

# Hunting for pigments in bacterial settlers of the Great Pacific Garbage Patch

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## Abstract

The Great Pacific Garbage Patch, a significant collection of plastic introduced by human activities, provides an ideal environment to study bacterial lifestyles on plastic substrates. We proposed that bacteria colonizing the floating plastic debris would develop strategies to deal with the ultraviolet-exposed substrate, such as the production of antioxidant pigments. We observed a variety of pigmentation in 67 strains that were directly cultivated from plastic pieces sampled from the Garbage Patch. The genomic analysis of four representative strains, each distinct in taxonomy, revealed multiple pathways for carotenoid production. These pathways include those that produce less common carotenoids and a cluster of photosynthetic genes. This cluster appears to originate from a potentially new species of the *Rhodobacteraceae* family. This represents the first report of an aerobic anoxygenic photoheterotrophic bacterium from plastic biofilms. Spectral analysis showed that the bacteria actively produce carotenoids, such as beta-carotene and beta-cryptoxanthin, and bacteriochlorophyll a. Furthermore, we discovered that the genetic ability to synthesize carotenoids is more common in plastic biofilms than in the surrounding water communities. Our findings suggest that plastic biofilms could be an overlooked source of bacteria-produced carotenoids, including rare forms. It also suggests that photoreactive molecules might play a crucial role in bacterial biofilm communities in surface water.

## INTRODUCTION

For more than 70 years, highly stable synthetic compounds collectively known as ‘plastics’ have been produced for commercial use, with much of it ending up as anthropogenic waste in aquatic environments across the world (Geyer et al., 2017). An estimated >4900 Mt, or around 60% of all plastic ever produced, has been

estimated to accumulate in our landfills and natural environments (Geyer et al., 2017). The exact amount of this discarded or littered plastic that ends up in marine environments is difficult to calculate, but an estimated 4.8–12 Mt ended up in the world’s oceans in 2010 alone (Jambeck et al., 2015), and it accumulates in remote locations where it is very difficult to remove (MacLeod & Arp, 2021). The plastic debris in the

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oceans is not evenly distributed, as currents and gyres lead to certain regions containing a higher concentration of plastic than others (Eriksen et al., 2014). Such a phenomenon occurs in the North Pacific Ocean, in a region between the California coast and the Hawaii islands, in what is often referred to as the Great Pacific Garbage Patch (GPGP) (Lebreton et al., 2018). At least 79 thousand tons of plastic pieces are found floating in the GPGP, and this amount is increasing exponentially (Lebreton et al., 2018). This and other areas of increased plastic debris in the world's oceans have become well-documented regions for the aggregation of obligate surface-dwelling metazoans (i.e., the neuston) as well as biofilm-forming microorganisms (Amaral-Zettler & Zettler, 2015; Haram et al., 2021; Chong & Spencer, 2023).

Whereas many studies have investigated the bacterial component of marine plastic biofilms in terms of membership and community structure, most studies to date have utilized culture-independent methods, such as the partial amplification of the 16S rRNA gene or metagenomics (Kesy & Oberbeckmann, 2019; Kesy & Labrenz, 2020; Amaral-Zettler et al., 2020; Basili et al., 2020; Oberbeckmann et al., 2021; Scales et al., 2021; Vaksmaa et al., 2021). Culture-independent methods are powerful in their potential to analyse whole microbial communities at once and get around the issue that most microorganisms are not readily cultivable using standard plating techniques (Steen, 2019). However, there are advantages to culture-dependent methods, such as the ability to distinguish between viable and dead bacteria and acellular DNA, and the ability to perform more complete descriptions of the taxonomic breakdown of the biofilm community. Importantly, the identification and attribution of expressed secondary metabolites from individual taxa is only possible through the cultivation of living organisms.

One of the defining attributes of floating plastic debris is its high exposure to light and UV radiation. Plastic polymers such as polyethylene (PE) and polypropylene (PP) are less dense than ocean water and tend to remain floating on the ocean's surface even after biofouling, except for when they occur in shapes with insufficient volume-to-surface ratios, such as films (Lebreton & Slat, 2018; Rynek & Tekman, 2024). While other materials can float on the ocean's surface, very few, if any, are as recalcitrant as plastic; a comprehensive study that mapped the GPGP found floating plastic pieces with legible manufacturer labels that dated the plastic as far back as the 1970s (Lebreton et al., 2018; Rynek & Tekman, 2024).

Due to their UV-protective qualities, the production of photoreactive molecules, like carotenoids, would be highly beneficial in a floating biofilm community. At least 324 different carotenoid molecules have been described in 179 different bacteria species, grouped into either C30, C40, C45, or C50 carotenoids, based

on the number of carbons (Yabuzaki, 2017). They can be further classified by whether they are composed of only carbon and hydrogen (carotenes) or whether they are also oxygen-bearing (xanthophylls) (Ram & Mitra, 2020). Further chemical modifications are also possible, such as glycosylation, which does not change the optical properties of the carotenoids but might serve functions when carotenoids are associated with photosynthetic reactive centres (Azai et al., 2020). The most common biologically produced carotenoids are non-glycosylated C40 carotenes such as phytoene, 15-cis-phytofluene, all-trans-phytofluene, lycopene,  $\beta$ -carotene, and  $\alpha$ -carotene (Yabuzaki, 2017). Despite their wide range in molecular structure, at their core, all carotenoids have similar functions- they protect against reactive oxygen species produced inside the cells, in particular related to UV light exposure, or are produced by organisms as photoreactive molecules that assist in light-harvesting processes and assist in maintenance of cell membrane fluidity at lower temperatures (Cogdell et al., 2000; Dieser et al., 2010).

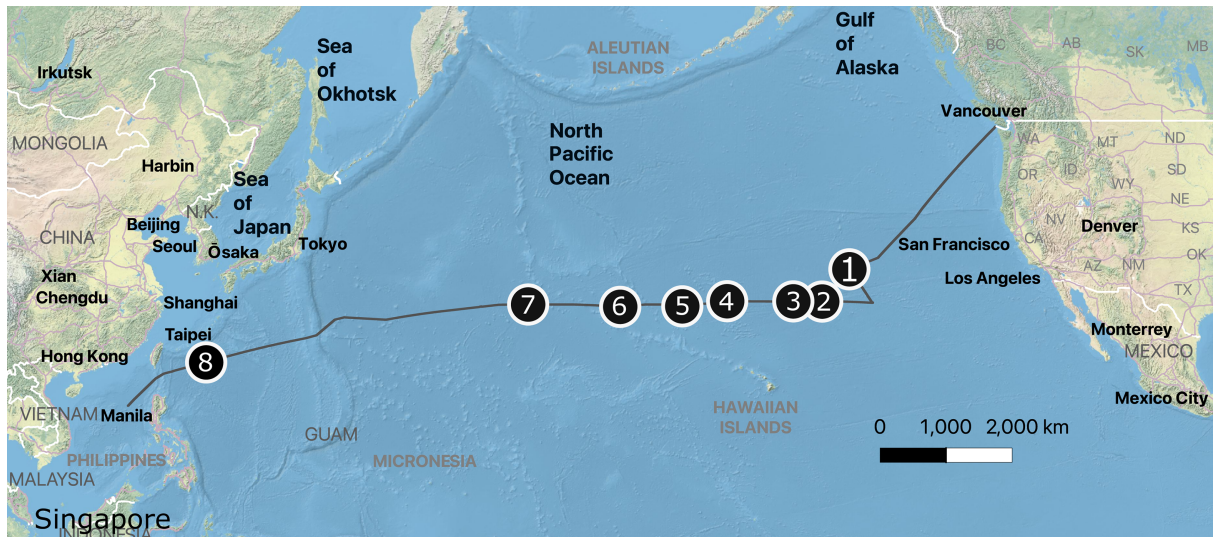
We hypothesized that organisms attached to plastic pieces on the ocean's surface would likely contain the biosynthetic pathways to produce these photoreactive molecules. To test this hypothesis, we combined culture-independent and culture-dependent community analysis, whole-genome sequencing, and HPLC-UV/Vis & MS profiling. We cultivated 67 bacteria isolates directly from biofilms attached to plastic sampled from the GPGP and focused on four isolates from taxonomically divergent families that produced photoreactive pigments. Whole genome sequencing allowed us to map their pigment-producing genes and perform an in-depth analysis of their enzymatic pathways. In addition, we looked for evidence of increased carotenoid-related genes in plastic biofilm-related metagenomes compared to the surrounding water.

## RESULTS

### Taxonomic composition of prokaryotic plastic settlers

A scientific expedition across the North Pacific Ocean was performed and multiple sampling stations were selected within the region of the GPGP and in background locations with expected lower loads of plastic debris (Figure 1). Plastic debris was sampled from surface water for whole-community sampling (cultivation-independent) and cultivation. Background water samples were also taken at each sampling station.

Whole community analysis based on the biofilm communities from surface water-sampled plastic revealed no significant differences in overall community structure based on polymer type (PE or PP) (Figure S1, Table S1). In contrast, biofilm communities on both PE



**FIGURE 1** Cruise transect across the North Pacific Ocean, from Vancouver to Singapore, performed on RV SONNE expedition SO268/3. Eight sampling locations were selected along the transect for plating, plastic biofilm community analysis, or both. For community analysis, water samples were also collected to determine the background water community.

and PP showed significant differences compared to both particle-associated (filtered through a 3- $\mu\text{m}$  filter) and free-living (filtered through a 0.22- $\mu\text{m}$  filter) water communities (Table S1). Plastic-associated communities were statistically different from water communities when both the centre of the data and the variance around the data, were compared (Figure S1, Table S1). While there were not enough plastic pieces taken from each station to perform statistical analysis, the overall communities did not appear to cluster based on the station (Figure S1).

Direct cultivation from plastic pieces was performed on the cruise using a range of incubation methods to target a wide diversity of bacteria. Heterogeneous colony phenotypes grew from surface water plastic debris sampled from the GPGP (Figure S2). Different coloured pigments were visible, ranging from red, orange, yellow, beige, and black (Figure S2). Based on a qualitative assessment, the amount of pigmentation on the plate did not appear to be influenced by the light conditions used during the initial incubation on the cruise. Sixty-seven isolates survived being passaged *in vitro* and were selected for full-length 16S rRNA gene sequencing and phylogenetic analysis (Figure 2). The majority of these 67 GPGP Bacteria (GPGPB) fell into the Proteobacteria ( $n = 52$ ) phylum, with the remainder being found in the Actinobacteriota ( $n = 11$ ) and Firmicutes ( $n = 4$ ) phyla (Figure 2). The most represented family in the cultivates was *Pseudoalteromonadaceae* ( $n = 30$ ), followed by *Micrococcaceae* ( $n = 8$ ) and *Halomonadaceae* ( $n = 5$ ). The rest of the isolates were assigned to the *Bacillaceae* ( $n = 4$ ), *Dermabacteraceae* ( $n = 3$ ), *Moraxellaceae* ( $n = 3$ ), *Vibrionaceae* ( $n = 3$ ), *Alteromonadaceae* ( $n = 3$ ), *Rhodobacteraceae*

( $n = 2$ ), *Stappiaceae* ( $n = 2$ ), *Erythrobacteraceae* ( $n = 2$ ), *Pseudomonadaceae* ( $n = 1$ ), and *Erwiniaceae* ( $n = 1$ ) families (Figure 2).

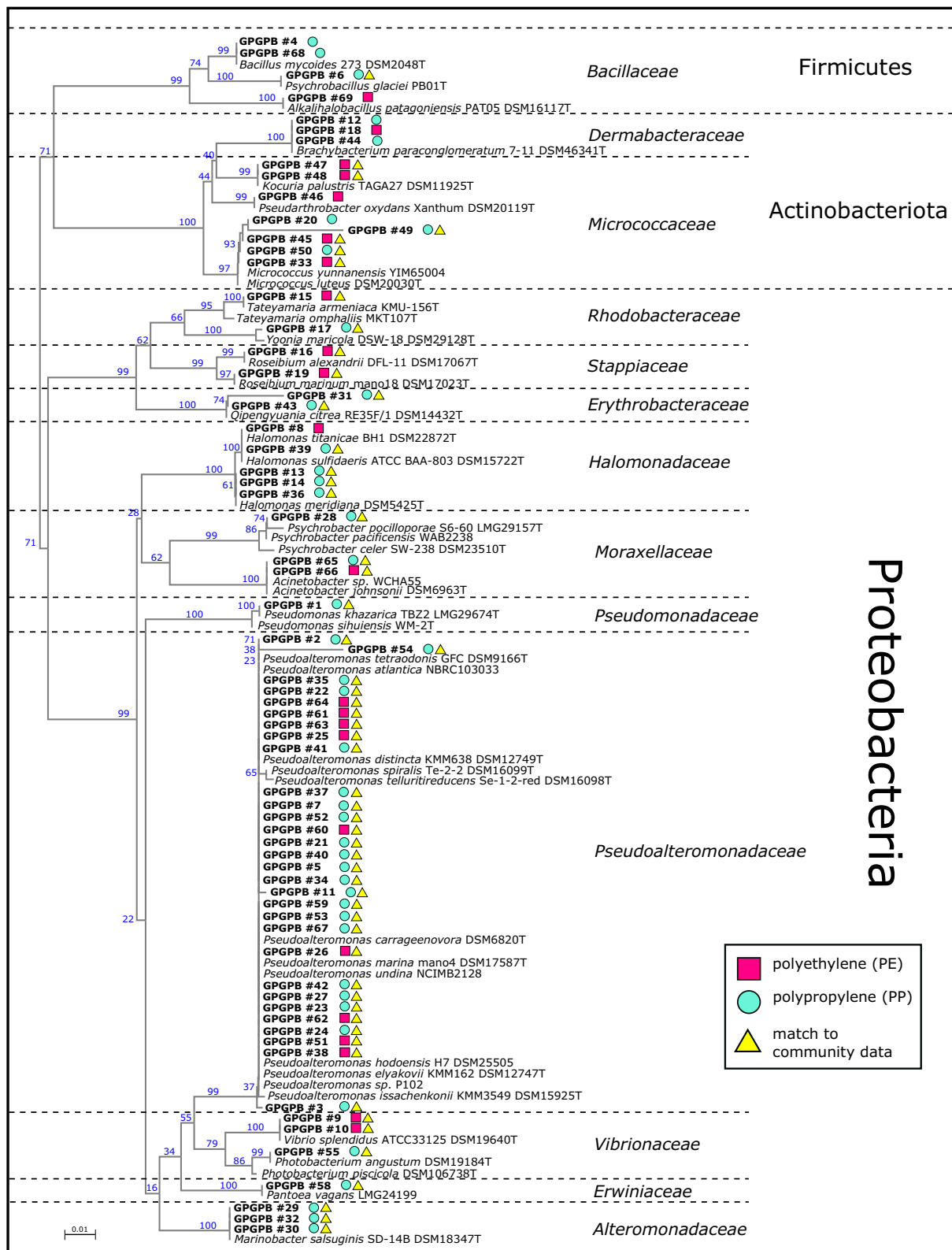
Overlap was found between the 16S rRNA gene sequences of the cultured GPGP isolates and the 16S rRNA amplicon whole community data. Then, 60 out of the 67 cultivated GPGPBs matched with at least one taxonomic unit from the community analysis at  $\geq 95\%$  sequence identity across the aligned portion of the 16S rRNA gene (Figure 2). However, since the community data amplifies just the V3-V4 variable region of the 16S rRNA gene ( $\sim 400$  bp), amplicon sequence variants (ASVs) can only be resolved down to the genus level. Thus, to say that a GPGP cultivate matched the same ASV is simply to state that they belong to the same genus and would likely be grouped into the same taxonomic unit.

## Genomic profiling of four pigmented isolates

Four cultivates from four different families—GPGPB12, 17, 31, 33—that remained pigmented during passage in the laboratory and whose pigmentation was present during growth on solid and liquid media were selected for further analysis (Figure 3).

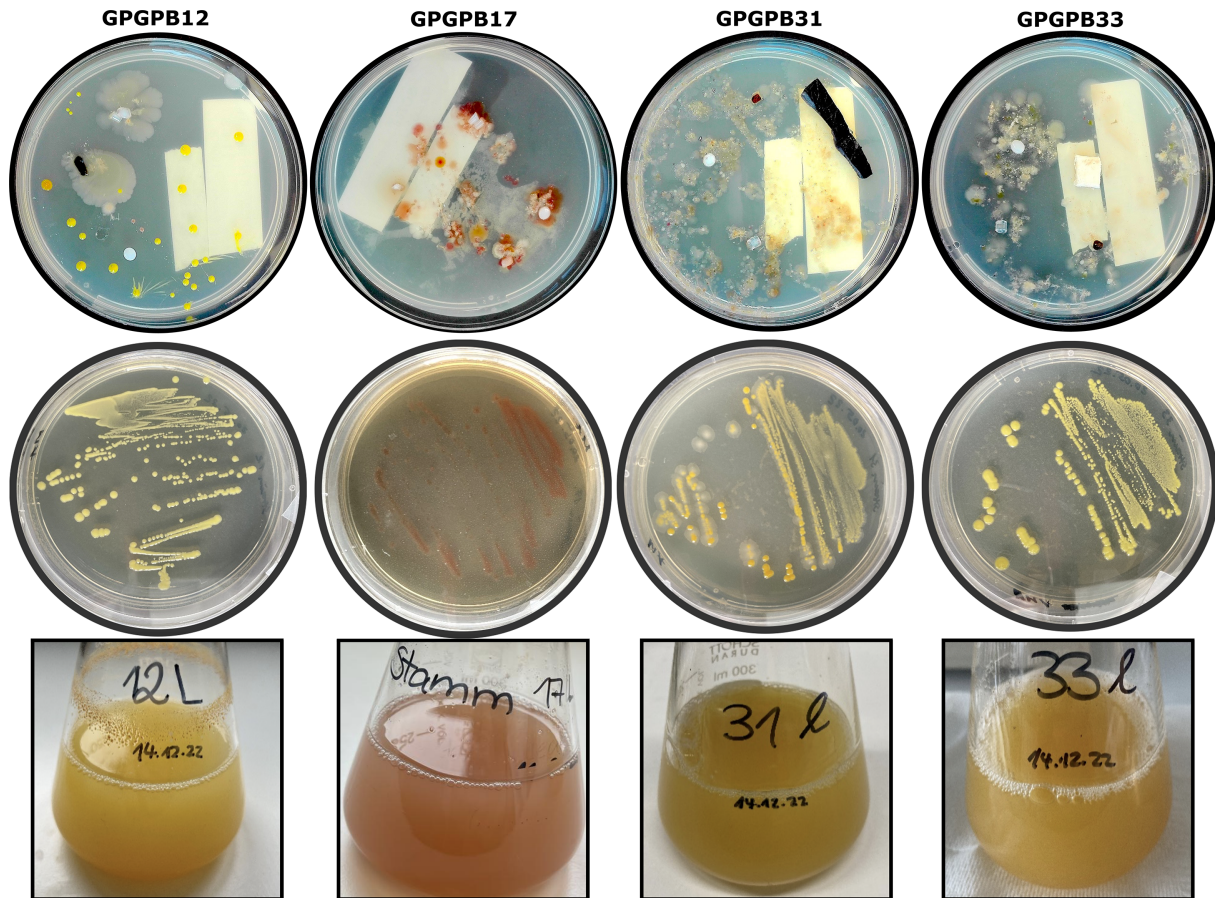
Analysis of the whole genomes of GPGPB12, 17, 31, and 33 was performed to obtain species-level assignments and detailed metabolic information. While a phylogenomic tree placed GPGPB12 as a *Brachy bacterium paraconglomeratum*, GPGPB31 as a *Qipenyguania citrea*, and GPGPB33 as a *Micrococcus yunnanensis*, the closest taxonomic placement the tree





**FIGURE 2** Phylogenetic tree of the 67 Great Pacific Garbage Patch Bacteria (GPGPB) isolates and their nearest neighbours. Tree built on the whole 16S rRNA gene sequence. Labels on the right of the tree indicate the corresponding taxonomic family and phylum. The neighbour-joining algorithm was used to construct the tree. Confidence values based on 1000 bootstraps are displayed in blue. A teal circle indicates that the corresponding GPGPB grew up from a polypropylene (PP) piece, while a magenta square indicates it grew up from a polyethylene (PE) piece. Yellow triangles represent that the corresponding GPGPB was found in culture-independent community data (Figure S2).





**FIGURE 3** The original plate (first row), passaged plate (second row), and liquid broth (third row) of the four pigmented Great Pacific Garbage Patch Bacteria (GPGPB), 12, 17, 31, and 33 isolates, respectively.

**TABLE 1** Percent average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) between the four pigmented whole genomes isolated from GPGP plastic debris and the most closely related whole genome publicly available on NCBI. Since the most closely related *Yoonia* genome showed less than 90% ANI and 70% dDDH with GPGPB17, a representative for every *Yoonia* species with a sequence whole genome was included for comparison.

Genome A	Genome B	ANI (%)	dDDH (%)
GPGPB12	<i>Brachybacterium paraconglomeratum</i> KCTC 9916	95.42	58.8
GPGPB17	<i>Yoonia maricola</i> DSM 29128	80.37	22.5
	<i>Yoonia rosea</i> DSM 29591	78.17	20.6
	<i>Yoonia vestfoldensis</i> DSM 16212	74.01	18.9
	<i>Yoonia vestfoldensis</i> SKA53	73.98	—
	<i>Yoonia sediminilitoris</i> DSM 29955	73.95	19.5
	<i>Yoonia sediminilitoris</i> CECT 8284	73.95	—
	<i>Yoonia vestfoldensis</i> SMR4r	73.90	—
	<i>Yoonia tamlensis</i> strain DSM 26879	73.86	18.9
	<i>Yoonia maritima</i> DSM 101533	73.35	18.5
	<i>Yoonia maritima</i> YPC211	73.24	—
GPGPB31	<i>Qipengyuania citrea</i> CGMCC	97.81	81.8
GPGPB33	<i>Micrococcus yunnanensis</i> DSM 24531	97.39	83.3

could assign to GPGPB17 was within the *Yoonia* genus (Figure S3). Average nucleotide identity (ANI) analysis agreed with the results from the phylogenomic tree and revealed that GPGPB17 did not share more than 81% ANI with any previously sequenced *Yoonia* genome

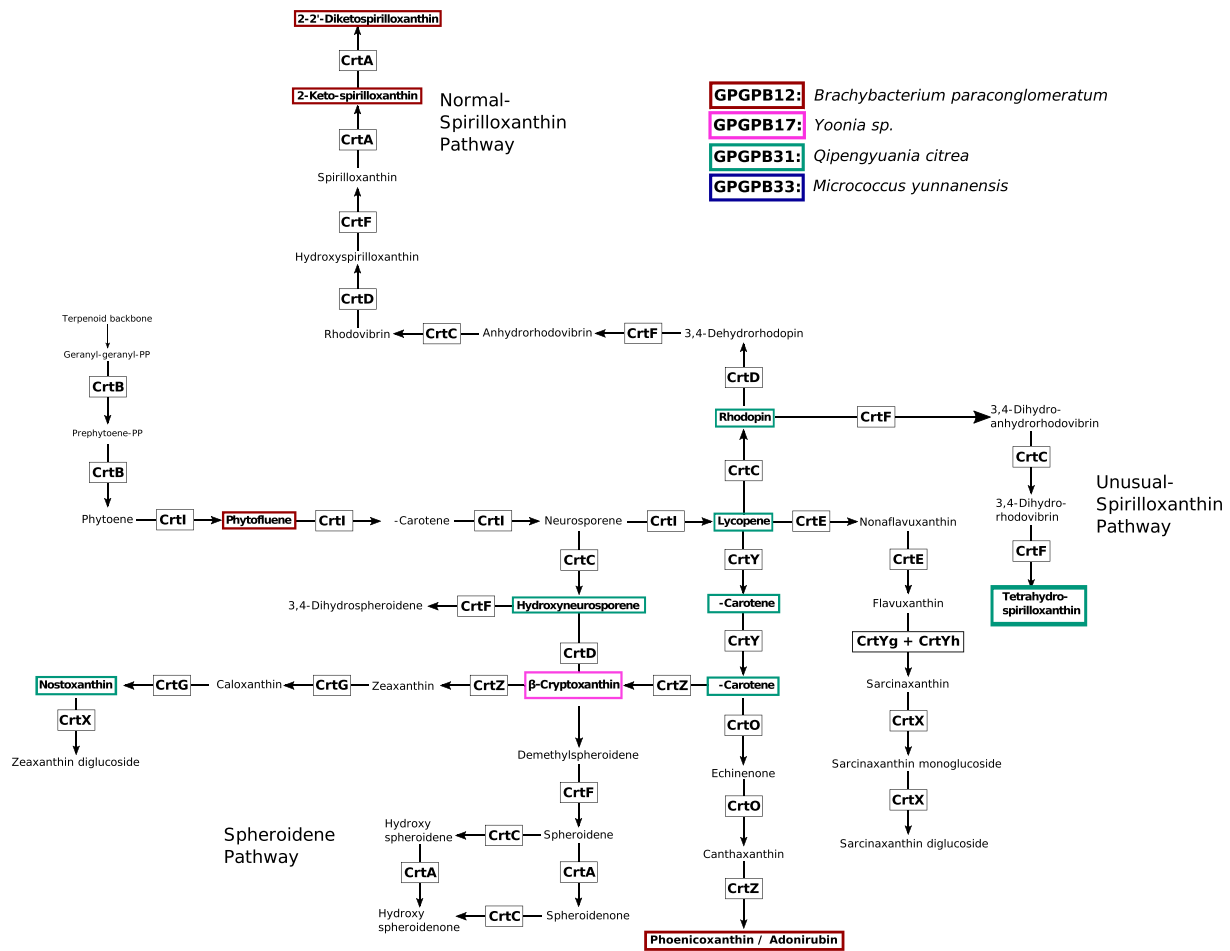
(Table 1). Two bacterial genomes should share an ANI of 94% or higher to be considered members of the same species (Jain & Rodriguez-R, 2018). However, digital DNA–DNA hybridization (dDDH) analysis differed slightly from the results of the ANI analysis and the



phylogenomic tree. While dDDH also clearly showed that GPGPB17 fell below the 70% cutoff to be considered the same as any currently known *Yoonia* species (18.5–22.5%), GPGPB12 also fell below the dDDH

species cutoff when compared to *B. paraconglomeratum* KCTC 9916 (58.8%) (Table 1) (Auch & von Jan, 2010).

Genetic analysis of the annotated whole genomes of the four pigmented strains was performed to detail



**FIGURE 5** Relevant carotenoid biosynthesis pathways based on KEGG. The pathways were included either because the corresponding enzymes were found in one of the four genomes or because a carotenoid molecule in that pathway was expressed and identified. Expressed and identified carotenoids are bolded and bordered by a coloured box: maroon for Great Pacific Garbage Patch Bacterium (GPGPB)12, pink for GPGPB17, green for GPGPB31, and blue for GPGPB33.

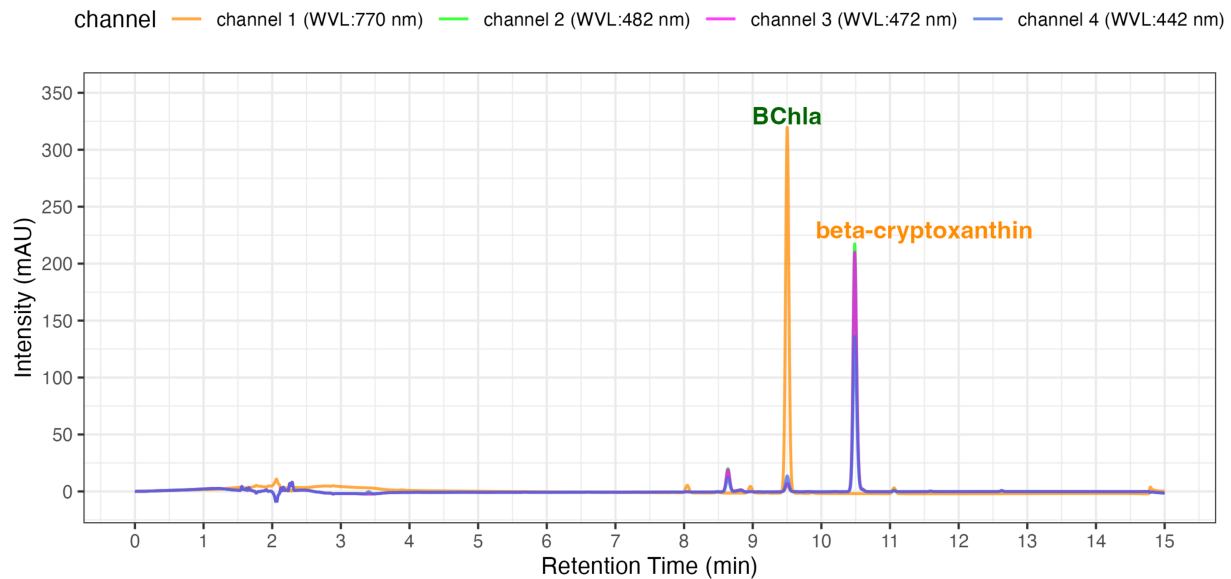
**TABLE 2** Measured mass ( $m/z$ ) and molecular formulas of identified carotenoids. The most abundant carotenoids per strain are indicated in bold.

Strain	Carotenoid	Major ion ( $m/z$ )	Formula
<b>GPGPB12</b>	Keto-spirilloxanthin	610	$C_{42}H_{58}O_3$
	Phytofluene	542	$C_{40}H_{62}$
	<b>Phytofluene</b>	<b>542</b>	<b><math>C_{40}H_{62}</math></b>
	Diketospirilloxanthin <sup>a</sup>	626	$C_{42}H_{56}O_4$
<b>GPGPB17</b>	<b><math>\beta</math>-Cryptoxanthin<sup>b</sup></b>		<b><math>C_{40}H_{56}O</math></b>
<b>GPGPB31</b>	<b>Nostoxanthin/Tetrahydrospirilloxanthin</b>	<b>601</b>	<b><math>C_{40}H_{56}O_4/C_{42}H_{64}O_2</math></b>
	Rhodopin/Hydroxyneurosporene	555/557	$C_{40}H_{58}O/$ $C_{40}H_{60}O$
	Non-distinguishable mix of ( $\alpha$ , $\beta$ , $\gamma$ -) carotenes/ lycopene		$C_{40}H_{56}$

<sup>a</sup>A mixed elution that also contained a molecule with a mass similar to Adonirubin.

<sup>b</sup> $\beta$ -Cryptoxanthine was identified solely via HPLC-UV/VIS and comparison to an internal standard.



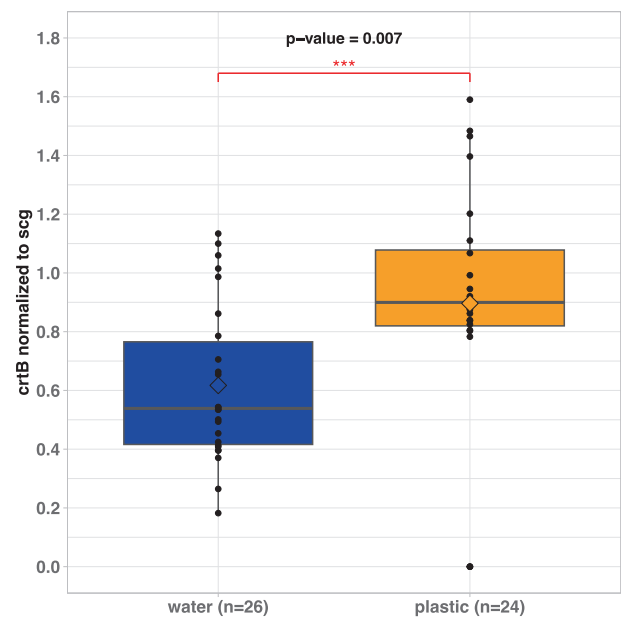


**FIGURE 6** HPLC-UV/Vis of Great Pacific Garbage Patch Bacteria (GPGPB)17.

their exact pigment and phototrophic-related gene elements. Each of the four strains contained a different assortment of genes related to carotenoid production (Figure 4, Table S2). For GPGPB12 (*B. paraconglomeratum*) and GPGPB17 (*Yoonia* sp.), all the identified pigment-related genes occurred together in gene clusters. In addition, for GPGPB17 (*Yoonia* sp.), its carotenoid-related genes occurred alongside genes related to the biosynthesis of bacteriochlorophyll *a* (BChla), in what is referred to as a photosynthetic gene cluster (PGC) (Brinkmann & Göker, 2018). In contrast, both GPGPB31 (*Q. citrea*) and GPGPB33 (*M. yunnanensis*) contained small carotenoid gene clusters around the rate-limiting enzyme-encoding gene *crtB*, with additional carotenoid-related genes scattered throughout their genomes (Figure 4, Table S2).

### Targeted metabolomics and relevance of carotenoid production

Targeted metabolomics was combined with the reconstruction of known carotenoid biosynthesis pathways based on genomic information to determine the identity of the produced carotenoids (Figures 4 and 5, Tables 2, S2). Metabolic reconstruction of possible carotenoid pathways in GPGPB12 (*B. paraconglomeratum*) showed that it contained the carotenoid-related enzymes phytoene synthase (CrtB), phytoene desaturase (CrtI) and lycopene beta-cyclase (CrtY). In addition, a thiamine thiazole synthase predicted in GPGPB12 showed medium homology (95% query coverage and 30% sequence identity) to the beta-carotene ketolase (CrtO) of the Gram-positive bacteria *Gordonia paraffinivorans* (GAC84397.1), suggesting this enzyme might have functionality within carotenoid biosynthesis. Based on these enzymes, GPGPB12



**FIGURE 7** *crtB* gene counts in publicly available metagenomes in plastic biofilms (orange) and surface water communities (dark blue) in the North Pacific. CrtB counts were normalized by the single-copy marker gene (*scg*) abundance after correcting for gene length.

should be able to produce C40 carotenes, which also corresponds to its yellow pigment when grown in vitro (Figure 5). HPLC-UV/Vis analysis showed that GPGPB12 produced six elution peaks whose absorption spectrum was in the range characteristic of carotenoids; however, none matched the included standards (Table S3). Additional analysis with mass spectrometry (MS) revealed the identity of two of these carotenoid molecules to be phytofluene (542 *m/z*), another to be 2-keto-spirilloxanthin (610 *m/z*) and one elution appeared to contain two



molecules, 2,2-diketoxipirilloxanthin (626.6  $m/z$ ) and adonirubin (581.1  $m/z$ ) (Table 2). Two carotenoid molecules could not be identified by either method. Interestingly, no proteins from the GPGPB12 whole genome matched with any homology protein sequences of CrtZ, which is required for the biosynthesis of adonirubin, or CrtC, CrtD, and CrtF, all of which are required for the production of 2-keto-spirilloxanthin and 2,2-diketoxipirilloxanthin.

Similar to GPGPB12, the genome of GPGPB31 (*Q. citrea*) was annotated to contain the enzymes CrtB, CrtI, and CrtY in a central carotenoid cluster. In addition to these enzymes, GPGPB31 was also found to have genes to produce the enzymes CrtZ, CrtG, and CrtE (Figure 4, Table S2). HPLC-UV/Vis revealed this isolate had six elution peaks within the absorption spectrum characteristic of carotenoids (Table S3). MS measured the masses characteristic of nostoxanthin or tetrahydrospirilloxanthin (601  $m/z$ ), rhodopin (555  $m/z$ ), and hydroxy-neurosporene (557  $m/z$ ) (Table 2). One additional elution peak appeared to contain a mixture of carotenes ( $\alpha$ ,  $\beta$ -, and/or  $\gamma$ -carotene) and lycopene with overlapping UV spectra. The molecules within the other three elution peaks could not be identified with either HPLC-UV/Vis or MS. While GPGPB31 contains the predicted enzymes necessary to produce nostoxanthin, homology searches using known CrtC proteins could not locate this enzyme within the GPGPB31 genome, even though this enzyme is considered necessary for the production of tetrahydrospirilloxanthin, rhodopin, and hydroxy-neurosporene (Figure 1).

In addition to the carotenoid-related enzymes in GPGPB31, GPGPB33 (*M. yunnanensis*) was annotated to contain the carotenoid-related enzymes CrtX and CrtR (Figures 4 and 5, Tables 2, S2 and S3). Instead of the traditional beta-cyclase (CrtY), GPGPB31 contained CrtYg and CrtYh, which are two C50 beta-cyclase subunits (Figure 4, Table S2). Altogether, this provides GPGPB33 with the potential to produce less common glycosylated C50 xanthophylls, such as zeaxanthin diglucoside and sarcinaxanthin diglucoside (Figure 5). While HPLC-UV/Vis determined GPGPB33 had two elution peaks within the absorption spectrum characteristic of carotenoids, it could not be identified either through comparison to internal standards or through MS (Table S3).

Based on its annotated carotenoid-related genes, carotenoid production in strain GPGPB17 (*Yoonia* sp.) is the most complex of the four strains analysed, with multiple separate enzymatic pathways possible. Instead of the gene to produce CrtY, it contained the gene for an acyclic carotenoid 1,2-hydratase (CrtC). This, along with the genes for the production of CrtA, CrtD, and CrtF, allows it to branch down the normal and unusual spirilloxanthin pathways, as well as the spheroidene pathway (Figures 4 and 5, Table S2). Thus, GPGPB17 had the potential to produce numerous carotenoid biomolecules, such as spirilloxanthin and its derivatives and spheroidene and its derivatives

(Figure 5). Despite its large genetic potential for the biosynthesis of various carotenoids, HPLC-UV/Vis absorption spectrum analysis revealed that GPGPB17 produced only two molecules within the absorption spectrum characteristic of a carotenoid (Table S3). A comparison to an internal standard revealed the identity of this molecule to be the C40 carotenoid  $\beta$ -cryptoxanthin (Table 2, Figure 6). HPLC-UV/Vis analysis also further confirmed the production of BChla.

The gene *crtB* encodes phytoene synthase, the enzyme required for the production of any carotenoid pigments. Thus, the presence of the *crtB* gene in a genome is highly indicative of whether carotenoid biosynthesis is possible. An analysis was performed to evaluate the prevalence of the *crtB* gene across other described species within the genus of each of the four cultivated GPGP bacteria (Figure S4) and across publicly available plastic and water metagenomes sampled from the North Pacific (Figure 7, Table S4). The *crtB* gene occurs in multiple genomes of taxonomically related strains, suggesting that carotenoid production might be widespread in the corresponding genera of the four GPGPB (Figure S4). Furthermore, our analysis revealed that *crtB* is statistically more prevalent in surface-sampled plastic metagenomes than those of the surrounding water communities in the North Pacific (Figure 7, Table S4).

## DISCUSSION

### Plastic settlers often display pigmentation and belong to species typically associated with eukaryotes

Plastic pollution represents a relatively recent but ubiquitous and permanent constituent of marine ecosystems and comes with its own unique array of environmental inputs to which marine organisms must adapt. There is perhaps no better place to study this anthropogenically introduced portion of marine ecosystems than the GPGP, as it contains a high portion of floating plastic pieces that have potentially been trapped there for decades (Lebreton et al., 2018). Our whole community analysis showed that the biofilm communities of floating plastic pieces from the GPGP were significantly different from those of the surrounding water and that there was no differentiation in the plastic community based on polymer type (Figure S1, Table S1), findings that agree with previous research on marine and brackish water-sampled plastic pieces (Scales et al., 2021). Other factors, such as spatial and temporal parameters, as well as biofilm interactions, hence seem to be shaping these specific microbial communities on floating marine plastic debris.

By directly cultivating the biofilm from plastic pieces from the surface waters of the GPGP, we were able to identify and analyse plastic-associated bacteria at the



species level, conduct in vitro analysis, perform whole-genome sequencing and perform targeted metabolomics on selected produced secondary metabolites. Our research has revealed that many bacterial species associated with higher marine organisms are present in the cultivable portion of the GPGP plastic biofilm, including multiple *Pseudoalteromonas* species, *Psychrobacter pocilloporae*, *Tateyamaria omphalii*, *Roseibium alexandrii*, and *Pantoea vagans*, *Vibrio splendidus* (Biebl & Pukall, 2007; Kurahashi & Yokota, 2007; Brady & Venter, 2009; Beaz-Hidalgo & Balboa, 2010; Zachariah et al., 2016), which are otherwise found associated with species such as pufferfish, bivalves, seaweed, and marine algae (Simidu et al., 1990; Sawabe, 2000; Higgins et al., 2007) (Figure 2). Recent research has found that plastic pollution not only alters aquatic ecosystems at the prokaryotic level but at the eukaryotic level as well (Haram et al., 2021; Chong & Spencer, 2023). More research needs to be done to understand how plastic debris alters community structure and functioning across taxonomic domains, as this could modify ocean food webs and nutrient cycling.

A potential selective trait of floating plastic is its high exposure to light and UV radiation. Pigment biomolecules are in many cases associated with both light harvesting and protection against UV radiation, and thus, it is not surprising that there was a wide range of pigmentation in isolates grown directly from GPGP-sampled plastic (Figure S2). However, many isolates across all taxonomic groups lost the pigmentation that was visible on the original culture plate while being isolated in a laboratory environment. As with many secondary metabolites, the production of carotenoids is a regulated process. Some carotenoids are produced constitutively, while others are induced by light, or have triggers that are not understood (Takano, 2006). To increase the complexity, temperature also plays a role in bacterial carotenoid production, as carotenoids can influence membrane fluidity (Akulava et al., 2024; Dieser et al., 2010). We selected four plastic-associated isolates for in-depth analyses that displayed constitutive pigment production, under laboratory conditions.

The four isolates (Figure 3) were sequenced, and a genetic analysis of the pigmented biomolecule synthesis pathways was performed. The four isolates originated from different taxonomic families—GPGPB12-*Dermabacteraceae*, GPGPB17-*Rhodobacteraceae*, GPGPB31-*Erythrobacteraceae*, and GPGPB33-*Micrococcaceae* (Table 1, Figure S3), and each contained the entire enzymatic pathway to produce at least one carotenoid molecule (Figures 4 and 5, Table S2), suggesting that selective pressures promoted maintenance of these particular metabolic pathways across bacterial taxa despite costs associated with these traits. In addition, GPGPB17, a likely new species of *Rhodobacteraceae* within the *Yoonia* genus (Table 1), contained a PGC

that included bacteriochlorophyll *a* (BChl*a*), making it capable of a form of photosynthesis (Figures 4–6, Tables 2, S2 and S3). The presence of multiple carotenoids across taxa, and a phototrophic isolate, strongly underlines the importance of light management in these plastic-associated biofilms.

## Diverse carotenoid production in plastic-associated bacteria

While carotenoid production was shared between all four isolates, each contained a different repertoire of carotenoid-related genes and produced different identified carotenoid molecules. GPGPB12, *B. paraconglomeratum*, had the genetic repertoire to produce enzymes required to synthesize multiple C40 carotenes, such as phytoene, phytofluene,  $\zeta$ -carotene, neurosporene, lycopene,  $\gamma$ -carotene and  $\beta$ -carotene (Figures 4 and 5, Table S2). However, of these only phytofluene production was confirmed via MS (Table 2). Phytofluene is the second step in carotenoid metabolism, after phytoene (Figure 5). Phytofluene is mostly known for being one of the few colourless carotenoids and being found in such foods as tomatoes and carrots, however, its unique function inside prokaryotes is not generally understood (Meléndez-Martínez et al., 2015). Additional carotenoids identified through our methods were produced through the normal spirilloxanthin pathway (Figure 5, Table 2), although the enzymes currently believed necessary for this could not be identified using traditional homology searches against previously identified protein sequences. There are two possible explanations for this. One is that other enzymes in *B. paraconglomeratum* are fulfilling the functions of CrtC, CrtD, CrtF, CrtZ, and CrtO. For example, CrtI and CrtD are both desaturases and as the metabolic maps of carotenoid production make very clear, enzymes are regularly reused in different steps of these pathways (Figure 5). In addition, another enzyme, a thiamine thiazole synthase, has 30% amino acid identity to CrtO, which might indicate some overlap in function, however, sequence analysis alone is unlikely to determine whether it could carry out the functions of CrtO in the synthesis pathway. Another possibility is that these enzymes do exist in *B. paraconglomeratum* but are so different at the DNA and amino acid levels to previously identified versions of these genes and gene products that they do not come up in homology searches. *B. paraconglomeratum* is a high H + C gram-positive bacteria, and this type of bacteria is little studied, not just with regard to their carotenoid pathways. No carotenoid-related enzyme sequence was available to use for the genomic searches from within *B. paraconglomeratum*'s genus, family (*Dermabacteraceae*), or order (*Micrococcales*). Our results hopefully provide enough evidence that studying





carotenoid production in *Brachy bacterium* bacteria is a realm of research worth pursuing in the future.

More is known about carotenoid product pathways within the genus of GPGB31, *Qipengyuania citrea*. In members of the *Qipengyuania* genus, carotenoid-related gene clusters have been divided into four types (Liu & Pei, 2022; Tareen, 2022). Based on the identified genes and gene products, the carotenoid gene cluster within GPGB31 represents type I. In previous research, type I-related carotenoid clusters were associated with the production of the yellow C40 xanthophyll nostoxanthin, which agrees with GPGB31's colony phenotype, carotenoid gene mapping, metabolic pathways reconstruction, and identified secondary metabolites (Figures 3–5, Tables 2 and S2) (Liu et al., 2022). There is evidence that, in addition to its UV-related antioxidant capabilities, nostoxanthin can potentially protect against reactive oxygen species produced in relation to salt stress, an attribute that would be beneficial in the salty environment of the Pacific Ocean (Kikukawa et al., 2021; Jiang & Seo, 2023). Further identified molecules in GPGB31 were rhodopin and hydroxyneurosporene (which is sometimes also referred to as chloroxanthin). While hydroxyneurosporene production has been reported for other Proteobacteria (Sandmann, 2023), this is the first known description in *Qipengyuania*. Finally, the production of C40 carotenoids that occur early in the metabolic pathways was verified for *Qipengyuania* by the detection of a mixture of carotenes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) and lycopene.

Genomic analysis revealed that GPGB33 (*M. yunnanensis*) can produce a couple of additional carotenoid products that were not found in the other isolates, such as CrtX and CrtR (Figure 4, Table S2). Also, unlike GPGB12 and GPBP31, we were not able to positively identify any of the carotenoid molecules that GPGB33 produced (Table 2, Table S3). The methods for secondary metabolite characterization included in this study rely on previously identified carotenoids (i.e. available standards), which can lead to identification bias. In addition, carotenoids often have overlapping elutions, measured absorption spectra, and nearly identical measured mass, adding additional difficulties to identification. However, due to the genomic metabolic mapping, it is possible to deduce that GPGB33 has the genetic capability to produce C50 carotenoids, in particular, the C50 xanthophylls sarcinaxanthin and sarcinaxanthin derivatives, sarcinaxanthin monoglucoside and di-glucoside (Figures 4 and 5, Table S2). So far, the production of sarcinaxanthin, and its derivatives, have only been described in very few bacteria, including marine bacteria such as the *Micrococcus* species *luteus* and *yunnanensis*, and *Kocuria palustris* (Roman et al., 2010; Osawa & Ishii, 2010; de Cássia Dias Mendes-Silva et al., 2021). In *Micrococcus*, the genes to produce the C50 beta-cyclase (CrtY) are split into two subunits (CrtYg and CrtYh), which work

together to catalyse the reaction of flavuxanthin to sarcinaxanthin (Figure 4) (Netzer Roman et al., 2010). Potentially, CrtYg and CrtYh can also catalyse the reaction of lycopene to  $\gamma$ - and then  $\beta$ -carotene. As with the other carotenoids discussed thus far ( $\gamma$ -carotene,  $\beta$ -carotene, and nostoxanthin), the C50 xanthophylls are known primarily for their antioxidant and UV-protective qualities, with sarcinaxanthin even being patented as a potential sunscreen (US patent: US20130078203A1) (de Cássia Dias Mendes-Silva et al., 2021). Future research could study these less-known carotenoids and use marine biofilms as a model to explore new photoreactive molecules.

## A new species of aerobic anoxygenic phototrophic bacteria

The photoreactive properties of the carotenoid identified in GPGB17 are more complex than those of the other isolates. GPGB17, a potentially new species in the *Yoonia* genus (Table 1), was found to contain a PGC, where carotenoid-related genes are grouped with genes related to phototrophy, such as those necessary to produce BChla (Figures 4 and 5, Table S2) (Brinkmann et al., 2018). The close association between these genes within the genome is due to the important interactions between the two groups of pigment molecules in photoheterotrophic bacteria, including members of the *Rhodobacteraceae* family. In these bacteria, carotenoids both protect against the production of reactive oxygen species when molecular oxygen interacts with BChla and assist directly in the harvesting of light (Niedzwiedzki & Dilbeck, 2015). In the latter function, the carotenoid molecule absorbs light within the spectral range of 450–550 nm, which is otherwise inaccessible to BChla, and transmits some of this absorbed energy to membrane-bound photochemical reaction centres (Niedzwiedzki & Dilbeck, 2015). In addition, carotenoids can also assist in the structural stabilization of the photoreactive centres (Ashikhmin et al., 2017). PGCs, like those found in GPGB17, are scattered throughout genomes in the *Rhodobacteraceae* family and are even potentially spread through horizontal transfer on extrachromosomal plasmids (Brinkmann et al., 2018). The consortium of carotenoid-related genes in these PGCs allows for multiple possible carotenoid biosynthesis pathways, such as the normal and unusual spirilloxanthin pathways, as well as the spheroidene pathway (Figure 5). Phototrophic bacteria have been known to switch between different carotenoids in these pathways under different settings, such as in *Rhodobacter sphaeroides*, which switches between spheroidene and spheroidenone production depending on whether it is grown anaerobically or aerobically, respectively (Šlouf et al., 2012). Certain *Rubribacterium* bacteria produce spheroidene and



spirilloxanthin at the same time as BChla, though their functions regarding its photoreactive centre have not been elucidated (Pujalte et al., 2014). In this analysis, absorbance analysis revealed one large carotenoid-related peak alongside a BChla peak in GGPB17, suggesting that this species of *Yoonia* produced one primary carotenoid when grown aerobically in light (Figure 6, Table 2). Comparison to a standard revealed this carotenoid to be  $\beta$ -cryptoxanthin, a carotenoid synthesized from  $\beta$ -carotene through CrtZ (Figure 5), which has been reported in another marine Alphaproteobacterium, *Erythrobacter* sp. kj5.

The inclusion of a PGC in GGPB17 implies that this potentially new species of *Yoonia* is an anoxygenic phototrophic (AAP) bacterium. AAP bacteria carry out respiration on organic carbon substrates and derive a significant portion of their energy requirements from phototrophy, depending on the absence or presence of light, respectively (Koblížek, 2015). In light exposure AAP bacteria reduce their respiration while also increasing ATP concentration, indicating that phototrophy is more efficient than, and inhibitory towards, respiration in AAP bacteria (Hauruseu and Koblížek, 2012; Piwosz, Villena-Alemany and Mujakić, 2022). When light decreases, respiration can increase again, allowing AAP bacteria to thrive in both high-light and low-light conditions (Piwosz, Villena-Alemany and Mujakić, 2022). Such a trait would provide an advantage over chemoheterotrophic bacteria under carbon limitation. In marine environments, AAP bacteria play an important role in the 'microbial loop' through the recycling of organic matter and influencing overall bacterial growth efficiency (Koblížek, 2015). While other studies have pointed to the presence of photosynthetic-related genes and proteins in bacterial plastic biofilm communities (Oberbeckmann et al., 2021; Bos & Kaul, 2023), this is, to the best of our knowledge, the first description of an AAP bacterium associated with plastic in the marine environment.

### Carotenoid production in association with a plastic-lifestyle

We postulated that carotenoid production could be a physical adaptation that is selected for, or enriched, in surface-water plastic biofilms due to the high level of exposure to light and UV radiation in this environment. Indeed we confirmed that the *crtB* gene, encoding for phytoene synthase, was found in significantly higher quantities in plastic biofilms than in surrounding water communities (Figure 7). Biofilms on natural, or any type of non-plastic, pelagic surfaces from the North Pacific, or similar environment, could not be included in this analysis because no such data were available. Nevertheless, our analysis emphasizes the high relevance of carotenoid-producing bacteria in plastic biofilms in the marine environment.

## CONCLUSIONS AND FUTURE DIRECTIONS

Four distinct bacterial species that were isolated from the plastic sampled from the GGP exhibited the remarkable ability to produce a diverse range of carotenoids. One of these isolates was revealed to potentially be a new species of *Yoonia* sp., which was identified as photoheterotrophic due to its production of BChla, indicating it was an aerobic AAP bacterium. For the majority of identified carotenoids, this represents the first known published reference of the carotenoid in the corresponding bacterial species. In addition, genomic analysis could not locate all gene/gene products required to produce some of the identified carotenoid molecules. Overall, this points to the lack of knowledge on carotenoids and how their production functions across prokaryotes. Future work would benefit from the availability of standards for a suite of these pigments. Further, transcriptomics could help explain the plasticity in pigment production and the seemingly complex ways in which carotenoid production is regulated in plastic-associated marine bacteria. The production of carotenoids and the ability to perform anoxygenic phototrophy would be advantageous traits for organisms inhabiting floating plastic particles of the North Pacific, as it enables them to absorb light and offers protection against stress caused by UV radiation. We encourage increased research into the connection between photo-reactive molecules and microbial communities of surface water plastic pollution. In addition, more research is needed to better understand how prevalent AAP bacteria are in this habitat and what effect this type of energy harvesting has on carbon and nutrient cycling both within the biofilm and in the surrounding marine environment.

## EXPERIMENTAL PROCEDURES

### Pacific Ocean expedition

The SO268/3 transit of the R/V SONNE occurred between 31.05.2019 and 05.07.2019 from Vancouver, Canada, to Singapore. Along this transect at mostly 30° North, eight sampling stations were selected. Net sampling, consisting of a custom-made scoop apparatus, was used to capture macroplastic (>5 mm) from the water surface, whereas the cat sampling, consisting of a Neuston catamaran that has a trapping net with a mesh size of 330  $\mu$ m, was used to sample microplastics (<5 mm) from the water surface (Rynek et al., 2024).

All plastics in this study were determined to be composed of either PE or PP using the following method. Polymer identification of most macroplastics and microplastics was performed by ATR-FTIR spectroscopy



with an Agilent Cary 620 FT-IR microscope equipped with a Ge ATR crystal connected to a 15× objective. Measurements were performed with 16 co-added scans between 400 and 4000  $\text{cm}^{-1}$  and a spectral resolution of 4  $\text{cm}^{-1}$ . Measured spectra were assigned to either PE or PP by matching them with the SiMPle software (v. 1.01, database version 1.0.1). Raw spectra and first derivatives were used equally weighted for spectral comparison in the ranges of 3300–2700  $\text{cm}^{-1}$  and 1900–1250  $\text{cm}^{-1}$ . Polymer identification of microplastics of 2–5 mm was done using a microPHAZIR RX Analyser (Thermo Fisher).

## Plastics for culture-independent analysis

Biofilms were sterilely sampled from an area of 16 × 16 mm on the scoop-sampled macroplastics using alcohol-flamed sterile scalpels and scraped directly into microcentrifuge tubes. Additionally, at each station, 1 L of water was subsequently filtered first through a 3  $\mu\text{m}$  filter (3  $\mu\text{m}$  Isopore TSTP 04700 Millipore, Merck KGaA, Frankfurt, Germany), to capture the particle-attached water community, and then through a 0.22  $\mu\text{m}$  filter (0.22  $\mu\text{m}$  Isopore GTTP04700 membrane filters Millipore, Merck KGaA, Frankfurt, Germany), to capture the free-living water community. Filters were then transferred to microcentrifuge tubes. Tubes containing biofilms and filters were frozen at  $-80^\circ\text{C}$  until DNA extraction was performed after the expedition.

## Cultivation

Prior to the expedition, artificial seawater (ASW) plates were prepared to contain 40 g sea salt, 14 g peptone (Difco), 1 g yeast (Difco), and 15 g bacto-agar (Difco) per 1 L MilliQ water. Before the agar solidified, PE and PP pieces sterilized by soaking overnight in 70% ethanol were added to each plate to give the cultivates a solid surface in the agar to form biofilms. During the expedition, plastic pieces were collected from surface water. Microplastic pieces from the cat sampling were rinsed with sterile seawater and placed directly on the plate. Macroplastics from the scoop were either (i) cut into smaller pieces with sterile scissors, the cut pieces rinsed in sterile seawater and plated, or (ii) the biofilm was scraped off using alcohol-flamed sterile scalpels into sterile seawater, and the biofilm solution was plated. Three control plates were incubated alongside the experimental plates. Incubation was done with different cultivation methods to capture a range of different organisms; full light and low light at room temperature (RT); no light at  $4^\circ\text{C}$ , and outside for direct sunlight. After the 35-day expedition, all plates were sent back to Germany at  $4^\circ\text{C}$  for analysis. Individual morphologies were described by sight. Each morphology was restreaked on ASW media and

incubated at RT until individual colonies formed. Individual colonies were inoculated in 20 ml liquid ASW and grown up until confluence. Half of the grown-up liquid culture was frozen with 20% glycerol and kept at  $-80^\circ\text{C}$  for long-term storage, while the other half was centrifuged at 10,000 rpm for 10 min, had the supernatant removed and stored at  $-20^\circ\text{C}$  until DNA extraction.

## Whole-community profiling, genomic extraction, and sequencing

For the cultivates, DNA extraction of whole bacterial cells was performed using the Macherey Nagel NucleoSpin Microbial DNA Mini kit following the manufacturer's protocol. For each cultivar, the pellets from three tubes of 2 ml bacterial culture were pooled. Final elution was done with a 100  $\mu\text{l}$  elution buffer. DNA concentration was measured using a Nanodrop (Thermo Fisher). Amplification of the 16S rRNA gene was performed using the 27F (GAGTTTGATCMTGGCTCAG) and 1492R (GGYTACCTTGTTACGACTT) primers (Frank et al., 2008). The following PCR protocol was used: initial melt  $95^\circ\text{C}$  for 1 min, cycle melt  $95^\circ\text{C}$  for 30 s, anneal  $58^\circ\text{C}$  for 30 s, elongation  $72^\circ\text{C}$  for 1.5 min, cycle repeat to cycle melt step 29X (for a total of 30 cycles), and final elongation  $72^\circ\text{C}$  for 5 min. The full-length (Sanger) sequencing of the 16S rRNA gene was performed by LGC Genomics GmbH using their microtitre plate Premium Run service on 20  $\mu\text{l}$  of non-purified PCR products. For the Sanger sequencing, three primers were used, to achieve complete, high-quality 16S rRNA gene sequences: 27F, 1492R (sequences listed above), and 519F (CAGCAGCCGCGGTAATAC) (Mao et al., 2012). The consensus sequence between the forward and reverse sequences was determined in FinchTV Version 1.5.0.

For culture-independent, whole community analyses, DNA was extracted from scraped biofilms and water filters using the Macherey Nagel DNA nucleospin soil kit (Nucleo Spin Soil kit Macherey-Nagel, Düren, Germany), as per the manufacturer instructions. DNA concentration was measured using a nano Qubit (ThermoFisher). Next-generation Illumina Sequencing was performed on the Illumina MiSeq platform using a V3 kit for 2 × 300bp paired-end reads with a sequencing amount of 20 million reads, using the 341F (forward 5'-CCTACGGGNGGCWGCAG-3') and 785R (reverse 5'-GACTACHVGGGTATCTAAKCC-3') primer set (Thijs et al., 2017). The raw sequence reads were provided by LGC Genomics GmbH (Germany), and the demultiplexed and primer-clipped reads were used as primary input data for the subsequent bioinformatic pipeline. Bioinformatics was performed using Qiime2 on the EVE cluster (miniconda environment) (Bolyen et al., 2019). Reads were truncated at a Quality Score of Q20, and dada2 was used for denoising, quality





filtering, chimera checking, and paired-end read joining (Callahan et al., 2016). Chimeras were removed, and the taxonomic assignments were performed with the SILVA database (release 132) after a scikit-learn naïve-bayes classifier was trained for the reads. Data were exported for further statistical analyses in R. The R package phyloseq was used for data visualization. Chloroplasts, mitochondria, samples with fewer than 10,000 sequences, and ASVs with 0 counts were removed from the data. The Bray-Curtis distance metric, which takes into account both relative sequence abundances and the presence/absence of individual ASVs, was used to measure the dissimilarity of each sample and was displayed via principal coordinate analysis. To determine whether communities were different in species composition across the habitats, we used two statistical measurements (Table S1). The first, permutational multivariate analysis of variance, measures whether there are statistical differences in the centroids of data from different sample types in Euclidean space (Anderson, 2017). The second measures whether there are statistical differences in the dispersion, or variance, around the centroids between data from different sample types (Anderson, 2001). They are both ways of asking whether multivariate data in Euclidean space differ from each other. *P* values were obtained based on 999 permutations. The results of this analysis are displayed in Table S1.

### Taxonomic classification of the GPGPB isolates

The initial classification of the isolates was performed using publicly available databases containing collections of bacterial nucleotides. Full-length 16S sequences were first compared to NCBI's standard Nucleotide collection (nr/nt) using blastn (Altschul et al., 1990), followed by a 16S-based ID search against EzBioCloud's database (Yoon et al., 2017). To create a phylogenetic tree, complete 16S rRNA gene sequences of the top results from Blastn and EZBioCloud were obtained. The 16S rRNA sequences of nearest neighbours and GPGPB were aligned using multiple alignments via the fast Fourier transformation sequence alignment tool (Kato et al., 2002). Neighbour joining was performed on all conserved sites using the Jukes-Cantor substitution model, with the bootstrap set to 1000 resamples.

To determine if a cultivated GPGP bacterium was also found in the community data, the ASV representative sequences were used to query the full-length 16S rRNA sequences from each strain. Any ASV that matched the query 16S rRNA sequence by at least 95% identity and 100% query coverage was considered a potential candidate for being of the same taxon as the cultured isolate.

### Whole-cell genomic DNA extraction and whole-genome sequencing

For whole-genome sequencing, DNA was extracted using the Macherey Nagel Microbial DNA kit according to the manufacturer's instructions, and DNA concentrations were measured with Qubit. Oxford nanopore sequencing (ONT) library preparation was done using the rapid barcoding kit (RBK110) and, in the case of GPGPB17, the ligation sequencing kit (LSK109). Sequencing was performed on the Gridlon using R9.4.1 flow cells, base-calling was performed using guppy v4.3.4 in the high-accuracy mode for RBK110 and guppy v5.0.16 in the super-accurate mode for LSK109. The assembly of the ONT reads was performed using flye v2.9, resulting in complete genomes (1 contig per replicon) except in the case of GPGPB33 (Kolmogorov et al., 2019). Polishing of long read assemblies was done using first the long read data with medaka v1.4.3 (<https://github.com/nanoporetech/medaka>). Paired-end MiSeq Illumina reads were used for the final polishing. Illumina reads were adapter- and quality-trimmed with bbduk version 39.01 (Bushnell, 2014) and mapped against the genome assembly with bwa-mem (Li, 2013). Then Pilon version 1.24 was used to correct indels and substitution errors (Walker et al., 2014). The final assembly was taxonomically classified with GTDB-tk version 2.1.0 against the Genome Taxonomy Database release 207 (Chaumeil et al., 2019).

### Whole-genome annotation and phylogenomics tree

Gene prediction and annotation of the GPGPB strains was performed with PROKKA version 1.14.6 (Seemann, 2014). To obtain a more comprehensive description of their metabolic capabilities, additional databases were searched: KEGG release 105 (Kanehisa and Goto, 2000), NCBI nr (date accessed 10.12.2022), and InterPro version 5.60-92.0 (Jones et al., 2014). Furthermore, biosynthetic gene clusters and membrane-bound features were identified with antiSMASH version 6.1.1 (Blin et al., 2021) and DeepTMHMM version 1.0.20 (Hallgren et al. 2022), respectively. To explore the evolutionary relationships between the GPGPB and other pigment-bearing bacterial strains within their broader taxonomic context, a phylogenomic tree was reconstructed from a multi-locus sequence alignment of single copy marker genes using the de-novo workflow of GTDB-tk (Chaumeil et al., 2019) with the LG + gamma protein model.

### Extraction of pigments and HPLC analysis

Each strain was streaked onto marine agar (Difco 2216) from glycerol stocks and cultured aerobically at RT (20–



23°C) in the dark until individual colonies formed. A single colony, or group of phenotypically identical colonies, was used to inoculate a 5 ml liquid preculture in a glass culture tube. The preculture was incubated at 28°C for 4 days (strains GPGP12, 17, 33) or 10 days (strain GPGP31), and then 2 ml of it (1%) was transferred into the primary cultures. The primary cultures were grown aerobically in 200 ml of marine broth (Difco 2216) for 6–7 days at RT in ambient daylight on a benchtop shaker (GFL 3017), aerated at 130 rpm.

*Erythrobacter longus* DSM 6997 was obtained directly from DSMZ's culture collection and grown up in marine agar (Difco 2216) as specified, after which it was grown up and maintained the same as the GPGPB isolates. *E. longus* DSM 6997 was selected as a reference strain because it possesses both bacteriochlorophyll *a* and carotenoids, such as zeaxanthin (Ruivo et al., 2014). Bacterial cells were harvested by centrifugation (5250g, 20°C, 30 min). Pellets were resuspended with 3 ml of fresh medium and sonicated using a microtip in an ice bath for 7 min (HTU SONI 130, 1 s pulse, 2 s pause, amplitude 70%, total treatment time 21 min). The suspension was then centrifuged again (10 min 18,000g, RT), the supernatant discarded, and the pellet stored at –20°C. The subsequent extraction of molecules under dim light was based on Ruivo et al. protocols (Ruivo et al., 2014). Pellets were suspended in acetone:methanol 7:2 (v/v), sonicated in a bath (Branson 2210), vortexed, and then placed in a cooling block at –20°C to achieve a total extraction time of 15 min. Cell debris was then removed by pushing the extract through a 0.2 µm Millex Fluoropore PTFE filter into a brown glass vial.

HPLC analysis was immediately performed thereafter using a Dionex Ulti Mate 3000 system equipped with a photodiode array detector DAD UCI-50. The chromatographic separation of molecules was carried out using a C18 column for reversed-phase chromatography (Supelcosil LC-18\_DB). The column temperature was kept at 25°C by column oven TCC-3000SD. The solvent gradient followed the method of Goerick (2002), but only 20 µl of the sample was injected instead of 100 µl (Goerick, 2002). As another modification, an ion pair reagent was used to improve peak separation, consisting of 38.5 g ammonium acetate and 7.5 g tetrabutylammonium acetate in 500 ml of ultrapure water. The mobile phase consisted of acetonitrile:water:ion pair reagent in a ratio of 60:35:5 and pure acetone at a constant/different ratio/s. Pure standards used for comparison were bacteriochlorophyll *a* (Sigma-Aldrich 67H1784), zeaxanthin (Sigma-Aldrich 14681-1mg-F), beta-carotene (DHI-BatchNo:131), alloxanthin (DHI-BatchNo:allo-115), violaxanthin (DHI-BatchNo:viol-145), lycopene (Sigma-Aldrich 502-65-8), astaxanthin (Sigma-Aldrich 472-61-7), lutein (Sigma-Aldrich 57-83-0), and canthaxanthin (Sigma-Aldrich 514-78-3).

Analysis of chromatograms was performed using Chromeleon software version 7.2.10.24543 (ThermoFisher

Scientific). Extracted molecules were measured by their retention time and absorption maxima in the spectra from 200 to 800 nm. HPLC profiles of the standards were added to its internal library. The retention time of extracted molecules was compared to that of the standards as a first step. If there was a match, absorption spectra from 200 to 800 nm of the respective standard and sample peaks were compared as a second step. The Chromeleon software provided a match factor of the respective spectra, which should not be less than 900 for the clear identification of a pigment.

To gain additional information on the carotenoids produced by the four selected strains, 50 ml biomass of each strain was sent on ice to the company Analytice (Strasbourg, France). There, the samples were dried, solubilized in DMSO and injected in high-performance liquid chromatography (inverse phase) coupled to a UV detector (HPLC-UV) at 450 nm, using the standard compounds asthaxanthin (CAS 472-61-7), canthaxanthin (514-78-3), lutein(57-83-0)/zeaxanthin(144-68-3), lycopene (502-65-8), β-carotene (7235-40-7), and β-cryptoxanthin (472-70-8). Additionally, MS was carried out (HPLC-UV/MS) by Analytice to obtain the exact mass of the eluted carotenoid molecules.

## Quantification of *crtB* gene copies in environmental metagenomes from the North Pacific

To support our hypothesis that carotenoid biosynthesis may be a beneficial trait for plastic colonization, we quantified *crtB* gene copies in metagenomic read data sets from floating plastic debris (Bryant et al., 2016) and bulk surface (<50 m depth) water samples collected in the same geographic area during the TARA Ocean expedition and at the Hawaii Ocean Time Series station (Biller et al., 2018) (Table S4). Raw reads were downloaded from the European Nucleotide Archive and quality checked with fastqc v0.11.9 (Andrews et al., 2010). PhiX reads, and 3' sequencing adapters were removed with bbdut v39.01 (Bushnell, 2014), followed by quality trimming using a sliding window of 4 bp with an average quality threshold of 15. After quality trimming, reads either shorter than two-thirds of the initial read length or less than 100bp were discarded. The read\_qc module from MetaWRAP v1.3.2 was further used to detect and remove potential human contamination (Uritskiy, DiRuggiero and Taylor, 2018). Cleaned reads were assembled per sample using metaSPAdes v3.15.5 (Prijbelski & Antipov, 2020), retaining only contigs of at least 1000 bp (seqkit v2.3.1) (Shen et al., 2016).

In the case of the water samples, the metagenome assemblies were obtained directly from the Ocean Microbiomics Database v1, likewise removing short contigs (Paoli et al., 2022). Genes were predicted on



the assemblies using pprodigal v1.0.1 (<https://github.com/sjaenick/pprodigal>) (Hyatt et al., 2010) and functionally annotated with antiSMASH v6.1.1 (Blin et al., 2021) to identify crtB and with kofamscan v1.3.0 (Aramaki et al., 2020) to identify single copy marker genes. Clean reads were mapped to their respective assembly using bwa (Li, 2013), and read counts per gene were obtained with featureCounts v2.0.1 (Liao, Smyth and Shi, 2014). CrtB counts were normalized by the median abundance of 10 single-copy marker genes after correcting for gene length, as described in (Salazar et al., 2019).

## AUTHOR CONTRIBUTIONS

**Brittan S. Scales:** Conceptualization; investigation; writing – original draft; visualization; writing – review and editing; formal analysis; supervision; data curation; methodology. **Christiane Hassenrück:** Writing – review and editing; data curation; visualization; formal analysis. **Lynn Moldaenke:** Methodology; investigation. **Julia Hassa:** Writing – review and editing; data curation; supervision; formal analysis. **Christian Rückert-Reed:** Writing – review and editing; formal analysis; data curation. **Christoph Rummel:** Methodology; investigation; writing – review and editing; formal analysis. **Corinna Völkner:** Formal analysis; investigation; writing – review and editing; methodology. **Robby Rynek:** Writing – review and editing; investigation; formal analysis. **Tobias Busche:** Supervision; resources; data curation; methodology. **Jörn Kalinowski:** Data curation; supervision; resources; methodology. **Annika Jahnke:** Funding acquisition; project administration; supervision; conceptualization; writing – review and editing. **Mechthild Schmitt-Jansen:** Conceptualization; funding acquisition; writing – review and editing; supervision; investigation. **Katrin Wendt-Potthoff:** Conceptualization; funding acquisition; writing – review and editing; supervision; methodology; resources. **Sonja Oberbeckmann:** Conceptualization; funding acquisition; writing – review and editing; resources; supervision; project administration; validation; methodology.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

For the whole community dataset, raw sequence files, including sequencing controls, are available from the NCBI Short Read Archive (SRA) database (BioProject No. PRJNA837054): <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA837054>. The 16S rRNA Sanger sequences are available on NCBI's GenBank with the accession numbers PP494042-PP494110 and the whole genomes of isolates can be accessed within BioProject No. PRJNA925796: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA925796>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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