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Highlight

Pink- and orange-pigmented Planctomycetes produce saproxanthin-type carotenoids including a rare C_{45} carotenoid

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Summary

Planctomycetes are ubiquitous and environmentally important Gram-negative aquatic bacteria with key roles in global carbon and nitrogen cycles. Many planctomycetal species have a pink or orange colour and have been suggested to produce carotenoids. Potential applications as food colorants or antioxidants have been proposed. Hitherto, the planctomycetal metabolism is largely unexplored and the strain pigmentation has not been explored. For a holistic view of the complex planctomycetal physiology, we analysed carotenoid profiles of the pink-pigmented strain *Rhodopirellula rubra* LF2^T and of the orange strain *Rubinisphaera brasiliensis* Gr7. During LC–MS/MS analysis of culture extracts, we could identify three saproxanthin-type carotenoids including a rare C_{45} carotenoid. These compounds, saproxanthin, dehydroflexixanthin and 2′-isopentenyldehydrosaproxanthin, derive from the common carotenoid precursor lycopene and are characterized by related end groups, namely a 3-hydroxylated β-carotene-like cyclohexene ring as one end group and simple hydration on the other end of the molecule. Based on the observed molecule structure we present putative pathways for their biosynthesis. Results support Planctomycetes as a promising, yet mostly untapped source of carotenoids.

Introduction

Plants, bacteria, fungi and algae produce an impressive diversity of nearly 1200 carotenoids currently listed in the Carotenoids Database (Yabuzaki, 2017). Due to their anti-oxidative properties, they play a key role in the protection of the photosynthesis machinery in plants, algae and photosynthetic bacteria, including cyanobacteria and green sulphur bacteria. Furthermore, they can fine-tune the absorption properties of the photosynthesis apparatus, partly as a niche adaptation strategy towards different light conditions (Lichtenthaler, 1987). In this sense, carotenoids are considered as a borderline between primary and secondary metabolism. Interestingly, 311 of the 1182 carotenoids listed in the Carotenoids Database are produced by heterotrophic bacteria, supporting a more general role in the protection against oxidative stress, beyond photosynthesis (Gammone *et al*., 2015). Due to their natural anti-oxidative properties, carotenoids are also valuable compounds for commercial applications. Several carotenoids are approved by the European Union as food supplements with major application as colourants or anti-oxidants (Rao and Rao, 2007; Kallscheuer, 2018), while there is also substantial evidence for health-promoting effects of carotenoids as parts of the human diet (Concepcion *et al*., 2018). Several studies demonstrated that pigments derived from Planctomycetes are incorporated by *Daphnia magna*, a higher trophic level organism (Marinho *et al*., 2019) and evidenced the potential application of this bacterium to be used as single-cell-pigment for colour enhancement (Lage and Antunes, 2018).

Carotenoids belong to the large class of isoprenoids (or terpenoids) and their synthesis follows a concerted

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principle for carbon chain assembly, employing isoprene units as building blocks. A total of 1100 of the 1182 natural carotenoids are tetraterpenoids (C_{40} compounds), formed from eight isoprene monomers. The active form of these monomers, isopentenyl pyrophosphate, is produced by two known metabolic routes: the mevalonate pathway (starting from acetyl-CoA) and the non-mevalonate pathway (starting from pyruvate and glyceraldehyde 3-phosphate), of which the latter is also known as MEP/DOXP pathway (2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway) (Goldstein and Brown, 1990; Lichtenthaler, 2000).

While nearly all plants produce carotenoids, the capability for carotenoid production in bacteria appears to be more restricted to free-living species naturally dwelling in environments with frequently changing conditions such as soil or seawater. Many such species fall within the phylum Planctomycetes. Planctomycetes are a group of Gram-negative–like aquatic bacteria that are ubiquitous, often found associated with phototrophs such as macro- and micro-algae and that play environmentally important roles in global carbon- and nitrogen cycles (Wiegand *et al*., 2018). Several planctomycetal species have a red, pink or orange colour; however, the pigmentation of species in this phylum has not yet been investigated in detail. In this study, we thus analysed the major carotenoids of two planctomycetal strains: the pink species *Rhodopirellula rubra* LF2^T and the orange *Rubinisphaera brasiliensis* Gr7 (Lage and Bondoso, 2011; Bondoso *et al*., 2014). We further analysed the genomes of these organisms aiming at genes coding for enzymes putatively involved in the related biosynthetic pathways, which is the basis for further investigating the ecological and physiological relevance of these natural products in Planctomycetes.

Results and discussion

To examine the carotenoid profiles of Planctomycetes, we extracted and analysed carotenoids from the strains *R. rubra* LF2^T (pink) and *R. brasiliensis* Gr7 (orange) (Fig. 1), which were isolated in northern Portugal from macroalgae surfaces at the coasts of Foz, Porto and Aveiro respectively (Bondoso *et al*., 2014; Lage and Bondoso, 2011). Cells were harvested in the exponential growth phase and solvent extracts of cell pellets were subjected to LC–MS/MS analysis.

During spectrometrical analysis, extracts from both strains yielded a peak at a retention time of 17.1 min with a typical carotenoid UV/Vis spectrum (Fig. 2A), for which the MS/MS spectrum led to no clear identification in the European MassBank (NORMAN MassBank). Manual analysis of the full mass spectrum (Fig. 2B) revealed a sodiated molecule $[M + Na]^+$ at *m/z* 590, and a molecular ion at *m*/z 568, corresponding to the molecular formula $C_{40}H_{56}O_2$. This is also further supported by the observed signal for $[M + Na\text{-}H_2O]^{+\bullet}$ of 572. Obtained fragments in an *in silico* fragmentation approach (Table S1) yielded saproxanthin as the most probable candidate molecule (Fig. 2C). Saproxanthin is a tetraterpene (C_{40}) characterized by a carotenoid β-cycle additionally hydroxylated at C3 as one end group and simple hydration of the most distant double bond at the other end of the molecule. Saproxanthin was already identified in the 1960s as the major pigment in the marine flexibacterium *Saprospira grandis* (Aasen and Liaaen-Jensen, 1966) and is also present in marine species of the family Flavobacteriaceae (Hameed *et al*., 2014). For further supporting our putative identification of this carotenoid in Planctomycetes, we searched for additional data obtained for saproxanthin in the literature. A UV/Vis spectrum indistinguishable from the one obtained in our study was published for Flavobacteriaceae bacterium 04OKA-13-27 (Shindo *et al*., 2007). During the comparison of the spectra, we not only focused on the major peaks in the UV/Vis spectrum but also took characteristic peak 'shoulders' into consideration. The published spectrum was ultimately shown to belong to saproxanthin, thereby also substantially consolidating identification in our study. Saproxanthin shows potent antioxidative properties rendering it also interesting for commercial applications (Shindo *et al*., 2007).

An ion at 16.4 min with $[M + H]^+$ of 581 in the *R. rubra* LF2^T extract indicated the presence of a second compound in this strain. The observed mass differs from that of saproxanthin by 12 Da. According to our *in silico* fragmentation approach (Fig. 3A, Table S2) presence of an additional keto group at position 4 of the cyclohexene β-cycle of saproxanthin and presence of an additional double bond in the ring is likely. Taken together, this information suggests dehydroflexixanthin to be the compound in question ($C_{40}H_{52}O_3$, 580.4 Da, Fig. 3B). It was already shown earlier that dehydroflexixanthin is formed from the naturally produced carotenoid flexixanthin by auto-oxidation, leading to the introduction of the double bond between C2 and C3 in the cyclohexene ring (Coman and Weedon, 1975). Flexixanthin ($C_{40}H_{54}O_3$, 582.4 Da) was identified in the 1960s in *Flexibacter* species and represents the major carotenoid in these microorganisms (Nakagawa, 2015). Taking the published information on spontaneous oxidation of flexixanthin into account it is likely that flexixanthin is the actual carotenoid also produced in *R. rubra* LF2^T.

A third peak from the extract of *R. rubra* LF2^T eluted at 20.9 min and gave a UV/Vis spectrum similar to saproxanthin, but an $[M + H]^+$ signal of 635 (Fig. 4A and B). The difference of 66 Da indicates the presence of an additional isoprene unit in this compound. Ligation of

Fig. 1. Colours of the two investigated strains. The photographs show liquid cultures and cells streaked on agar plates of pink-pigmented *R. rubra* LF2^T and orange-pigmented *R. brasiliensis* Gr7.

isoprene (C_5H_8) to saproxanthin ($C_{40}H_{56}O_2$) would yield a compound with the sum formula $C_{45}H_{64}O_2$ and an $[M + H]^+$ signal of 637, which differs in 2 Da from the observed signal at 635. *In silico* fragmentation (Table S3) provides evidence that this peak corresponds to 2′-isopentenyldehydrosaproxanthin, a derivative of the auto-oxidation product of saproxanthin (explaining the 2 Da difference in mass) additionally harbouring an isopentenyl residue at the C2′-position (Fig. 4C). Presence of 2′-isopentenylsaproxanthin was reported earlier in the marine flavobacterium *Jejuia pallidilutea*, which was isolated in Japan and South Korea (Lee *et al*., 2009; Takatani *et al*., 2014). Taking the observed auto-oxidation into account, it is likely that 2′-Isopentenylsaproxanthin is also the actual compound produced by *R. rubra* LF2^T. This compound is a rare C_{45} carotenoid, of which only 12 compounds are listed in the Carotenoids Database. In the few known examples of C_{45} saproxanthin-type carotenoids, isopentenylation took place at C2′, which is in accordance with the observed fragment ions obtained in our study.

Next, we aimed at identifying the underlying biosynthetic pathway for the three identified carotenoids from the common carotenoid precursor lycopene. To this end,

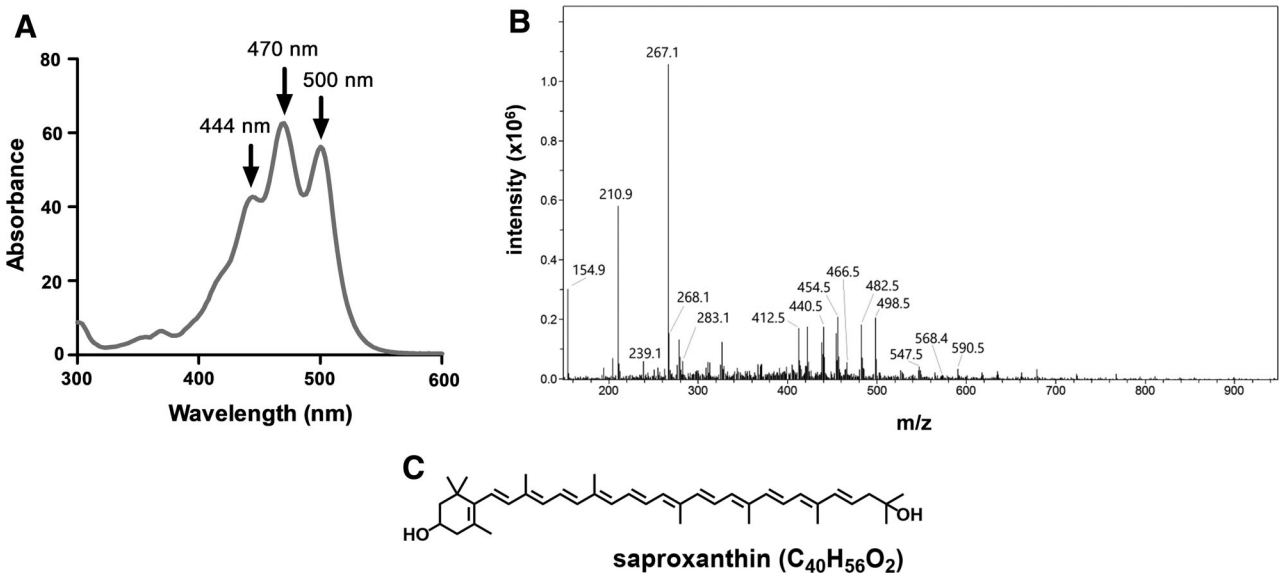

Fig. 2. Collected data leading to the identification of saproxanthin. The UV/Vis spectrum (A), full mass spectrum (B) and structural formula (C) of saproxanthin are depicted.

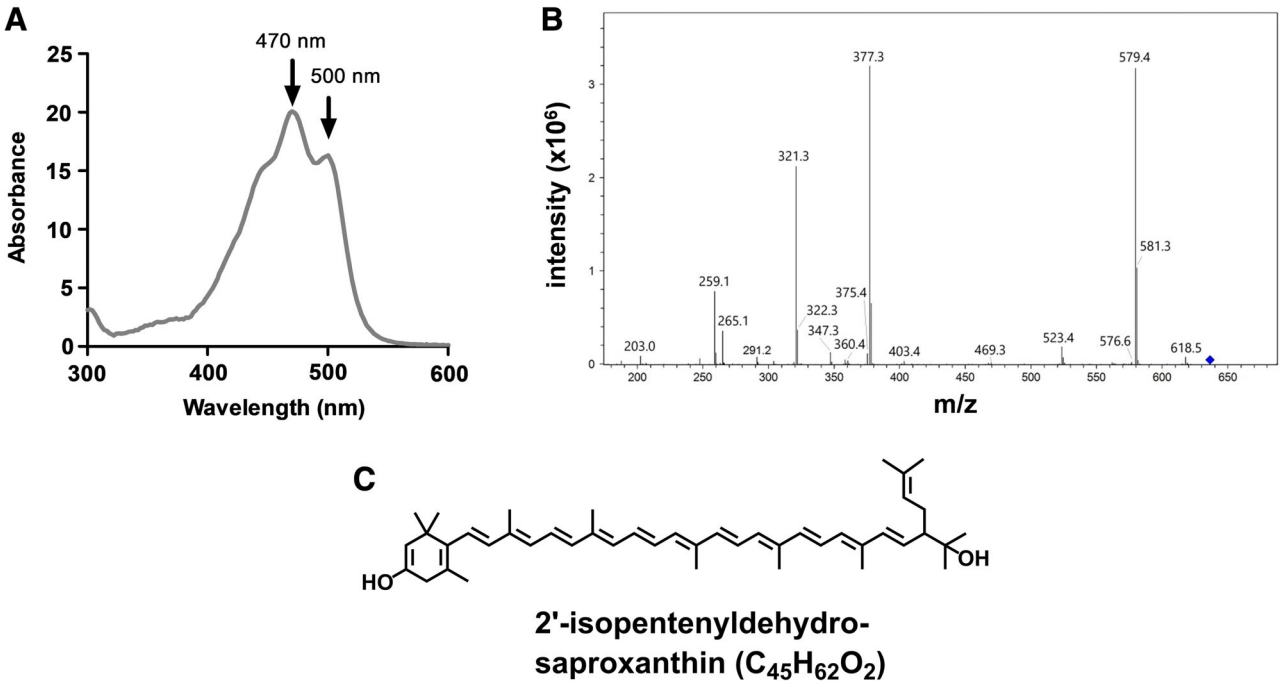

Fig. 3. Collected data leading to the identification of dehydroflexixanthin. The MS/MS spectrum of *m/z* 581 (A) and structural formula (B) of dehydroflexixanthin are depicted.

we analysed genome data also taking previously described pathways in Flexibacteria and Flavobacteria into account. Our analysis focused on the published genomes of *R. brasiliensis* DSM 5305^T and of *R. rubra* SWK7 (Klindworth *et al*., 2014; Scheuner *et al*., 2014). It is reasonable to argue that 1′-hydroxytorulene (myxocoxanthin) could be an intermediate of the planctomycetal pathway as this compound harbours the

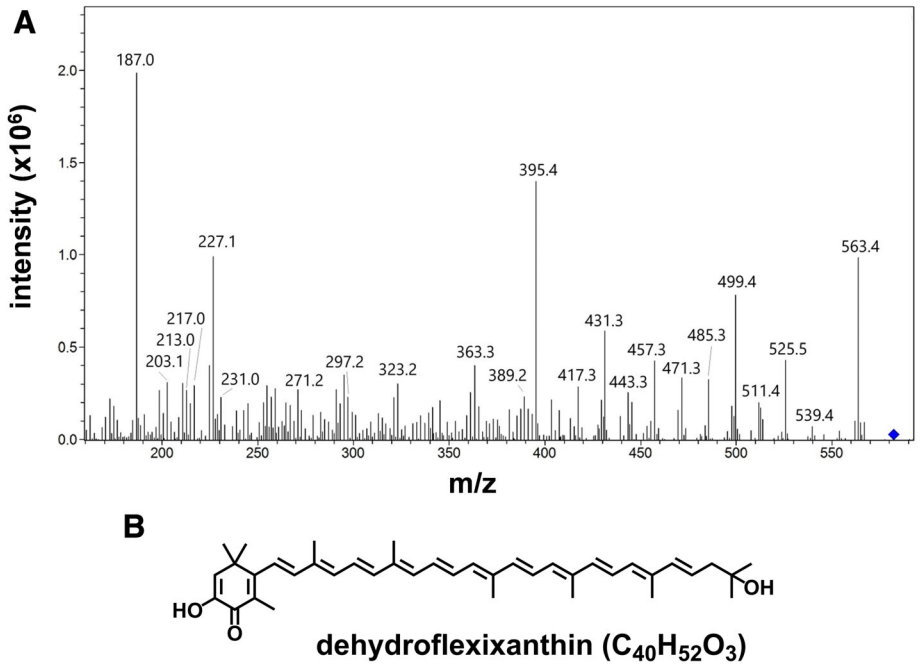

Fig. 4. Collected data leading to the identification of 2′-isopentenyldehydrosaproxanthin. The UV/Vis spectrum (A), MS/MS spectrum of *m/z* 635 (B) and structural formula (C) of 2′-isopentenyldehydrosaproxanthin are depicted.

modified end groups and serves as a common precursor for production of the three identified compounds (Fig. 5). Production of 1′-hydroxytorulene from lycopene requires the activity of a carotenoid 1,2-hydratase, a carotenoid 3,4-desaturase and a carotenoid β-cyclase. These reactions were already proposed for the flexixanthin biosynthetic pathway in the marine bacterium *Algoriphagus* sp. (Tao *et al*., 2006). Further conversion of 1′-hydroxytorulene to flexixanthin *via* deoxyflexixanthin is also in accordance with the pathway proposed in *Algoriphagus* sp., which involves a carotenoid ketolase and a carotenoid 3-hydroxylase (Fig. 5).

Conversion of 1′-hydroxytorulene to saproxanthin requires a carotenoid 3-hydroxylase activity for the introduction of the hydroxy group at C3. Subsequent isopentenylation of saproxanthin leading to 2′-isopentenylsaproxanthin is catalysed by an elongase (isopentenyltransferase) (Fig. 5). The responsible enzyme might be a homologue of the lycopene elongase LyeJ, which was identified in the bacterioruberin pathway of the archaeon *Haloarcula japonica* (Yang *et al*., 2015). As the natural substrate of LyeJ is lycopene, the exact order in which elongase, hydratase and cyclase catalyze modification reactions at the end group for ultimately yielding 2′-isopentenylsaproxanthin remains to be elucidated.

For getting a first insight into how the carotenoid biosynthetic pathway in Planctomycetes could be encoded, we performed sequence analyses based on local alignments and Hidden Markov Models with various enzymes known to synthesize the identified compounds in other microorganisms. For enzymes in the proposed pathway

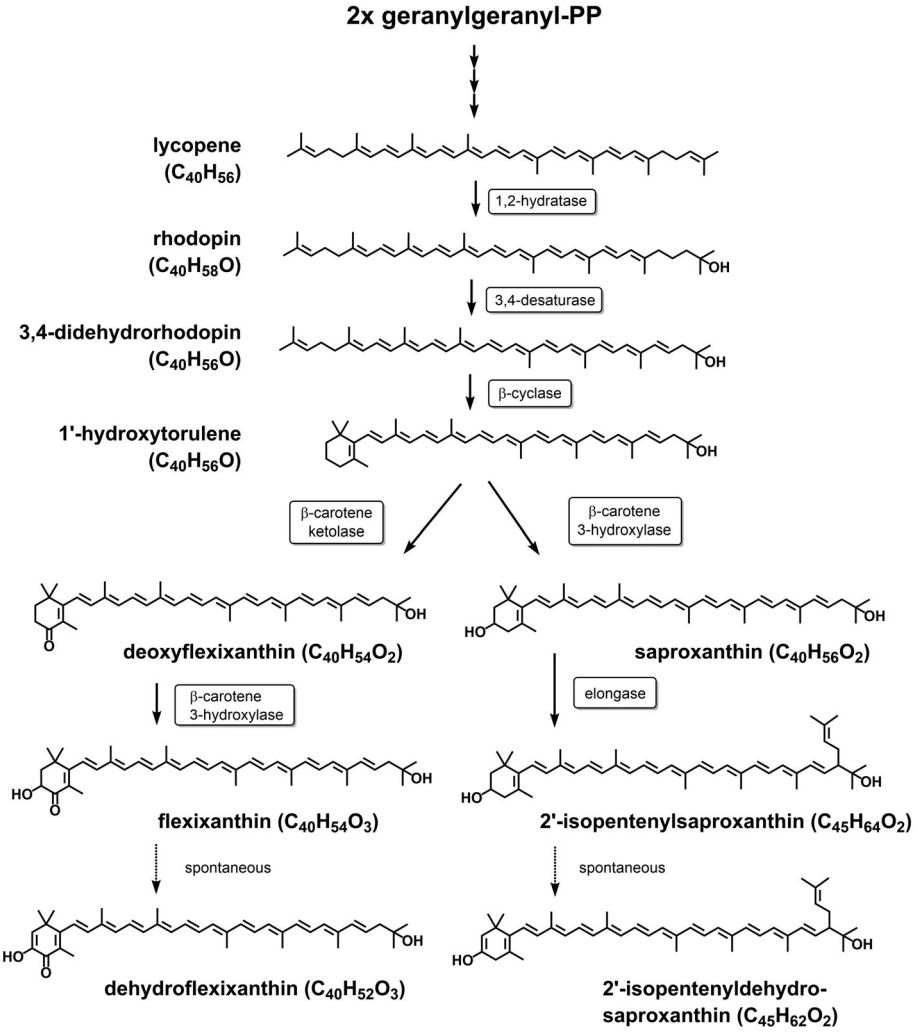

Fig. 5. Postulated pathway for production of the carotenoids identified in *R. rubra* LF2^T and *R. brasiliensis* Gr7. The postulated metabolic route leading to saproxanthin, 2′-isopentenyl(dehydro)saproxanthin and (dehydro)flexixanthin from the common carotenoid precursor lycopene is shown. The precursor geranylgeranyl pyrophosphate (geranylgeranyl-PP) is produced from isopentenyl-PP obtained from the MEP/DOXP pathway in planctomycetal strains. Three arrows indicate multiple reaction steps that are not depicted in detail.

starting from lycopene, produced from the MEP/DOXP pathways in both species, our analysis yielded no hits in *R. brasiliensis* DSM 5305^T and *R. rubra* SWK7 (Table 1). In *R. brasiliensis* DSM 5305^T, we could not even identify an enzyme candidate for phytoene desaturase, which is responsible for biosynthesis of the common carotenoid precursor lycopene and which is present in *R. rubra* SWK7 (Table 1). Taken together, we could not find candidate enzymes of the carotenoid biosynthesis pathway in the two investigated Planctomycetes so far, although the presence of identified compounds implies that enzymes for such a pathway must be present. Structural organisation and domain architecture of involved enzymes might be different from canonical ones, thereby providing a possible explanation of why these enzymes escaped our analysis. Planctomycetes are amongst the bacterial phyla with the most predicted genes of unknown function (40%–50%) and at the current stage, despite using state-of-the-art bioinformatic tools, the carotenoid biosynthesis pathway in Planctomycetes remains undiscovered. We must, therefore, stress that the shown pathway (Fig. 5) was postulated based on information from microorganisms known to produce these compounds. However, as the required reactions are basically given based on the end groups of the final compounds, we assume that they might be similar in the here investigated species (although the order of reactions may differ).

The probable lack of phytoene desaturase activity in *R. brasiliensis* DSM 5305^T is particularly interesting as it might be part of the explanation for differences in colony colours (pink or orange). This, however, will have to be addressed in follow-up studies. Remarkably, our observations remain astonishing when taking into account that genes coding for enzymes of the MEP/DOXP pathway responsible for the formation of the acyclic carotenoid precursor phytoene were easily identified with high significance parameters (Table 1).

Based on the UV/Vis spectra and information from the literature saproxanthin, 2′-isopentenylsaproxanthin and flexixanthin have yellow to orange colour and it is thus likely that the orange colour of *R. brasiliensis* Gr7 results from the presence of mixtures of these compounds. At this stage, it remains to be elucidated which compounds are responsible for the pink colour. There are in principle three theories for explaining this observation: (i) the pink to red colour is caused by a pathway intermediate of the postulated pathway (e.g. lycopene), (ii) pink strains form additional—yet to identify—carotenoids, or (iii) the compound causing the pink colour is not a carotenoid or escaped the analysis due to the formation of complexes, e.g. with proteins (Lakshman and Okoh, 1993). In *Flexibacter ruber*, it was observed that colonies have a red colour, although the yellow to orange flexixanthin was identified as the major carotenoid (Whitman, 2010). A similar situation might also explain the colony colour in the investigated planctomycetal strain LF2^T. Spectroscopic properties are typically also influenced by additional parameters, such as pH or components of the used cultivation medium. Either way, the observed differences in the carotenoid composition or even their absence

Table 1. Genes relevant for the production of the three identified carotenoids from precursors of the primary carbon metabolism.

Gene	Annotation	Accession number	
		R. brasiliensis DSM 5305^T	*R. rubra* SWK7
Non-mevalonate pathway			
dxs	1-deoxy-D-xylulose-5-phosphate synthase	ADY60203.1	EMI45825.1
dxr	1-deoxy-D-xylulose 5-phosphate reductoisomerase	ADY62041.1	EMI47130.1
ispD	2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase	ADY58900.1	EMI41460.1
ispE	4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase	ADY59769.1	EMI43255.1
ispF	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase	ADY62145.1	EMI41605.1
ispG, *gcpE*	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	ADY59897.1	EMI45477.1
ispH	4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase	ADY61028.1	EMI41209.1
Carotenoid biosynthesis			
crtE	Geranylgeranyl pyrophosphate synthase	ADY60202.1	EMI45824.1
crtB	Phytoene synthase	ADY58198.1	EMI41211.1
crtI	Phytoene desaturase	Not found	EMI45088.1
crtC	Acyclic carotenoid 1,2-hydratase	Not found	Not found
crtD	Carotenoid 3,4-desaturase	Not found	Not found
crtY	Lycopene β-cyclase	Not found	Not found
crtW	β-Carotene ketolase	Not found	Not found
crtZ	β-Carotene hydroxylase	Not found	Not found
lyeJ	Lycopene elongase/lycopene 1,2-hydratase	Not found	Not found

The GenBank accession numbers are given for genes identified in the genomes of *R. brasiliensis* DSM 5305^T and *R. rubra* SWK7.

among many white colony-forming planctomycetal stains is particularly interesting from both an ecological and physiological perspective.

In this study, we were able to identify three carotenoids present in two pigmented planctomycetal strains, thus contributing to improved characterization of bioactive molecules with potential biotechnological relevance in this phylum of aquatic bacteria (Graca *et al*., 2016; Jeske *et al*., 2016).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information