL web platform (https://itol.embl.de/) (Letunic & Bork, 2019).

## Protein phylogenetic analysis

For the RPA replication initiation protein phylogeny, amino acid sequences were aligned using MAFFT v7.271 (Katoh et al., 2002) and the alignment was trimmed with Trimal v1.2 (*-automated1*). IQ-TREE tool was also used to generate an ML tree with 1000 ultrafast bootstrap replicates and the best-fit amino acid substitution model LG + F + R8. The phylogenetic tree was rooted at the midpoint and visualized with iTOL.

## Comparison of GC content

The GC content of plasmids and chromosomes was calculated with in-house scripts in Python v3.7.1. GC correlation between plasmids and chromosomes was calculated with the scipy.stats.pearsonr function from Python. Plots and graphs were generated with the R package ggplot2 v3.3.6. For GC content analysis of the adjacent region of replication initiation protein genes, the Kruskal–Wallis test was applied, using the Python base function scipy.stats.kruskal.

## Characterization of the planctomycetal plasmid proteome

Plasmid and chromosomal proteomes of the *Planctomycetota* phylum were mapped to Pfam (Mistry et al., 2021) and eggNOG v5.0 (Huerta-Cepas et al., 2019) databases. Hidden Markov Models (HMM) for 19,632 protein families contained in Pfam-A 35.0 database released in November 2021 were downloaded (http://pfam.xfam.org/) and used to search for protein families in our dataset with the *hmmscan* function of HMMER suite v3.1b2 (Eddy, 2011) (parameters *-E 0.0001 - -domE 0.0001 - -incE 0.0001 - -incdomE 0.0001*). Only hits covering at least 80% of the protein profile were recorded in Supplementary Table S3. eggNOG-mapper v2.1.9 (http://eggnog-mapper.embl.de/) was used to identify putative COGs with the bacteria-optimized database (*e*-value $\leq 10^{-4}$ and 80% subject coverage). To search for plasmid replicons we used several tools: PlasmidFinder (Carattoli et al., 2014), DoriC 10.0 (Luo & Gao, 2019), and blastp (*e*-value $\leq 10^{-5}$) against the replication initiation protein database implemented in PLACNETw (Vielva et al., 2017) (Supplementary Table S6). A blastp (*e*-value $\leq 10^{-5}$) search was also used to detect putative toxin-antitoxin (TA) systems for the 6 subtypes available in the TADB 2.0 database (Xie et al., 2018). Antibiotic resistance genes were screened based on CARD (McArthur et al., 2013) through a blastp search (50% amino acid identity, 60% sequence coverage, *e*-value $\leq 10^{-5}$). AMRFinderPlus (Feldgarden et al., 2021) was also used to screen for antimicrobial and metal resistance genes using default parameters and the "*–plus*" option, which includes virulence factors and stress-response genes (biocides, metal, and heat resistance). Metal resistance genes were also screened based on the latest available Bacmet database (March 2018) (Pal et al., 2014) consisting of 753 confirmed metal resistance genes, using a blastp search with criteria set to include hits with ≥50% identity and ≥60% coverage. Additionally, the CRISPRCasTyper tool (Russel et al., 2020) was employed to search for putative CRISPRCas operons in the planctomycetal plasmid dataset using default parameters and 80% HMM profile coverage. MOBScan (Garcillán-Barcia et al., 2020) was used to search for the presence of relaxases both in plasmid and chromosomal genomes, while MacSyFinder (Abby et al., 2014) was employed to detect putative mating pair formation homologues.

## Genome comparisons and clusterization methods

Average Nucleotide Identity with a 50% length threshold ($ANI_{L50}$) was used to determine the

similarity of the nucleotide sequences of the Planctomycetes plasmids with a 50% threshold in the length of the smaller genome in the pair (Redondo-Salvo et al., 2020). In that way, only plasmid pairs that exhibited ≥70% nucleotide identity in at least 50% of the smaller genome were considered for scoring $ANI_{L50}$, while those not fulfilling the threshold were assigned $ANI_{L50} = 0$. Blastn searches using planctomycetal plasmids as queries against the PLSDB database (version 2020_06_23) were used to detect similar plasmids in other phyla (*e*-value 0.001, 50% nucleotide identity, and 70% sequence coverage). HPCs were generated with MMseqs2 (Steinegger & Söding, 2017) at 30% identity and 60% alignment coverage or 99% identity and 100% coverage (parameters - *-cov-mode 0 –cluster-mode 0 - -cluster-reassign*). Bipartite networks containing plasmid and protein cluster nodes were constructed. Networks were visualized with Gephi v0.9 (https://gephi.org/). FastANI was used to calculate ANI values between planctomycetal chromosomes using default parameters (Galperin et al., 2015).

## RESULTS AND DISCUSSION

### Planctomycetal plasmid overview

The scarcity of genome sequence data for many bacterial phyla is one of the main impairments to assess the contribution of plasmids, a fundamental part of the accessory genome, to their host adaptation, fitness and survival. The current planctomycetal plasmidome encompasses 21 plasmids distributed in 10 isolates (Supplementary Tables S1 and S2). As expected because of its larger representation in the dataset, class *Planctomycetia* gathered most of the plasmids (20), mainly in members of the *Isosphaeraceae* family (order *Isosphaerales*) (Figure 1). Notably, all members of this taxonomic family contained plasmids, varying in number from one to five. Five isolates closely related contain most of the plasmids (16) (Supplementary Figure S1). The size of plasmids associated with Planctomycetes varies, ranging from 12.5 to 278 kb with a median size of 81.1 kb, following an unimodal distribution (Figure 2A).

The GC content of planctomycetal plasmids is relatively higher compared to plasmids found in most other

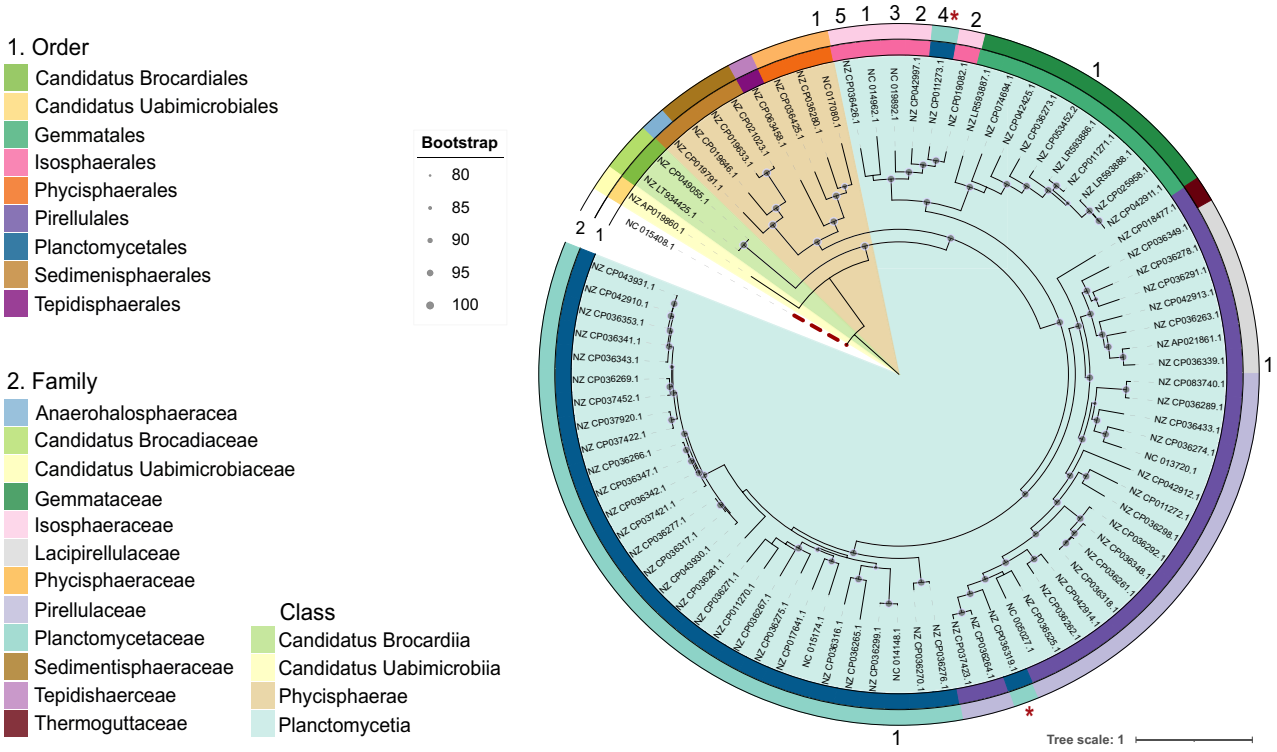


**FIGURE 1** Plasmid distribution on the *Planctomycetota* phylogeny. ML phylogeny based on universal bacterial markers retrieved with PhyloPhlAn from the 83 planctomycetal chromosomes. The outgroup (*Chlamydia pecorum* E58, NC_015408.1) is indicated by a discontinuous red line. Grey circles are placed on the branches supported by ultrafast bootstrap values ≥80%. The taxonomic metadata was retrieved from GenBank. The background colour indicates the *Planctomycetes* classes, while taxonomic orders and families are shown in the inner and outer coloured rings, according to the legend. The number of plasmids contained in the corresponding host is indicated. Incongruencies in the phylogenetic position of two isolates regarding the taxonomic metadata retrieved from GenBank are indicated by asterisks and information on their assignment to the *Isosphaeraceae* family reported by (Ivanova et al., 2017; Peeters et al., 2020; Vitorino & Lage, 2022) is provided in Supplementary Table S1.

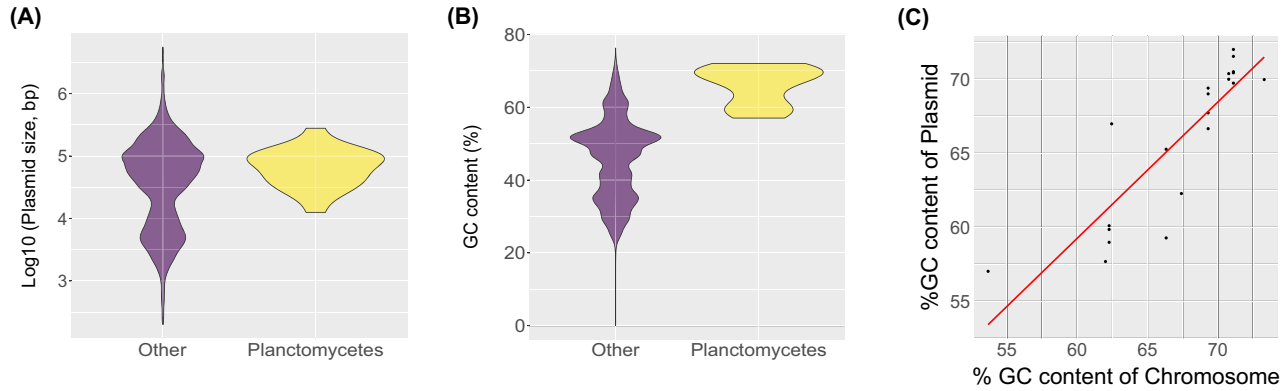


**FIGURE 2** Comparison of planctomycetal plasmids with those from other bacterial phyla and the cohabiting chromosome. Distribution of the planctomycetal plasmids (yellow) versus plasmids from other phyla (purple) by (A) size and (B) GC content. (C) GC correlation between planctomycetal plasmids and the chromosome of the plasmid-containing isolate. The red line indicates the linear regression model of the data. Planctomycetes plasmid GC content is significantly correlated with that of the cohabiting chromosome (Pearson's correlation coefficient $r = 0.86$, $p$-value $= 4.72e{-}07$).

phyla, with values ranging from 57% to 72% and a median GC content of 67.7% (Figure 2B). Furthermore, there is a significant correlation between the GC content of planctomycetal plasmids and that of the cohabiting chromosome, as evidenced by a high Pearson's correlation coefficient of 0.86 ($p$-value $= 4.72e{-}07$) (Figure 2C). This pattern aligns with the general trend observed in plasmid-host relationships in other phyla, as described previously (Almpanis et al., 2018). Since sequences recently introduced into a bacterial genome often bear unusual sequence characteristics and gradually tend to adopt the average nucleotide composition of the host genome (Lawrence & Ochman, 1998), the GC similarity between plasmids and their hosts is an indicator of long co-evolution between them. As also previously observed in plasmids originated from other phyla (Almpanis et al., 2018; Nishida, 2012), the planctomycetal plasmids are richer in AT than their cohabiting chromosomes, on average 1.5% higher. A higher energy cost and limited availability of G and C over A and T/U have been suggested as a basis for these differences (Rocha & Danchin, 2002).

## Biological functions encoded in the Planctomycetes plasmidome are very diverse

To explore the functions associated with plasmids hosted in Planctomycetes, the plasmid-encoded proteins (1375) were mapped to the Protein Families (Pfam) and Cluster of Orthologous Group (COG) databases. Six hundred and ninety-two of them were assigned to 456 Pfam families and 653 proteins to 20 out of the 23 different COG categories (Figure 3A; A and B), including 155 proteins assigned to the poorly characterized COG category S of 'function unknown', and other 53 proteins assigned to COGs without any category (Supplementary Table S3). Proteins assigned to Pfam families per plasmid ranged from 17% to 79% and those assigned to COGs from 12% to 96%. Taking both annotation methods together, 57% of the plasmid proteome could be assigned to a protein family (Pfam and/or COG) with a known function. It has been previously reported that 40%–50% of the complete planctomycetal proteome is annotated as hypothetical (Wiegand et al., 2020), a number higher than for most phyla (Galperin et al., 2015). Proteins involved in replication, recombination and repair (129); membrane biogenesis (76); transcription (63); signal transduction (46) and inorganic ion transport and metabolism (44) were the most frequent COGs detected, and most of them were found more than once per plasmid (Supplementary Table S3).

Planctomycetes have been reported to specialize in sugar and secondary metabolism, with a notable emphasis on the degradation of high molecular weight sugars. This specialization is attributed to the presence of gene clusters encoding carbohydrate-active enzymes (CAZymes) (Andrade et al., 2017). Remarkably, it has been conjectured that the presence of these gene clusters, generally encoded in the chromosome, may be a contributing factor to the relatively larger genomes observed within this taxonomic phylum (median 7.2 Mb) (Wiegand et al., 2020). Notably, the COG analysis identified 20 proteins in 8 plasmids as putative CAZymes: 4 glycosyl hydrolases (GH) and 16 glycosyl transferases (GT) belonging to 4 and 6 CAZy families, respectively (Supplementary Table S3). These proteins are included in COG categories related to carbohydrate transport and metabolism (4 proteins belonging to COG category G); cell wall, membrane and/or envelope biogenesis (15 proteins to COG M), and energy production and conversion coupled with carbohydrate transport and metabolism

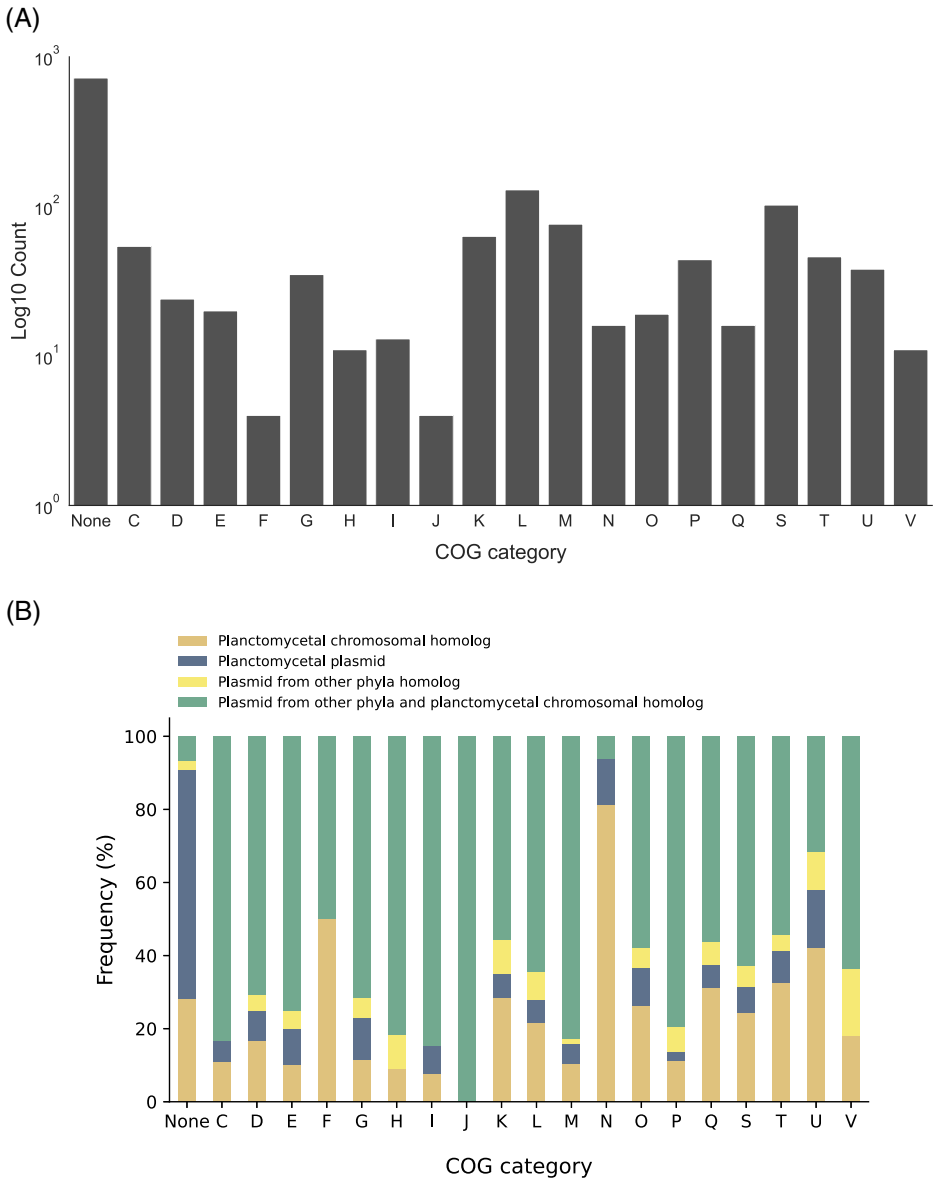


**FIGURE 3** Specific and shared proteins of the planctomycetal plasmid proteome. (A) Abundance of each COG category detected in the planctomycetal plasmid proteome. (B) For each COG category, the percentage of planctomycetal plasmid proteins with homologues in planctomycetal chromosomes (orange), in plasmids from other phyla (yellow), in both planctomycetal chromosomes and plasmids from other phyla (green), or without homologues in any of them (dark blue) is shown. List of COG categories: C: energy production and conversion; D: cell cycle control, cell division, chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme transport and metabolism; I: lipid transport and metabolism; J: translation, ribosomal structure and biogenesis; K: transcription; L: replication, recombination and repair; M: cell wall/membrane/envelope biogenesis; N: cell motility; O: post-translational modification, protein turnover, chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; S: function unknown; T: signal transduction mechanism; U: intracellular trafficking, secretion and vesicular transport; V: defense mechanisms.

(1 protein to COG categories C and G). The most abundant CAZy families detected were GT26 ($n = 5$), GT4 ($n = 4$), GT2,GT4 ($n = 3$), and GT2 ($n = 2$). The presence of CAZy proteins was already reported in plasmids pPALBO1 and pPL62-1 (Ivanova et al., 2017). A higher proportion of CAZymes encoded in plasmids are detected in isolates exhibiting a lower number of chromosomally-encoded CAZymes, which suggests that plasmids are likely complementing these metabolic pathways (Supplementary Table S4). An exceptional illustration can be found in the case of *Tautonia plasticadhaerens* EIP (NZ_CP036426.1), which encompasses 7 CAZymes within its chromosome. Additionally, it accommodates five plasmids, three of which, namely pEIP_2 (NZ_CP036428.1), pEIP_4 (NZ_CP036430.1), and pEIP_5 (NZ_CP036428.1), were identified to contain 1, 7, and 3 CAZymes, respectively.

## The currently available planctomycetal plasmidome is very diverse

Bacterial plasmids have been recently classified into Plasmid Taxonomic Units (PTUs), based on their average nucleotide identity, $ANI_{L50}$ (Garcillán-Barcia et al., 2023; Redondo-Salvo et al., 2020). To analyse the similarity of Planctomycetes plasmids, we first calculated the pairwise $ANI_{L50}$ values between them. Notably, despite prior observations of homologous regions and synteny conservation in certain plasmids of the *Isosphaeraceae* planctomycetes (Ivanova et al., 2017), all pairwise $ANI_{L50}$ values equalled 0, indicating a high level of diversity in the plasmid backbones.

Subsequently, we undertook a comprehensive analysis of the entire open reading frame dataset (ORFeome) derived from the 21 planctomycetal plasmids, a total of 1375 plasmid-encoded proteins, employing a clustering approach at 30% identity and 60% coverage, to identify remotely related homologues. This analysis yielded 1053 distinct homologous protein clusters (HPCs). To visually represent the relationships between plasmid genomes and HPCs, we constructed a bipartite network, as illustrated in Figure 4. In this network, connections between the two types of nodes (plasmid genomes and HPCs) were established when a member of a given protein cluster was present in a particular plasmid. As a result, plasmids sharing a greater number of HPCs tended to cluster together within the network. It is noteworthy that the resulting network displayed a sparse distribution, with only 135 protein clusters being shared by at least two plasmids, depicted as black nodes in Figure 4A. Conversely, 918 HPCs remained as singletons, each linked solely to a specific plasmid.

Several HPCs emerged as central hubs within the network, connecting multiple plasmids (Figure 4A). Notable examples of these hub proteins included the replication initiation protein RPA (PF10134.12, 40% average identity, present in 12 plasmids), the partition Walker A motif-containing ATPase ParA (PF01656.26, with an average identity of 34%, associated with 13 plasmids), the centromere-like binding protein ParB (PF02195.21, with an average identity of 34%, present in 8 plasmids), and a tyrosine recombinase phage integrase (PF00589.25, with an average identity of 43%, observed in 9 plasmids).

Within the network, three plasmids originating from the *Isosphaeraceae* family (pPL62-1, pPALBO1, and pOJF2_1) exhibited the most extensive interconnections, sharing a total of 37 HPCs. They were hosted in isolates with the highest ANI values between genome pairs containing plasmids (78%–80%) (Supplementary Figure S1). Nevertheless, we observed no clear correlation between the number of proteins shared by plasmid pairs and the genomic ANI value of their hosts. The shared HPCs among these three plasmids encompassed a range of functions, including ABC transporters (PF00005.30, PF01061.27), as well as several CAZymes, such as glycosyl transferases (PF00534.23, PF06165.14, PF13439.9, PF13524.9, PF13579.9) and glycosyl hydrolases (PF17167.7). This observation aligns with the previously reported diverse repertoire of CAZymes associated with plasmids pPL62-1 and pPALBO1 (Ivanova et al., 2017). Notably, pPL62-1 and pPALBO1 have been previously documented to exhibit a high degree of similarity, sharing up to 51 homologous loci (Ivanova et al., 2017). This shared genetic content explains their substantial connectivity within our bipartite network, with 46 HPCs in common. In contrast, at the other end of the spectrum, plasmid pPL62-4 shared a single HPC, and pPLIM01 remained entirely isolated within the network. Precisely in the most divergent planctomycetal plasmid, pPLIM01 of *Planctopirus limnophila*, an essential gene, encoding a protein of unknown function (WP_013112526.1) was recently identified by transposon-directed insertion site sequencing (Rivas-Marin et al., 2023). This plasmid is enriched in phage-related genes and could thus be a phage-plasmid (Pfeifer & Rocha, 2024).

## Planctomycetal plasmids and chromosomes share a large proportion of biological functions

Plasmid and chromosome proteins were clustered at 30% identity and 60% coverage. The majority of the plasmid proteins (823 out of 1375) grouped with chromosomal homologues and distributed in 581 mixed HPCs (Figure 4B and Supplementary Figure S2A,B). The most abundant shared HPCs included glycosyl transferases (PF13692.9, PF00534.23, PF13641.9, PF13439.9, PF13524.9), epimerases (PF01370.24) and DDE transposases (PF13546.9, PF13586.9) (Supplementary Table S3). Thus, 552 plasmid proteins, distributed in 502 HPCs do not have a chromosomal homologue in Planctomycetes.

When comparing the Pfam content of planctomycetal plasmids and their host genomes, it was observed that 13 plasmids exhibited a substantial overlap, sharing at least 50% of the Pfam groups with the cohabiting chromosome (Supplementary Figure S2A). In contrast, 8 plasmids were characterized by a marked absence, ranging from 60% to 95%, of the Pfam content that was not present in the chromosome of their respective hosts. Among the protein families present in plasmids but absent in their hosts we found glycosyl transferases (PF03808.16, PF10111.12, PF13439.9, PF13506.9, PF13524.9, PF13579.9, PF13632.9, PF13641.9, PF13692.9; $n = 22$ proteins), ParA partition protein (PF01656.26, PF13614.9; $n = 19$ proteins), tetratricopeptide repeat (PF00515.31, PF07721.17, PF13374.9,

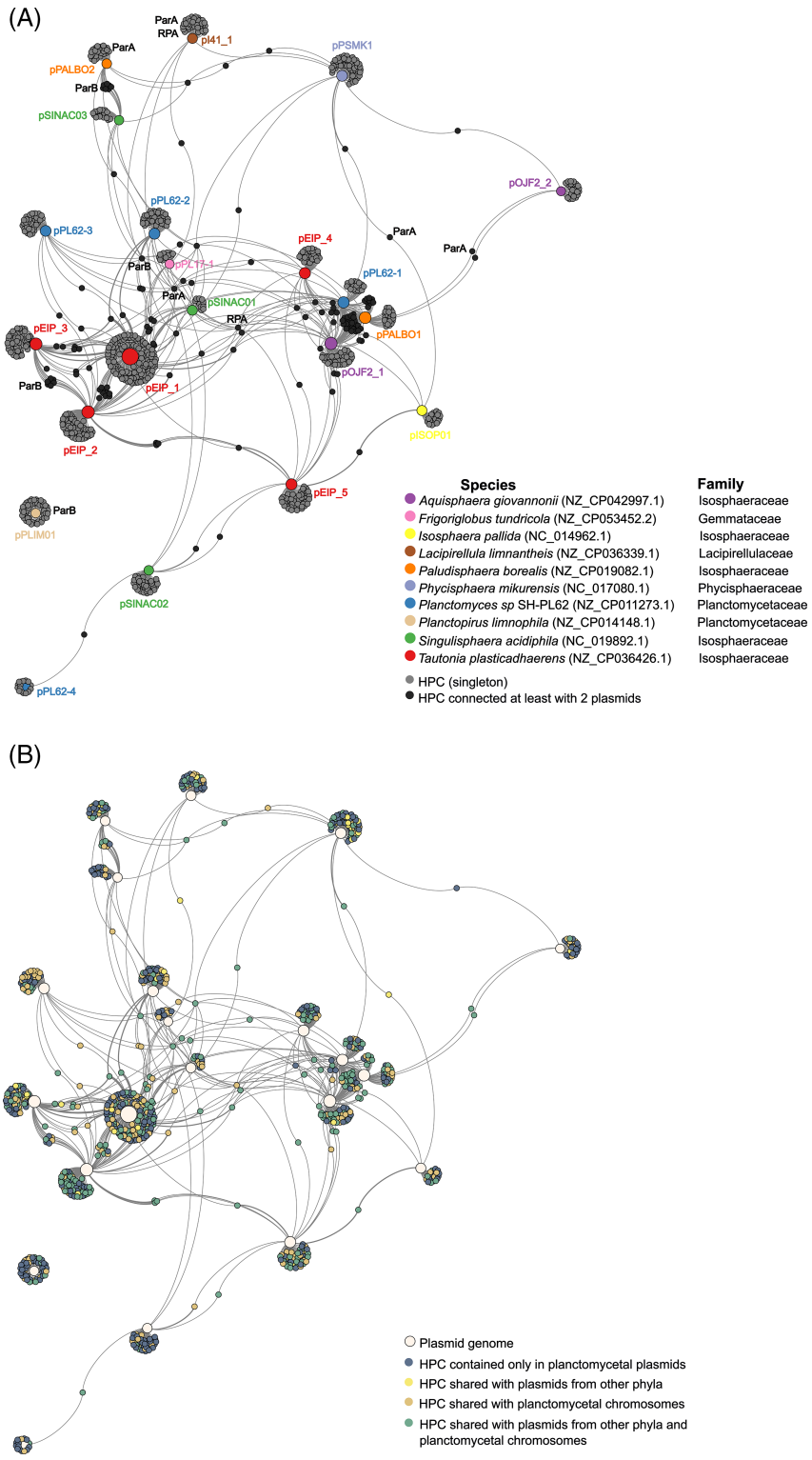


**FIGURE 4** Proteome network of the Planctomycetes plasmidome. Bipartite network of homologous protein clusters at 30% identity and 60% coverage of planctomycetal plasmids. Nodes representing protein clusters are small, while nodes representing plasmids are larger and scaled according to plasmid size. (A) Protein nodes are grey-coloured, except for those containing members from at least two plasmids, which are depicted in black. Clusters containing RPA initiators and partition proteins (ParA, ParB) are indicated. Plasmids are coloured by their host taxonomic family. (B) The colour of the protein nodes indicates if members of the HPC have homologues in the planctomycetal chromosome and/or in plasmids from other phyla.

PF13424.9, PF13428.9, PF13432.9; $n = 16$ proteins), epimerases (PF00908.20, PF01370.24; $n = 14$ proteins), homeodomain-like domains (PF13565.9; $n = 13$ proteins), phage integrases (PF00589.25, PF02899.20; $n = 13$ proteins), ParB partition protein (PF02195.21; $n = 12$ proteins), response regulator receiver domain (PF00072.27; $n = 11$ proteins), and DDE superfamily endonucleases (PF13358.9; $n = 8$ proteins). Nevertheless, all these Pfam families were found chromosomally encoded in other Planctomycetes.

## Recent gene transfer events between plasmids and chromosomes in planctomycetes

Instances of homology, particularly when subjected to rigorous criteria, serve as diagnostic indicators of recent genetic exchange events, inherently impeding the accumulation of substantial nucleotide alterations. In line with this approach, the proteomes from plasmids and chromosomes were subjected to a clustering process characterized by a 99% identity threshold and 100% coverage criterion. As a result of this analysis, we observed the presence of 12 proteins out of 1375 encoded within planctomycetal plasmids that were concurrently identified in the chromosome of their host (Supplementary Figure S4). Notably, these identified proteins primarily belonged to insertion sequences, including DDE transposases of the IS*701* (PF13546.9), IS*66* (PF03050.17), IS*4* (PF01609.24), and IS*4*/*5* (PF13340.9) families, as well as the IS*66* transposase accessory protein (PF05717.16), and four orphan ORFs. IS elements are recognized for facilitating the intracellular mobility of DNA, affecting not only their own genes but sometimes also those in their proximity (Partridge et al., 2018). Out of the IS-related genes, no other genes in the ISs vicinity seem to have been recently interchanged. Our results indicate the recent occurrence of transposition events between both genomic platforms in Planctomycetes.

## Shared functions found in plasmids within planctomycetes and those originating from other bacterial phyla

Subsequently, we examined the evolutionary connections between plasmids originating from Planctomycetes and those from other phyla. First, to test whether the planctomycetal plasmids were similar to other bacterial plasmids, each plasmid was blastn-searched against the PLSDB database (version 2020_06_23), encompassing a total of 34,513 bacterial plasmids. Remarkably, no hits were retrieved with the sole exception being the 15 planctomycetal plasmids already included in PLSDB, which were identified when queried using the same plasmid sequences. This result is congruent with the fact that most plasmids do not reach a host range beyond the phylum (Redondo-Salvo et al., 2020).

Next, the proteomes of planctomycetal and RefSeq212 plasmids were clustered at 30% identity and 60% coverage. This analysis unveiled 545 proteins derived from planctomycetal plasmids, distributed in 375 HPCs, that exhibited homology to counterparts in plasmids circulating out of Planctomycetes (Figure 4B, Supplementary Figure S3, and Supplementary Table S3). A majority of these mixed HPCs, 82% of the clusters, contained a single member from the planctomycetal plasmidome, in agreement with the low degree of connectivity between planctomycetal plasmids. Notably, a substantial majority of these shared proteins (535 out of 545) could be confidently classified into specific Pfam or COG groups (Supplementary Table S3, Figure S3B). The most populated HPCs included homologues of the DDE superfamily endonuclease (PF13358.9), partition ParA protein (PF01656.26), phage integrases (PF00589.25), glycosyl transferases (PF00534.23), epimerases (PF01370.24), AAA+ ATPases (PF13614.9), homeodomain-containing (PF13565.9, PF13614.9), and RHS-containing (PF05593.17) proteins. These putative far-related homologues are found in a variety of plasmids from different PTUs, 97% of which exhibited a host range restricted to different taxonomic ranks into the *Enterobacterales* order (host range I-IV) (Supplementary Table S5). Notably, 42% of HPCs shared between planctomycetal plasmids and chromosomes lacked homologues in plasmids from other phyla. This suggests a high proportion of functions specific to Planctomycetes encoded in their plasmids.

## Planctomycetal plasmid modules

Plasmid backbones comprise distinct modules that encompass different functions in the biology of these mobile genetic elements, such as replication, stability, adaptation, and propagation. We searched for the presence of proteins encoded in these different modules in the Planctomycetes plasmid dataset.

### Plasmid replication

Plasmids can replicate autonomously because they contain an origin of replication (*oriV*) and generally encode the replication initiation protein (RIP) responsible for recognizing the cognate *oriV* and initiating plasmid replication (del Solar et al., 1998). Initial attempts to type plasmid replication regions by using prototypes from *Enterobacteriaceae* and Gram-positive bacteria as implemented in PlasmidFinder (Carattoli et al., 2014),

or the origin of replication database implemented in DoriC 10.0 (Luo & Gao, 2019), did not yield any hit in the planctomycetal plasmidome. This suggests that the replication regions of these plasmids differ significantly from those in other phyla. This finding is consistent with previous attempts to introduce IncQ, IncP and pBBR replicons into Planctomycetes via conjugative matings, which failed to produce plasmid replicants (Jogler & Jogler, 2013). We then retrieved RIPs from the Planctomycetes plasmids by carrying a blastp search against known RIP families (Supplementary Table S6). For 14 out of the 21 planctomycetal plasmids a putative RIP was detected, 13 belonged to the RPA family (PF10134.12) and 3 to the HTH_36 family (PF00239.24), and two of the plasmids encoded both RIPs (Supplementary Table S2). These RPA homologues matched Pfam PF10134.12 with coverage values ranging from 58% to 93%. The average percentage identity of the 13 RPA proteins detected in Planctomycetes plasmids was 37.5%.

RPA is a single-stranded DNA binding protein that is required for multiple processes in DNA metabolism, including DNA replication, DNA repair, and recombination (del Solar et al., 1998; Lilly & Camps, 2015) identified as a replication initiation protein in plasmid pBTK45 (GenBank Acc. no. EU585932.1) of the betaproteobacterium *Tetrathiobacter kashmirensis* strain WGT (Dam et al., 2009). We reconstructed the RPA gene phylogeny using the proteins retrieved with pfam PF10134.12 from the complete bacterial plasmid dataset to study the context of these planctomycetal RIPs regarding those from other phyla. A total of 530 RPA sequences were included in the phylogenetic tree (Figure 5). The RPA proteins detected in Planctomycetes clustered together in a separated clade from those present in the other phyla. Besides, RPA proteins from Planctomycetes did not cluster at 30% identity and 60% coverage with the homologues out of this phylum. These facts suggest a large divergence of this subset of RPA replication proteins and its specialization in

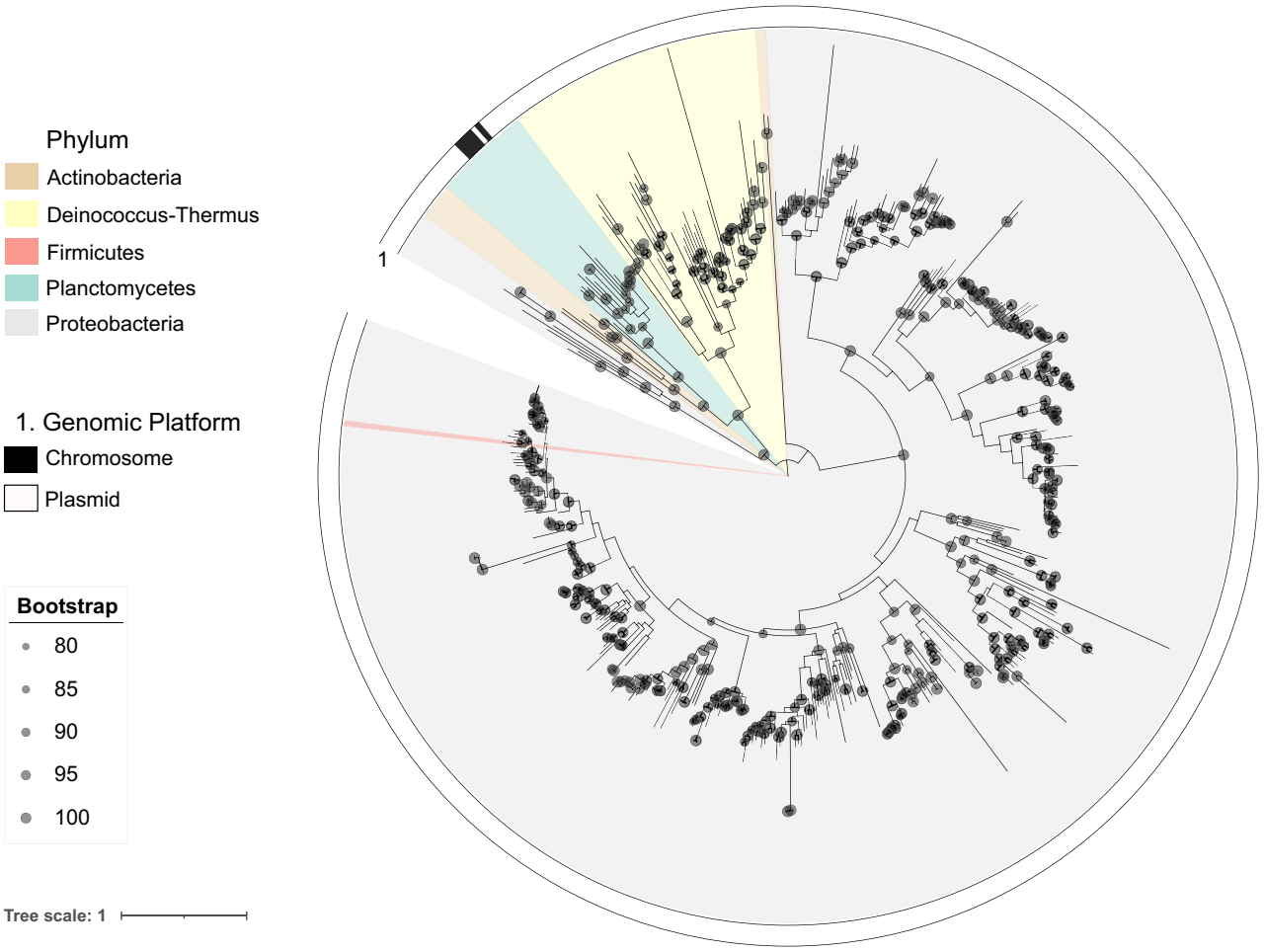


**FIGURE 5** RPA protein phylogeny. Maximum likelihood tree based on 530 RPA amino acid sequences retrieved from plasmids in the RefSeq212 database, 13 from planctomycetal plasmids and 5 homologues detected in planctomycetes chromosomes, using the best-fit amino acid substitution model LG + F + R8. The tree was rooted at the midpoint. Grey circles are placed only on the branches supported by ultrafast bootstrap values ≥80%. For each RPA homologue, genome and protein accession numbers are included at the tips. The different host phyla are shown in colours according to the legend. RPA homologues detected in planctomycetes chromosomes are depicted in black in the outer ring.

Planctomycetes, without recent interchanges with other hosts. The closest planctomycetal RPA homologues out of this phylum were found in Actinobacteria. Five RPA homologues were detected in the Planctomycetes chromosomes: two in *Lacipirellula parvula*, two in *Gemmata obscuriglobus*, and one in *Fuerstiella marisgermanici*. These chromosomal RPA homologues are located in a separate, more recent clade from most RPA encoded in plasmids, except for the RPA contained in plasmid pPL17-1, hosted in *Frigoriglobus tundricola*, which is intermingled with the chromosomal ones. It suggests that chromosomal RPA proteins are derived from plasmids that are integrated into the chromosome.

Generally, the *oriV* sequences are located in close proximity to the replication-related genes and are enriched in A + T content to facilitate DNA unwinding (del Solar et al., 1998; Lilly & Camps, 2015). In the Planctomycetes plasmids, there is generally a decrease in the GC content in the 500 bp region located immediately upstream of the genes encoding RPA initiators (on average 10% lower than the total plasmid GC content) (Supplementary Figure S5), suggesting a putative *oriV* sequence in that location. The *oriV* regions generally contain sequences repeated in tandem (iterons) that are recognized by the plasmid-encoded RIP and are critical to controlling the plasmid replication (del Solar et al., 1998). In the Planctomycetes plasmids where a member of the RPA replication protein family was identified, we performed a search for tandem repeats and found different kinds of iterons, either upstream, or into the *rpa* gene (Supplementary Table S7).

## Plasmid stability

Segregational stability is essential for the plasmid maintenance in bacterial populations. The active partition is one of the plasmid segregation mechanisms encoded by the plasmids (Baxter & Funnell, 2014). Plasmid-encoded partitioning loci encode at least a *cis*-acting centromere-like site and two *trans*-acting proteins, a DNA-binding protein that binds specifically to the centromere-like site and an NTPase that forms filamentous structures (Ebersbach & Gerdes, 2005). The ATPase ParA (PF01656.26) and the DNA-binding protein ParB (PF02195.21) were detected in 19 and 12 plasmids, respectively. In 11 of these plasmids, the ParA and ParB homologues were detected in close genetic proximity, resembling a classical type I partition system (Supplementary Table S2, Figure 6). The average percentage identity of the detected ParA and ParB proteins was 29.5% and 34.4%, respectively.

Another strategy to maintain plasmid stability is post-segregational killing (Gerdes et al., 1986). This mechanism relies on toxin-antitoxin (TA) systems, which consist of a stable toxin that poisons the host cell and a much less stable cognate antitoxin that counteracts this detrimental effect, both components encoded by genes generally linked (Harms et al., 2018; Jurėnas et al., 2022). Plasmids encoding TA systems ensure their preservation in the bacterial population since cells lacking the plasmid, and thus the antidote, are eliminated (Díaz-Orejas et al., 2017). TA modules can be classified into eight different groups according to the molecular pattern of antitoxin and the mechanism of toxin neutralization (Jurėnas et al., 2022). The TADB 2.0 database (Xie et al., 2018) was used to retrieve putative TA modules in the Planctomycetes plasmid dataset. Among the different groups of TA, only type II TA proteins were detected. In type II TA systems, a direct protein–protein interaction between toxin and antitoxin blocks the action of the toxin (Gerdes et al., 1986). Putative type II TA modules were detected in 4 plasmids: AbiEii (PF08843.14)/AbiEi (PF17194.7 + PF11459.11), RelE (PF06296.15)/MqsA (PF15731.8), HigB (PF09907.12)/HigA (PF01381.25), and Gp49 (PF05973.17)/HTH_37 (PF13744.9) (Supplementary Table S2).

## Plasmid adaptation

Plasmid adaptative modules confer a beneficial advantage to the cells harbouring them within a specific environment. Typically, these traits are associated with resistance to antimicrobials or metals, degradative pathways, and virulence factors. Besides the above-mentioned detection of CAZymes potentially involved in the metabolism of complex carbohydrates, we also screened the planctomycetal plasmids searching for antimicrobial resistance genes using different databases, and no hits meeting the threshold cutoff were identified. This contrasts with the fact that Planctomycetes are naturally resistant to many antibiotics (Cayrou et al., 2010; Godinho et al., 2019). Congruently, when searching in the Planctomycetes chromosomal dataset, 142 putative antimicrobial resistance traits were retrieved. Antibiotic efflux pumps were the most frequently detected, such as MexF (70), RosA (11), AbeS (11), KdpE (10), MexK (7) and MexQ (5) (Supplementary Table S8). Metal resistance genes were also searched. Four hits based on criteria of ≥50% identity and ≥60% coverage were detected in two plasmids (Supplementary Table S2). Copper (*actP*); nickel and cobalt (*cnrA*); and cadmium, zinc and cobalt (*czcP*) resistances in plasmid pPSMK1 (NC_017081.1) from *Phycisphaera mikurensis* NBRC 102666. Chromate resistance (*chrA1*) was retrieved in pEIP_2 plasmid (NZ_CP036428.1) from *Tautonia plasticadhaerens*. Finally, we searched for defence mechanisms against other MGEs using CRISPRCasTyper. However, no putative CRISPR-Cas operons were found. Instead, in seven plasmids individual protein genes functionally

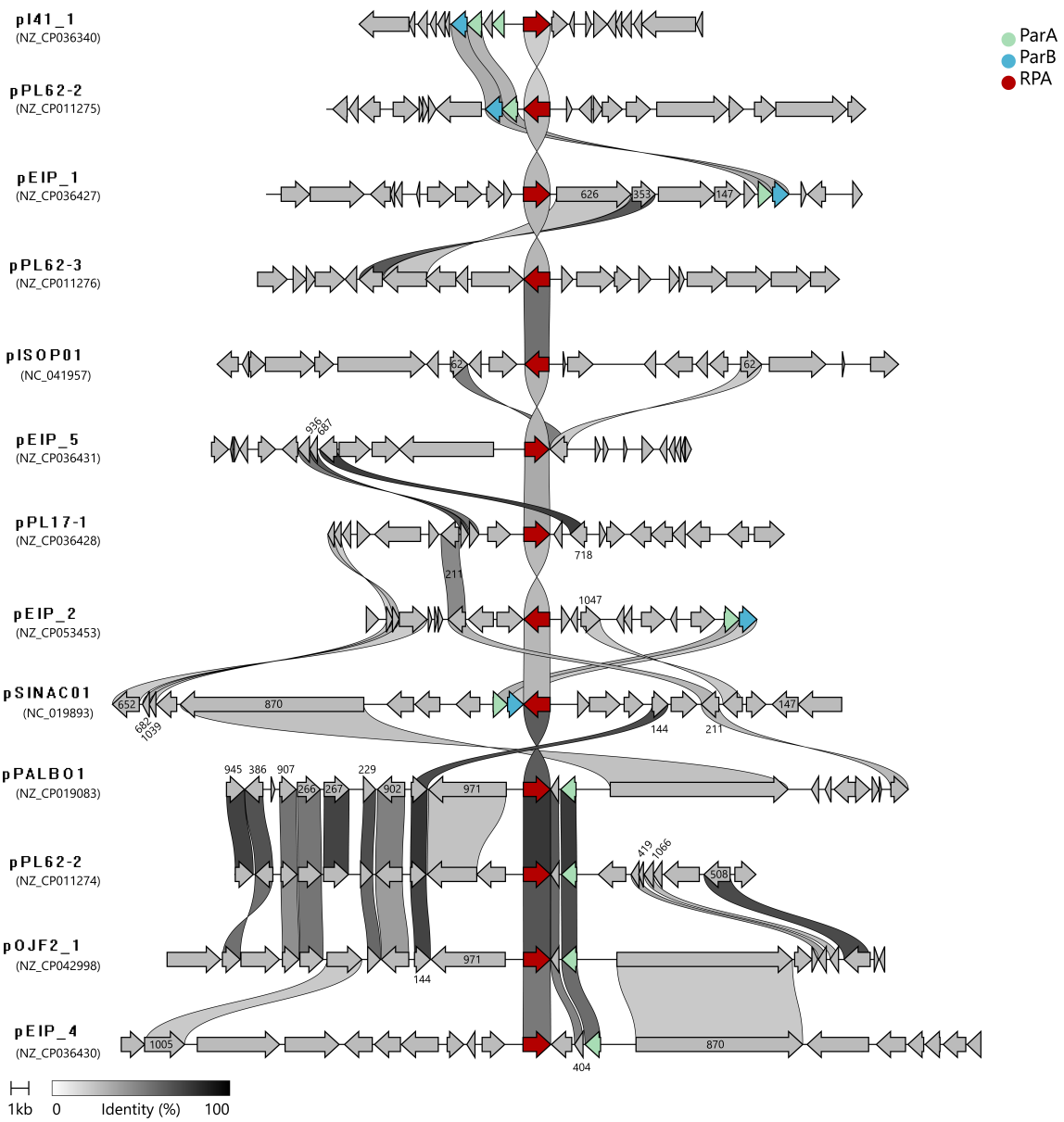


**FIGURE 6** Genetic organization of the *rpa*, *parA* and *parB* homologues detected in planctomycetes plasmids. The genetic vicinity or the replication initiation gene *rpa* (10 genes upstream and downstream *rpa*) is compared between 13 planctomycetal plasmids by using clinker (Gilchrist & Chooi, 2021) with default parameters. Relevant genes are coloured according to the legend and for others shared by several plasmids, the HPC number is indicated (see Supplementary Table S3).

linked to CRISPR-Cas systems were detected (Supplementary Table S2). Additionally, two orphan CRISPR arrays, with no Cas protein associated, were detected: one in pISOP01 plasmid (NC_014957.1) from *Isosphaera pallida* ATCC 43644, and the other in the pEIP_3 plasmid (NZ_CP036429.1) from *Tautonia plasticadhaerens* (Supplementary Table S2).

## Plasmid transfer

Plasmid transfer from a donor to a recipient cell via conjugation is another key feature of plasmid biology. Following introduction into the new host, the plasmid fate is determined by its replication capability, which may result in its retention as a self-replicating entity or its integration into the host chromosome (Guglielmini et al., 2011). Plasmids and integrative elements are categorized according to their potential to be transmitted by conjugation (Garcillán-Barcia & de la Cruz, 2013). They are classified as conjugative if they contain the entire conjugative apparatus, including relaxase and mating pair formation (MPF) systems. On the other hand, they are labelled as mobilizable if they only carry the coding information for a relaxase. Phylogenetically, relaxases are organized into nine distinct

MOB families (Garcillán-Barcia et al., 2020), and the MPF systems are classified into eight different types (Guglielmini et al., 2014).

A chromosomal relaxase gene (*traI*) was identified in a previous genomic analysis of *Gemmata obscuriglobus* (Jenkins et al., 2002). We identified a relaxase gene in four distinct plasmids, three of them relaxases falling within the $MOB_F$ class and the remaining one classified as $MOB_P$ (Supplementary Table S9). Out of these plasmids, only one, pPSMK1, hosted in *Phycisphaera mikurensis* (class Phycisphaerae), was found to encode a putative $MPF_T$ system, which is the most abundant type in the phylum Proteobacteria (Guglielmini et al., 2014). The genetic arrangement of this putative conjugative system is illustrated in Supplementary Figure S6. Notably, this putative conjugative system lacks the VirB1 and VirB7 components. VirB1 is a cell wall hydrolase that was found to be non-essential for T-DNA transfer (Berger & Christie, 1994; Fullner, 1998). VirB7 is a small fast-evolving lipoprotein (Guglielmini et al., 2014), which could explain why it went unnoticed.

Relaxases were also detected in Planctomycetes chromosomes, suggesting the presence of Integrative and Mobilizable Elements (Supplementary Table S9). In multiple cases, two relaxases were detected in the same chromosome, with 29 instances of $MOB_F$ relaxases distributed across 21 chromosomes, and one instance of $MOB_P$ relaxase found within a single chromosome. A coupling protein (T4CP) was often detected along with the relaxase. No complete MPF system was detected in chromosomes, although some MPF proteins were found in 38 chromosomes, suggesting that no Integrative and Conjugative Elements were present in the chromosomal dataset.

## CONCLUSIONS

Planctomycetal plasmids seemed to have been largely coevolved with their hosts, as indicated by the high correlation between the GC content of the plasmids and their cohabiting chromosomes. Forty-three per cent of the plasmid proteome could not be assigned either to a Pfam or COG category, highlighting the underexplored potential of distinct planctomycetal plasmids in offering proteins with diverse and novel functions. A large portion of the proteins encoded in the plactomycetal plasmidome (60%) showed to have a far-related chromosomal homologue. This and the fact that the functionally characterizable part of the planctomycetal plasmidome comprises a wide range of bacterial gene functions suggest that most types of functions can be captured on plasmid DNA and become mobilized. Consistent with this, transposases from various IS elements have been identified to undergo recent mobilization events between planctomycetal chromosomes and their co-resident plasmids, providing a pathway for crosstalk between both genomic platforms. Besides, site-specific tyrosine recombinases were found in a large portion of planctomycetal plasmids, which could facilitate gene capture and/or plasmid integration into the chromosome. Specifically, the discovery of RIPs integrated into the chromosome underscores the potential for stabilizing genes typically carried in mobile platforms.

Planctomycetal plasmids showed high diversity in gene content, a characteristic anticipated by the limited dataset of complete planctomycetal genomes. They were also very different from plasmids out of Planctomycetes, although remote homology could be detected in 40% of the planctomycetal proteome. In summary, 36% of the proteins composing the planctomycetal plasmidome (498 planctomycetal plasmid proteins, grouped in 451 HPCs) lacked a far-related homologue either in plasmids from other phyla or planctomycetal hosts.

We found that replication initiation proteins belonging to the RPA family are abundant in planctomycetal plasmids, and regions with characteristics compatible with origins of replication were identified in the proximity of these replication genes. Moreover, active type I partition systems were identified in several of these plasmids. These biological parts could be harnessed to design shuttle vectors able to be stably maintained in Planctomycetes. Finally, the very existence of mobilizable and conjugative elements in this phylum anticipates the possibility of bacterial conjugation among its members. To substantiate these findings, further experimental assays aimed at validating plasmid transfer via the putative planctomycetal conjugative system could offer valuable insights into the dynamics of bacterial conjugation within this phylum.

## AUTHOR CONTRIBUTIONS

**María del Mar Quiñonero-Coronel:** Conceptualization; investigation; writing – original draft; writing – review and editing; methodology; validation; visualization; formal analysis; data curation. **Damien Paul Devos:** Conceptualization; writing – review and editing; formal analysis. **M. Pilar Garcillán-Barcia:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; formal analysis; project administration; supervision.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=203682&assembly_level=3:3.

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## SUPPORTING INFORMATION

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