

***In vivo* and *in vitro* studies on the carotenoid cleavage oxygenases from *Sphingopyxis alaskensis* RB2256 and *Plesiocystis pacifica* SIR-1 revealed their substrate specificities and non-retinal-forming cleavage activities**

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Carotenoid cleavage oxygenases are nonheme iron enzymes that specifically cleave carbon–carbon double bonds of carotenoids. Their apocarotenoid cleavage products serve as important signaling molecules that are involved in various biological processes. A database search revealed the presence of putative carotenoid cleavage oxygenase genes in the genomes of *Sphingopyxis alaskensis* RB2256 and *Plesiocystis pacifica* SIR-1. The four genes *sala_1698*, *sala_1008*, *ppsir1_15490* and *ppsir1_17230* were cloned and heterologously expressed in carotenoid-producing *Escherichia coli* JM109 strains. Two of the four encoded proteins exhibited carotenoid cleavage activity. *S. alaskensis* RB2256 carotenoid cleavage oxygenase (SaCCO), which is encoded by *sala_1698*, was shown to cleave acyclic and monocyclic substrates. Coexpression of *sala_1698* in carotenoid-producing *E. coli* JM109 strains revealed cleavage activity for lycopene, hydroxylycopene, and dihydroxylycopene. The monocyclic substrate apo-8'-carotenal was cleaved *in vitro* by purified SaCCO at the 9'/10' and 11'/12' double bonds. The second enzyme, *P. pacifica* SIR-1 carotenoid cleavage oxygenase (PpCCO), is encoded by *ppsir1_15490*. PpCCO-mediated carotenoid cleavage requires the presence of either hydroxy or keto groups. PpCCO cleaved zeaxanthin, hydroxylycopene, and dihydroxylycopene, and also the C₅₀ carotenoids decaprenoxanthin, sarprenoxanthin and sarcinaxanthin, in carotenoid-producing *E. coli* JM109 strains. Whole cells of *E. coli* JM109 overexpressing *ppsir1_15490mut*, a mutant of *ppsir1_15490* with enhanced gene expression, were applied for the conversion of carotenoids. Analysis of the carotenoid cleavage products revealed a single cleavage site at the 13'/14' double bond for astaxanthin, and two cleavage sites at the 11'/12' or 13'/14' double bond for zeaxanthin, nostoxanthin, and canthaxanthin.

Introduction

Carotenoids are a class of natural pigments that are produced by all photosynthetic organisms and also by some nonphotosynthetic fungi and bacteria. They are structurally derived from the C₄₀ tetraterpene lycopene

(ψ,ψ-carotene; Fig. 1), which gives the stem name for the nomenclature of carotenoids [1]. The long conjugated double bond system of carotenoids causes their strong light absorption and antioxidative properties,

Abbreviations

PpCCO, *Plesiocystis pacifica* SIR-1 carotenoid cleavage oxygenase; SaCCO, *Sphingopyxis alaskensis* RB2256 carotenoid cleavage oxygenase.

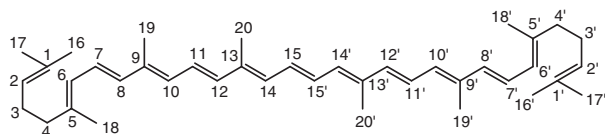


Fig. 1. Structure of lycopene (ψ,ψ -carotene) with numbering of the carbon atoms according to [1].

which are characteristics of carotenoids that directly correlate with their natural functions [2].

Apocarotenoids are carotenoid derivatives that are usually synthesized by oxidative cleavage of carotenoids. Carotenoid cleavage can occur by chemical oxidation or oxidation by nonspecific enzymes such as lipoxygenases or peroxidases [3–5]. Besides these unspecific mechanisms, there exists a group of non-heme iron enzymes, the carotenoid cleavage oxygenases, that selectively cleave carbon–carbon double bonds of carotenoids (reviewed in [6]).

Carotenoid cleavage oxygenase-mediated tailoring of carotenoids occurs either at a single double bond or simultaneously at two double bonds, and results in aldehyde or ketone cleavage products. Depending on the cleavage position(s), apocarotenoids have more or less shortened carbon skeletons as compared with their parent molecules. The presence of a sufficient number of conjugated double bonds maintains the color properties of the apocarotenoid. Bixin, also known as annatto, is a widely used food and cosmetic colorant that is extracted from the seeds of *Bixa orellana*. Cloning and functional characterization of the genes involved in the synthesis of bixin revealed that the first step in the synthesis of the colorant is mediated by a carotenoid cleavage oxygenase that targets the 5/6 and 5'/6' double bonds of lycopene [7].

Cleavage of carotenoids is also an important route to many scent and aroma compounds, such as the C_{13} ketone β -ionone, a major component of the violet scent [8]. AtCCD1 from *Arabidopsis thaliana* and its orthologs, which are found throughout the plant kingdom, cleave their carotenoid substrates at the 9/10 and 9'/10' double bonds, thereby giving rise to one C_{14} dialdehyde and two C_{13} ketones [9].

Another important class of apocarotenoid compounds are the retinoids (retinal, retinol, and retinoic acid). They are produced by central cleavage of C_{40} carotenoids that possess at least one unsubstituted β -end group or by cleavage of apocarotenoids with β -end groups and prenyl chains of sufficient length. Retinal plays a crucial role as a chromophore in photochemically reactive proteins (rhodopsins) in eukaryotes, archeons, and bacteria (reviewed in [10,11]). In

archeons and bacteria, rhodopsins (bacteriorhodopsins) were shown to function as light-driven ion transporters that are involved in ATP synthesis, light sensing, and osmotic homeostasis [12–14]. Bacteriorhodopsin was first isolated from the purple membrane of *Halobacterium salinarum* [15]. The retinal chromophore of *H. salinarum* bacteriorhodopsin was shown to be synthesized from β -carotene by bacteriorhodopsin-related protein (Brp) and bacteriorhodopsin-related protein-like homolog protein (Blh) [16].

To date, little is known about the carotenoid cleavage oxygenases from proteobacteria. Metagenomic experiments revealed the presence of a new type of rhodopsin (proteorhodopsin) in this phylum [17]. Moreover, Blh homologs responsible for retinal formation were found in several genomes isolated from oceanic surface water [18]. Investigations on carotenoid cleavage oxygenase homologs from *Novophingobium aromaticivorans* and *Bradyrhizobium* sp. revealed cleavage activities of the enzymes for stilbenes but not for carotenoid substrates [19].

We browsed proteobacterial genomes published in GenBank [20] for putative carotenoid cleavage oxygenase genes, and picked the marine α -proteobacterium *Sphingopyxis alaskensis* RB2256 and the γ -proteobacterium *Plesiocystis pacifica* SIR-1 for our investigations. The putative carotenoid cleavage oxygenase genes of the two strains were cloned and expressed in *Escherichia coli* JM109. Two enzymes with carotenoid cleavage activity were further characterized.

Results

Screening GenBank for putative carotenoid oxygenase genes

Elucidation of the 3D structures of the two carotenoid cleavage oxygenases ACO and VP14 revealed the basic structural motif of this enzyme family to be a propeller-like β -sheet arrangement [21,22]. The seven propeller blades comprise a tunnel in which the catalytic Fe^{2+} is coordinated by four highly conserved histidines. The knowledge about the common structural features of carotenoid cleavage oxygenases allowed a database-driven search for homologous enzymes. We performed a GenBank search for putative carotenoid cleavage oxygenases with the deduced amino acid sequence of NosCCD (Protein ID [NP_485149.1](#)), a carotenoid cleavage oxygenase from the cyanobacterium *Nostoc* sp. PCC 7120 [23,24], and BLAST software [25] in default settings. Among the results that were obtained for the phylum *Proteobacteria*, we picked *S. alaskensis* RB2256 and *P. pacifica* SIR-1 for our studies. Each

genome was found to contain two putative carotenoid cleavage oxygenase genes. The particular identities with and similarities to NosCCD of the encoded proteins are given in Table 1. The putative carotenoid cleavage oxygenases of *S. alaskensis* RB2256 have also been identified by the database search performed by Marasco & Schmidt-Dannert [19], but the carotenoid cleavage activities of the enzymes have not been investigated yet.

Two-plasmid system for *in vivo* tests on carotenoid cleavage activity

The putative carotenoid cleavage oxygenase genes were amplified by PCR and inserted into the pBR322-based and L-rhamnose-inducible vector pJOE5751.1 (Table S1). *E. coli* JM109 was cotransformed with different pBBR1MCS-2-based L-rhamnose-inducible carotenoid biosynthesis vectors (Table S1) and a carotenoid cleavage oxygenase vector. Strains with a particular carotenoid biosynthesis vector and an empty vector without the carotenoid cleavage oxygenase gene (pJH43.1) were used as controls. Gene expression of both vectors was

Table 1. Identities with and similarities to NosCCD of putative carotenoid cleavage oxygenases from *S. alaskensis* RB2256 and *P. pacifica* SIR-1. Identity: identical amino acids. Similarity: identical amino acids plus conservative substituted amino acids.

Putative carotenoid cleavage oxygenase	Protein ID	Identity with NosCCD (%)	Similarity to NosCCD (%)
Sala_1008	YP_616058.1	38	59
SaCCO (Sala_1698)	YP_616744.1	37	53
PpCCO (Ppsir1_15490)	ZP_01913312.1	27	41
Ppsir1_17230	ZP_01912480	36	55

simultaneously induced with 0.2% (w/v) L-rhamnose. Comparison of the cell pellets after 24 h revealed that coexpression of *sala_1698* and *ppsir1_15490* led to color loss of either all or certain carotenoid-producing *E. coli* JM109 strains, whereas coexpression of *sala_1008* and *ppsir1_17230* had no effect on the colors of the tested strains (Table 2; Fig. S1). Because of their carotenoid cleavage activities, Sala_1698 and Ppsir1_15490 were named *S. alaskensis* RB2256 carotenoid cleavage oxygenase (SaCCO) and *P. pacifica* SIR-1 carotenoid cleavage oxygenase (PpCCO), respectively.

SaCCO cleaves acyclic carotenoids *in vivo*

Because coexpression of *sala_1698* led to color loss of all tested carotenoid-producing *E. coli* JM109 strains (Table 2), conclusions regarding the specific cleavage activity of SaCCO for a particular carotenoid substrate could not be drawn. The loss of color might have been caused either by cleavage of every carotenoid or by cleavage of a common precursor of all tested carotenoids. To test which scenario was more likely, the cleavage activity of SaCCO on lycopene was investigated *in vivo*. In order to uncouple the induction of carotenoid biosynthesis and carotenoid cleavage oxygenase biosynthesis, *sala_1698* was inserted into the pBR322-based and L-arabinose-inducible vector pJOE5275.26 (pJH55.1; Table S1). This made it possible to first induce carotenoid biosynthesis with L-rhamnose and then separately induce the expression of *sala_1698* with L-arabinose. The control culture of *E. coli* JM109 pJOE5573.3/pJH55.1 without L-arabinose produced 1.45 µg of lycopene per 5×10^9 cells after 23.25 h (Fig. 2A). The culture of *E. coli* JM109 pJOE5573.3/pJH55.1 with L-arabinose produced lycopene until

Table 2. Bleaching of carotenoid-producing *E. coli* JM109 strains by coexpression of putative carotenoid cleavage oxygenase genes from *S. alaskensis* RB2256 and *P. pacifica* SIR-1.

Carotenoid biosynthesis plasmid	Produced carotenoid(s)	Bleaching of strains when coexpressed with			
		<i>sala_1008</i> (pJH41.1)	<i>sala_1698</i> (pJH42.1)	<i>ppsir1_15490</i> (pJH59.1)	<i>ppsir1_17230</i> (pJH60.2)
pJOE5573.3	Lycopene	–	+	–	–
pJH88.1	Lycopene Hydroxylycopene Dihydroxylycopene	–	+	+	–
pJOE5607.5	β-Carotene	–	+	–	–
pJH14.1	Zeaxanthin	–	+	+	–
pJH29.10	Decaprenoxanthin Sarprenoxanthin Sarcinaxanthin	–	+	+	–

L-arabinose was added. A decrease in lycopene content was measured 2 h after L-arabinose addition (0.04 μg of lycopene per 5×10^9 cells after 23.25 h). *In vivo* cleavage activity of SaCCO was also detected for hydroxylycopene and dihydroxylycopene (Fig. 2B,C).

The carotenoid content of the β -carotene-producing strain *E. coli* JM109 pJOE5607.5/pJH55.1 developed differently from that in the strains that produced acyclic carotenoids. Only a slight decrease in β -carotene content was measured in the 4 h following L-arabinose addition (Fig. 2D). Although the reduction in β -carotene content was more pronounced after 23.25 h, the final β -carotene content of the culture of *E. coli* JM109 pJOE5607.5/pJH55.1 was still clearly higher than the final carotenoid contents of the cultures that produced acyclic carotenoids. The results indicate that β -carotene is not cleaved or is only poorly cleaved by SaCCO *in vivo*. The production of β -carotene probably stopped because of degradation of its precursor, lycopene. Because no additional β -carotene was synthesized in the presence of SaCCO, the already synthesized β -carotene was thinned out upon cell growth, which developed from an $D_{600 \text{ nm}}$ of 1.98 at 5.25 h to 9.88 at 23.25 h.

SaCCO cleaves apo-8'-carotenal *in vitro*

In order to obtain detectable amounts of carotenoid cleavage products, *sala_1698* was expressed as a *Strep*-tag II fusion protein, purified with *Strep*-Tactin sepharose, and applied for *in vitro* carotenoid cleavage

assays. Cleavage activity of purified SaCCO was detected for apo-8'-carotenal, but not for β -carotene and zeaxanthin (data not shown). Lycopene was only very poorly converted by purified SaCCO *in vitro*. Two additional peaks, probably cleavage products of lycopene, could sometimes be detected at 450 nm in the HPLC chromatogram, although with insufficient reproducibility (data not shown). In contrast, apo-8'-carotenal conversion could already be observed optically by a change in the color of the reaction (Fig. 3A,B). The carotenoids of the assays were extracted and subjected to HPLC (system 1) and LC-MS (system 2) analyses. As compared with the HPLC chromatogram of the control (Fig. 3A), two additional peaks with shorter retention times than apo-8'-carotenal could be observed in the chromatogram of the apo-8'-carotenal cleavage assay with purified SaCCO (Fig. 3B).

Figure 3C–E shows the data of the LC-MS analyses of products 1–3. According to the measured molecular ion $[M + H]^+$ of m/z 351.10 (Fig. 3C) and the measured absorption maximum at 426 nm (in HPLC eluent, data not shown), product 1 was identified as apo-12'-carotenal. The mass spectrum of product 2 exhibited a mass peak of m/z 377.20 corresponding to the molecular ion $[M + H]^+$ of apo-10'-carotenal (Fig. 3D). The absorption maximum was measured at 448 nm (in HPLC eluent, data not shown). Product 3 is the substrate apo-8'-carotenal with the expected molecular ion $[M + H]^+$ of m/z 417.40 (Fig. 3E) and an absorption maximum at 461 nm (in HPLC eluent, data not shown).

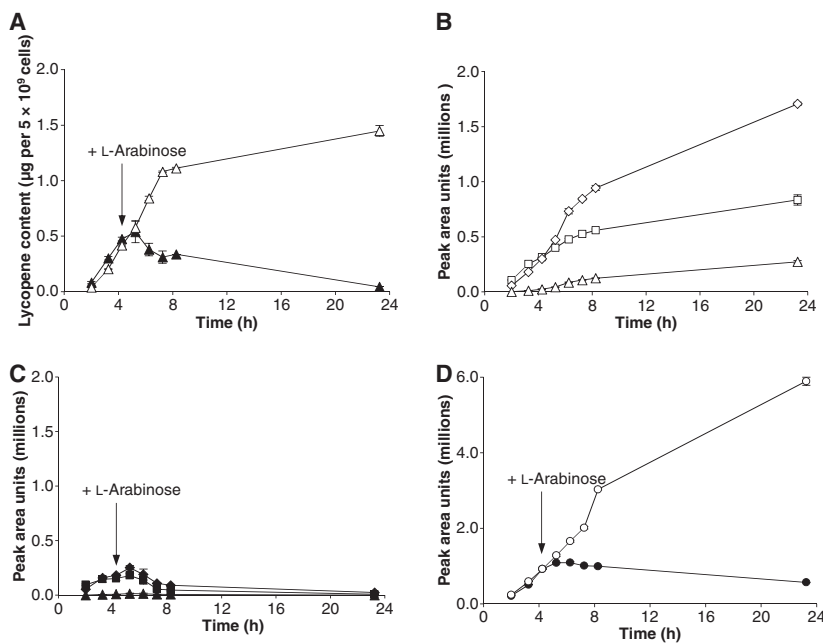


Fig. 2. Time-dependent carotenoid contents of *E. coli* JM109 cultures with and without coexpression of *sala_1698*. (A) *E. coli* JM109 pJOE5573.3/pJH55.1 (lycopene/SaCCO). (B, C) *E. coli* JM109 pJH88.1/pJH55.1 (hydroxylycopene, dihydroxylycopene, and lycopene/SaCCO). (D) *E. coli* JM109 pJOE5607.5/pJH55.1 (β -carotene/SaCCO). Empty symbols: cultures without L-arabinose (controls). Filled symbols: cultures with 0.2% (w/v) L-arabinose (induction of *sala_1698* expression). Triangles: lycopene. Squares: dihydroxylycopene. Rhomboids: hydroxylycopene. Circles: β -carotene. Time 0 represents the addition of 0.2% (w/v) L-rhamnose (induction of carotenoid biosynthesis).

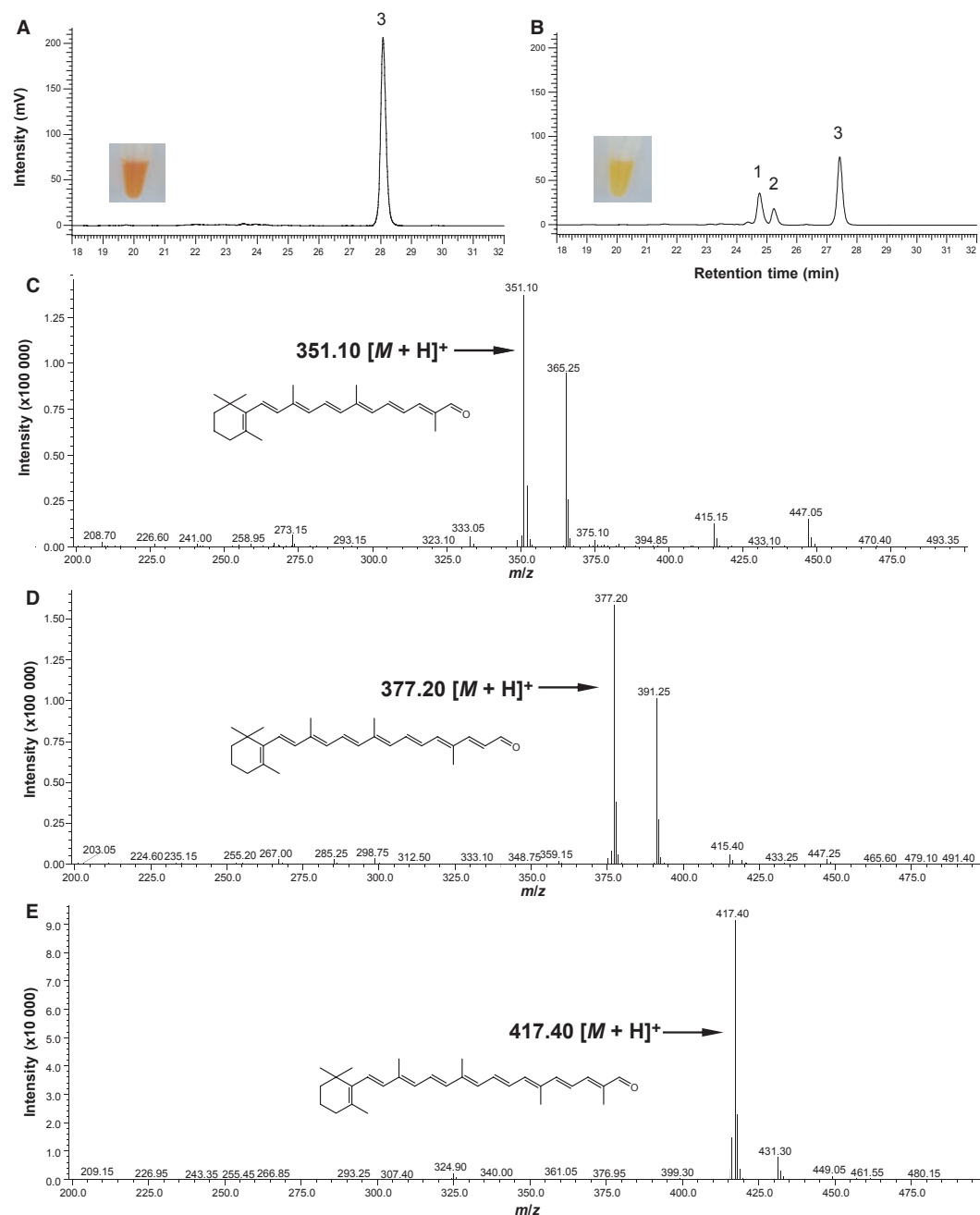


Fig. 3. LC-MS analysis of apo-8'-carotenal cleavage products synthesized by purified SaCCO *in vitro*. (A) HPLC chromatogram and a photograph of the reaction mixture without SaCCO (control). (B) HPLC chromatogram and a photograph of the reaction mixture with purified SaCCO (60 min, 30 °C). (C–E) Mass spectra and structures of (C) product 1 (apo-12'-carotenal), (D) product 2 (apo-10'-carotenal), and (E) product 3 (apo-8'-carotenal).

By means of the identified cleavage products apo-12'-carotenal and apo-10'-carotenal, two cleavage sites of the substrate apo-8'-carotenal at the 9'/10' and 11'/12' double bonds were derived (Fig. 4).

Apo-8'-carotenal cleavage assays were also used for kinetic analysis (Table 3) and determination

of the temperature and pH profiles of SaCCO (Fig. 5). Furthermore, it was observed that the presence of 0.3 mM FeSO₄ in the reaction buffer increased enzyme activity by ~1.5-fold, although the addition of FeSO₄ was not essential (data not shown).

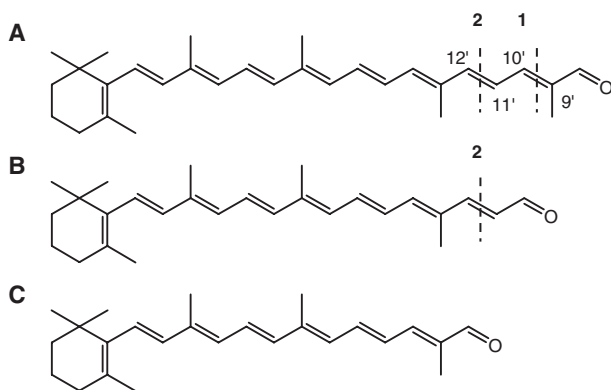


Fig. 4. Cleavage of apo-8'-carotenal by SaCCO. (A) Apo-8'-carotenal. (B) Apo-10'-carotenal. (C) Apo-12'-carotenal. The identified cleavage positions are indicated. Cleavage might occur imprecisely (position 1 or 2) or successively (first position 1, and then position 2).

Table 3. Kinetic parameters of SaCCO for its substrate apo-8'-carotenal. The values represent the mean $\pm$ standard deviations of four independent experiments.

Kinetic parameter	Value
K_m (μM)	122.9 ± 18.5
$V_{\max}$ ($\text{nmol mg}^{-1} \cdot \text{min}^{-1}$)	15.9 ± 3.0
k_{cat} (s^{-1})	4.9 ± 0.9
k_{cat}/K_m ($\text{nm}^{-1} \cdot \text{s}^{-1}$)	39.9 ± 7.0

PpCCO cleaves hydroxy carotenoids *in vivo*

The *in vivo* experiments with PpCCO were performed analogously to the experiments with SaCCO. *ppsir1_15490* was inserted into the pBR322-based and L-arabinose-inducible vector pJOE5275.26 (pJH62.1; Table S1) and combined with the different carotenoid biosynthesis vectors. Comparison of the carotenoid contents of *E. coli* JM109 pJOE5607.5/pJH62.1 (β -carotene) and *E. coli* JM109 pJH14.1/pJH62.1 (zeaxanthin) revealed that L-arabinose addition had an effect only on the zeaxanthin-producing culture. The zeaxanthin content of the culture of *E. coli* JM109 pJH14.1/pJH62.1 began to decrease

2 h after L-arabinose addition, whereas the control culture without L-arabinose continued to produce zeaxanthin (Fig. 6A). In contrast, no effect on the β -carotene production of *E. coli* JM109 pJOE5607.5/pJH62.1 was observed when L-arabinose was added (Fig. 6B).

Similar *in vivo* experiments were performed with *E. coli* JM109 pJOE5573.3/pJH62.1 (lycopene) and *E. coli* JM109 pJH88.1/pJH62.1 (lycopene, hydroxylycopene, and dihydroxylycopene). An L-arabinose-dependent decrease in carotenoid content could be observed only in the culture that produced hydroxylated lycopene derivatives in addition to lycopene (Fig. S2).

We also investigated the cleavage activity of PpCCO for hydroxylated C_{50} carotenoids. The vector pJH29.10 for the production of decaprenoxanthin, sarprenoxanthin and sarcinaxanthin (Fig. 7G–I) was constructed by insertion of *crtEb*, *crtYe* and *crtYf* from *Corynebacterium glutamicum* [26,27] into the lycopene biosynthesis vector pJOE5573.3 (Table S1). As was the case for the other hydroxy carotenoid-producing *E. coli* JM109 strains, an L-arabinose-dependent decrease in carotenoid content could be detected for the C_{50} hydroxy carotenoid-producing strain *E. coli* JM109 pJH29.10/pJH62.1 (Fig. S3).

The results indicate that unsubstituted acyclic and bicyclic carotenoids are not cleaved by PpCCO *in vivo*, whereas the presence of hydroxy groups enables PpCCO-mediated cleavage of both acyclic and bicyclic C_{40} and C_{50} carotenoids.

PpCCO cleaves hydroxy and keto carotenoids in whole cell catalysis with *E. coli* JM109 pJH66.1

PpCCO activity was lost immediately after cell integrity had been disturbed, and the enzyme was therefore not purified by affinity chromatography. We applied whole cells of *E. coli* JM109 pJH66.1 that overexpressed *ppsir1_15490mut* (*ppsir1_15490* with enhanced gene expression; see Experimental procedures) for the conversion of carotenoids. Uninduced cells of *E. coli* JM109

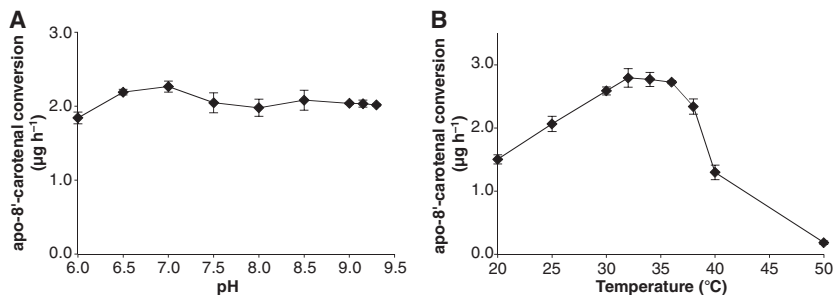


Fig. 5. Effects of pH (A) and temperature (B) on the conversion of apo-8'-carotenal by SaCCO.

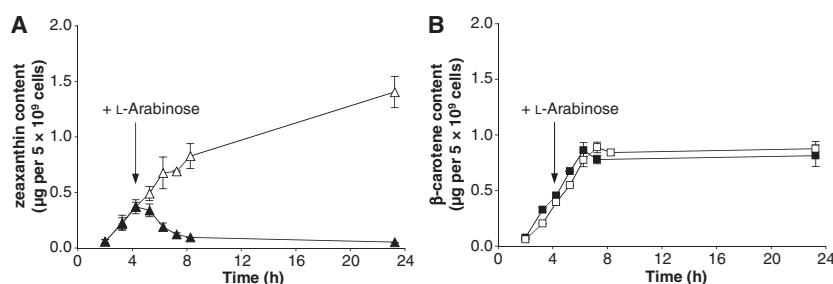


Fig. 6. Time-dependent carotenoid contents of *E. coli* JM109 cultures with and without coexpression of *ppsir1_15490*. (A) *E. coli* JM109 pJH14.1/pJH62.1 (zeaxanthin/PpCCO). (B) *E. coli* JM109 pJOE5607.5/pJH62.1 (β -carotene/PpCCO). Empty symbols: cultures without L-arabinose (controls). Filled symbols: cultures with 0.2% (w/v) L-arabinose (induction of *ppsir1_15490* expression). Squares: β -carotene. Triangles: zeaxanthin. Time 0 represents the addition of 0.2% (w/v) L-rhamnose (induction of carotenoid biosynthesis).

pJH66.1 were used as the control. Cleavage was found for canthaxanthin, astaxanthin, zeaxanthin, and nostoxanthin. In all cases, additional peaks could be observed in the HPLC chromatograms of the extracts from incubations with induced cells (data not shown). Only poor cleavage activity was observed for hydroxylycopene and dihydroxylycopene and the C_{50} carotenoids decaprenoxanthin, sarprenoxanthin, and sarcinaxanthin (data not shown).

The cleavage products of canthaxanthin, zeaxanthin, nostoxanthin and astaxanthin were purified by preparative TLC and subjected to LC-MS analysis on system 3. Several peaks could be observed in the HPLC chromatogram of the isolated canthaxanthin cleavage products (detection wavelength, 400 nm; Fig. 8B). According to the detected molecular ions $[M + H]^+$ of m/z 273.0 (peak 1), m/z 325.0 (peak 2), and m/z 365.1 (peak 4), the products were identified as apo-13'-canthaxanthinone, apo-14'-canthaxanthinal, and apo-12'-canthaxanthinal, respectively. The mass spectra of products 3 and 5 also showed molecular ions $[M + H]^+$ of m/z 325.0 and m/z 365.1, respectively (data not shown). Hence, products 3 and 5 are most likely isomers of products 2 and 4. The two cleavage products apo-14'-canthaxanthinal and apo-13'-canthaxanthinone probably arose from cleavage of canthaxanthin at the 13'/14' double bond (Fig. 7C). Apo-12'-canthaxanthinal is probably the bigger cleavage product that resulted from cleavage of canthaxanthin at the 11'/12' double bond (Fig. 7C). However, the smaller cleavage product, apo-11'-canthaxanthinal, was not detected.

LC-MS analysis of the isolated cleavage products of zeaxanthin gave the three corresponding cleavage products apo-13'-zeaxanthinone, apo-14'-zeaxanthinal, and apo-12'-zeaxanthinal (Fig. S4). As in the case of canthaxanthin, the smallest cleavage product of zeaxanthin, apo-11'-zeaxanthinal, was not detected. For nostoxanthin, only the two bigger cleavage products,

apo-12'-nostoxanthinal and apo-14'-nostoxanthinal, were detected (detection wavelength, 275 nm; Fig. S5). In the case of astaxanthin, the two cleavage products apo-14'-astaxanthinal and apo-13'-astaxanthinone were found (Fig. S6). The identified cleavage products consistently indicate that PpCCO cleaves canthaxanthin, zeaxanthin and nostoxanthin imprecisely at the 11'/12' or 13'/14' positions (Fig. 7A–C). The identified astaxanthin cleavage products indicate a single cleavage site at the 13'/14' double bond (Fig. 7D).

Discussion

The present study investigated the putative carotenoid cleavage oxygenases of the marine proteobacteria *S. alaskensis* RB2256 and *P. pacifica* SIR-1.

SaCCO cleaves acyclic and monocyclic substrates. Lycopene was shown to be efficiently cleaved by SaCCO *in vivo*, but the conversion of lycopene by purified SaCCO *in vitro* was quite poor. The same problem was described for MtCCO from *Mycobacterium tuberculosis*. The enzyme also exhibited lycopene cleavage activity *in vivo* that could not be detected by *in vitro* incubation with the purified enzyme [28].

In vitro carotenoid cleavage requires solubilization of the hydrophobic substrates. A major amount of lycopene was always precipitated during the micellization process (data not shown). Insufficient solubilization of lycopene is therefore a probable reason for the lack of conversion of this substrate *in vitro*.

Analysis of the cleavage products obtained from *in vitro* incubation of apo-8'-carotenal with purified SaCCO revealed two cleavage sites at the 9'/10' and 11'/12' double bonds. As the smaller cleavage products were not detected, it remains unclear whether the cleavage of apo-8'-carotenal occurs imprecisely or successively via the intermediate apo-10'-carotenal (Fig. 4). Imprecise cleavage of carotenoid substrates

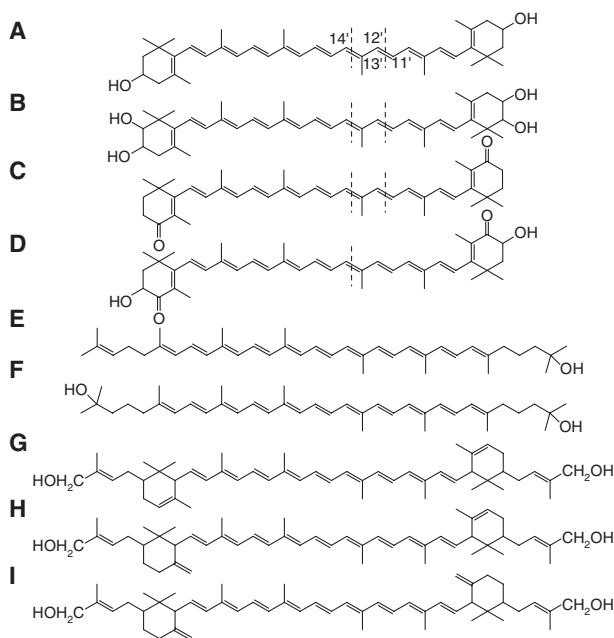


Fig. 7. Carotenoid substrates cleaved by PpCCO. (A) Zeaxanthin. (B) Nostoxanthin. (C) Canthaxanthin. (D) Astaxanthin. (E) Hydroxylycopene. (F) Dihydroxylycopene. (G) Decaprenoxanthin. (H) Sarpenoxanthin. (I) Sarcinaxanthin. The cleavage positions of substrates A–D have been identified, and are indicated; the cleavage positions of substrates E–I are unknown.

was also reported for MtCCO [28] and OsCCD1 [29]. This study revealed that PpCCO-mediated carotenoid cleavage also occurs imprecisely. Therefore, imprecise cleavage of apo-8'-carotenal by SaCCO can be assumed, rather than a successive cleavage.

The 9'/10' double bond cleaved by SaCCO is also targeted by various other known carotenoid cleavage enzymes [9,24,30,31], but cleavage of the 11'/12' double bond has been reported for none of them. Hence, SaCCO is especially interesting for the production of apo-12'-carotenal. The selectivity of the enzyme for the 11'/12' double bond might be improved by mutagenesis.

PpCCO cleaves acyclic and bicyclic C₄₀ and C₅₀ carotenoid substrates with hydroxy and/or keto groups. Owing to instability of the enzyme, purification of PpCCO by affinity chromatography was not an appropriate approach for the conversion of carotenoids *in vitro*. We also observed inactivation of SaCCO when the cells were disrupted with an ultrasonic homogenizer (data not shown). In contrast to PpCCO, an active SaCCO could be obtained when the cells were disrupted by high-pressure homogenization. Possible reasons for inactivation of PpCCO might be the loss or oxidation of the catalytic Fe²⁺ during cell disruption.

Carotenoid conversion with PpCCO was accomplished by using whole cells of *E. coli* JM109 overexpressing *ppsir1_15490mut*, a mutant of *ppsir1_15490* with enhanced gene expression. The identification of the cleavage products indicated that cleavage of zeaxanthin, nostoxanthin and canthaxanthin occurs imprecisely at the 11'/12' or 13'/14' double bonds. For astaxanthin, only one cleavage site, at the 13'/14' double bond, was found. We also observed that astaxanthin was converted more efficiently by PpCCO than the other tested substrates (data not shown). An influence of hydroxy groups on the cleavage specificity was also described for MtCCO. Hydroxylated substrates are predominantly cleaved at the 15/15' double bond, whereas unsubstituted substrates are cleaved at the 13/14 double bond [28]. Therefore, the selective cleavage of astaxanthin by PpCCO might be attributable to its specific functional groups.

In vivo cleavage activity of PpCCO was demonstrated for hydroxylycopene, dihydroxylycopene, and hydroxylated C₅₀ carotenoids. However, the substrates were only poorly cleaved in whole cell catalysis with *ppsir1_15490mut*-overexpressing *E. coli* JM109. As for lycopene, precipitation during micellization was also observed for hydroxylycopene and dihydroxylycopene, but not for the C₅₀ carotenoids (data not shown). In any case, the special structure of the C₅₀ carotenoids might also be a factor complicating the conversion of the substrates by PpCCO in whole cell catalysis with *E. coli* JM109 pJH66.1.

As already mentioned, some marine proteobacterial genomes were found to contain genes encoding retinal-forming carotenoid cleavage oxygenases [17,18]. Neither SaCCO nor PpCCO showed retinal formation. Furthermore, none of the genomes contains a proteorhodopsin homolog (BLAST search with [AAR05342.1](#)). Therefore, the two carotenoid cleavage oxygenases SaCCO and PpCCO are probably not responsible for the synthesis of a proteorhodopsin chromophore.

Another possible function of SaCCO and PpCCO in their natural hosts could be the regulation of their carotenoid contents in response to changing environmental conditions. The gene set found in the genome of *S. alaskensis* RB2256 and the analysis of its main carotenoid indicated that the strain produces nostoxanthin. SaCCO was shown to cleave only acyclic and monocyclic carotenoids. The bicyclic carotenoids zeaxanthin and β -carotene were not cleaved by purified SaCCO *in vitro*. Therefore, the function of SaCCO in *S. alaskensis* RB2256 is probably not the cleavage of nostoxanthin. This leads to the question of whether there is another enzyme that mediates the initial

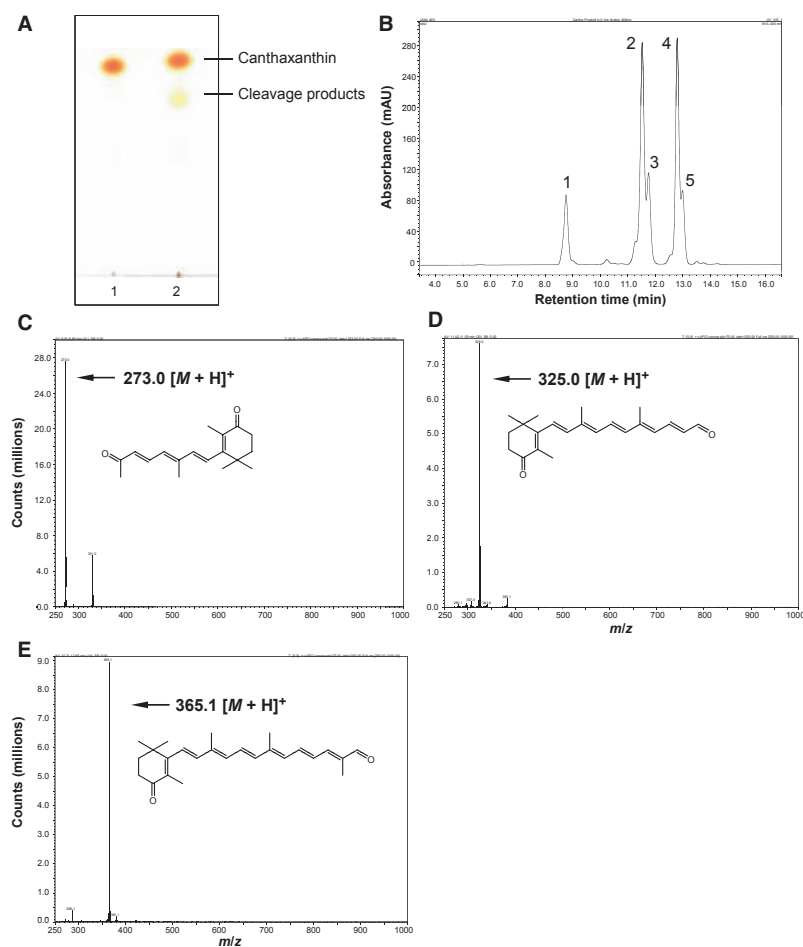


Fig. 8. Isolation and LC-MS analysis of canthaxanthin cleavage products synthesized by whole cell catalysis with *E. coli* JM109 pJH66.1. (A) Thin layer chromatogram of extracts from whole cell catalysis of canthaxanthin. Lane 1: control (2×10^{10} uninduced cells). Lane 2: canthaxanthin cleavage reaction (2×10^{10} induced cells). (B) HPLC chromatogram of isolated canthaxanthin cleavage products (detection wavelength: 400 nm). (C–E) Mass spectra and structures of (C) product 1 (apo-13'-canthaxanthinone), (D) product 2 (apo-14'-canthaxanthinal), and (E) product 4 (apo-12'-canthaxanthinal). mAU, milli absorbance units.

cleavage of nostoxanthin. The other putative carotenoid cleavage oxygenase of *S. alaskensis* RB2256, Sala_1008, showed no carotenoid cleavage activity in our tests. Besides enzyme-mediated mechanisms, apocarotenoids can also be formed chemically under conditions of oxidative stress [32]. The occurrence of apocarotenoids is therefore not necessarily associated with a specific function of the molecule. Apocarotenoid-cleaving enzymes, such as SaCCO, could be responsible for the degradation of damaged carotenoids. Apo-8'-carotenal conversion by SaCCO was considerably slower than that by other carotenoid cleavage oxygenases. Diox1 from *Synechocystis* sp. PCC 6803 and MtCCO convert apo-8'-carotenal with K_m values of 2.5 μM and 4.15 μM , respectively, and, in the case of MtCCO, with a k_{cat} of 392.7 s^{-1} [28,33]. Hence, apo-8'-carotenal might not be a natural substrate for SaCCO. It would be interesting to investigate the activity of the enzyme for hydroxylated β -end group apocarotenoids, which probably occur rather than apocarotenoids with unsubstituted β -end groups in *S. alaskensis* RB2256.

Experimental procedures

Media and cultivation of strains

The strains used in this study are listed in Table S2. *C. glutamicum* and *S. alaskensis* RB2256 were cultivated in LB (10 $\text{g}\cdot\text{L}^{-1}$ tryptone, 5 $\text{g}\cdot\text{L}^{-1}$ NaCl, 5 $\text{g}\cdot\text{L}^{-1}$ yeast extract) at 30 °C and 200 rpm. in Erlenmeyer flasks. Unless stated otherwise, recombinant *E. coli* JM109 strains were cultivated in LB with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, 46 mM sodium phosphate buffer (pH 7.8) and 0.46% (w/v) glycerol at 30 or 37 °C and 200 rpm. in Erlenmeyer flasks. *P. pacifica* SIR-1 was not cultivated in this study (chromosomal DNA was provided by Leibniz Institute DSMZ, Braunschweig, Germany).

Plasmid construction

All vectors used in this study are listed in Table S1. The enhanced green fluorescent protein genes of the basic vectors were replaced by PCR products with the carotenoid cleavage oxygenase or carotenoid biosynthesis genes, respectively. The genes were amplified with 1 U of Phusion Hot Start DNA polymerase (Finnzymes Oy, Vantaa,

Finland) and the oligonucleotides shown in Table S3. Chromosomal DNA of the strains was used as a template for PCR. The PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, München, Germany), and cut with the restriction enzymes (New England Biolabs, Frankfurt am Main, Germany) given in Table S3. For the construction of pJH29.10, the In-Fusion Dry-Down PCR Cloning Kit (Takara Bio Europe/Contech, Saint-Germain-en-Laye, France) was used. pJH43.1 was constructed by deleting the enhanced green fluorescent protein gene from pJOE5751.1. This was achieved by cutting the plasmid with *NdeI/HindIII*, isolating the bigger DNA fragment after separation of the fragments by agarose gel electrophoresis, end polishing with Klenow fill-in reaction, and subsequent religation.

Mutagenesis of *ppsir1_15490* for enhanced gene expression in *E. coli*

Expression of *ppsir1_15490* was improved by mutagenesis of the third base of each of the first four codons following the initiation codon ATG without changing the amino acid sequence. The wild-type gene was amplified and, at the same time, mutated with the oligonucleotides S5636 and S6169 (degenerate) (Table S3). The PCR product was purified with the GFX PCR DNA and Gel Band Purification Kit, digested with *NdeI* and *HindIII*, and then ligated with the *NdeI/HindIII* linearized vector pJOE5751.1. A color screen method was employed for the identification of plasmids with improved expression of *ppsir1_15490*. *E. coli* JM109 was cotransformed with pJH66 (*ppsir1_15490* mutants) and pJH14.1 (zeaxanthin). Transformants were selected on LB agar with 100 µg·mL⁻¹ ampicillin, 50 µg·mL⁻¹ kanamycin, and 0.2% (w/v) L-rhamnose (Fig. S7A). As a control, *E. coli* JM109 was cotransformed with pJH14.1 and pJH59.1 (wild-type *ppsir1_15490*). A clone with a lighter color than the control colonies was selected (Fig. S7B). The plasmid with the mutated *ppsir1_15490* was isolated, and the mutant sequence was elucidated by DNA sequencing. It was found that the wild-type sequence 3'-ATGCAGGCGCCCCGC-5' was changed to 3'-ATGCAAGCACCTCGA-5' in the mutant plasmid. The gene with improved expression was designated *ppsir1_15490mut*, and the corresponding plasmid pJH66.1 (Table S1).

In vivo carotenoid cleavage experiments

E. coli JM109 was simultaneously transformed with an L-rhamnose-inducible carotenoid biosynthesis plasmid and an L-arabinose-inducible carotenoid cleavage oxygenase plasmid. The strains were cultivated at 30 °C and 200 rpm, starting from an initial $D_{600\text{ nm}}$ of 0.022–0.025. After 1 h 50 min, carotenoid synthesis was induced by the addition of 0.2% (w/v) L-rhamnose. The expression of the carotenoid cleavage oxygenase genes was induced 4.25 h later by the

addition of 0.2% (w/v) L-arabinose. At different times, two parallel samples of 5×10^9 cells were harvested by centrifugation (4500 g for 7 min), and extracted with 200-µL portions of acetone until the cells became colorless. The acetone extracts were pooled and mixed with 1/5 volume H₂O and 1/5 volume hexane. After brief centrifugation (16 000 g for 1 min), the upper phase was isolated and the acetone/water phase was further extracted with small portions of hexane until the hexane extract remained colorless. The hexane extracts were pooled, and the solvent was evaporated in the vacuum centrifuge. The dried carotenoid extracts were resolved in 100 µL of acetone, and 20 µL of each sample was analyzed by HPLC on system 1.

Analysis of the main carotenoid of *S. alaskensis* RB2256

The genome of *S. alaskensis* RB2256 was browsed for putative carotenoid biosynthesis genes by a BLAST search with default settings and the amino acid sequences of the protein products of carotenoid biosynthesis genes with known functions. The putative genes *crtB* (*sala_3132*, Gene ID 4082388), *crtE* (*sala_2194*, Gene ID 4080152), *crtG* (*sala_3136*, Gene ID 4082392), *crtI* (*sala_3134*, Gene ID 4082390), *crtY* (*sala_3135*, Gene ID 4082391) and *crtZ* (*sala_2128*, Gene ID 4080103) were found. HPLC analysis of an extract from *S. alaskensis* RB2256 on system 1 revealed that the strain produces a main carotenoid (81%) with a shorter retention time than zeaxanthin (data not shown). The main carotenoid of *S. alaskensis* RB2256 was isolated by TLC on TLC silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany), with acetone and hexane (1 : 1) as mobile phase. Absorption maxima of the isolated carotenoid were measured at 453.0 and 479.7 nm in acetone, and at 451.0 and 477.2 nm in ethanol. The data correspond to the absorption maxima given for nostoxanthin [(2*R*,3*R*,2'*R*,3'*R*)-β,β-carotene-2,3,2',3'-tetrol] [34]. A molecular ion $[M + H]^+$ of m/z 601.1 was measured by LC-MS analysis on system 3 (Fig. S8). On the basis of the found set of carotenoid biosynthesis genes and the results obtained with HPLC and LC-MS analyses, we propose that the main carotenoid produced by *S. alaskensis* RB2256 is nostoxanthin (Fig. 7B).

P. pacifica SIR-1 *crtC* gene

The putative CrtC (Ppsir1_30569, Protein ID [ZP_01912132.1](#)) from *P. pacifica* SIR-1 and CrtC from *Myxococcus xanthus* [35] share 31% identity and 42% similarity. *E. coli* JM109 pJH88.1 (Table S1) produces three carotenoids, which were analyzed by LC-MS (Fig. S9). The two carotenoids that were synthesized from lycopene by CrtC showed molecular ions $[M + H]^+$ of m/z 573.3 (peak 1) and m/z 555.3 (peak 2). The absorption maxima were measured at 445, 473 and 503 nm (peak 1) and at 442, 472 and 503 nm (peak 2) (in HPLC eluent, system 2, data not shown). According to their masses and absorption maxima, the

substances were identified as hydroxylycopene (1,2-dihydro- ψ,ψ -carotene-1-ol) and dihydroxylycopene (1,2,1',2'-tetrahydro- ψ,ψ -carotene-1,1'-diol) [36].

Preparation of carotenoids for *in vitro* assays

β -Carotene and apo-8'-carotenal were purchased from Sigma Aldrich (Steinheim, Germany), and astaxanthin and canthaxanthin were gifts from the Institute of Food Chemistry, University of Hohenheim (Stuttgart, Germany). Zeaxanthin was extracted from *E. coli* JM109 pJH14.1. Lycopene was extracted from tomato puree. Nostoxanthin was extracted from *S. alaskensis* RB2256. Hydroxylycopene and dihydroxylycopene were extracted from *E. coli* JM109 pJH88.1. Carotenoid substrates were applied at a final concentration of 80 μM . For the preparation of carotenoid micelles, the substrates were first dissolved in 50 μL of dichloromethane (astaxanthin and canthaxanthin), 50 μL of acetone (β -carotene and lycopene), or 50 μL of ethanol (nostoxanthin and zeaxanthin), and then mixed with 50 μL of 4% (w/v) *n*-octyl- β -glucoside in ethanol. The solvent was evaporated in the vacuum centrifuge, and the residues were resuspended in 2 $\times$ incubation buffer I or II (see below).

In vitro carotenoid cleavage assays with purified SaCCO

E. coli JM109 pJH58.2 was cultivated in 40 mL of LB with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin at 37 °C and 200 rpm. Gene expression was induced with 0.2% (w/v) L-rhamnose at a $D_{600\text{ nm}}$ of 0.3. The culture was then incubated at 30 °C and 200 rpm. overnight. The 54.5-kDa SaCCO-*Strep*-tag II fusion protein (C-terminal fusion) was purified with *Strep*-Tactin sepharose (IBA, Göttingen, Germany), according to the manufacturer's protocol. The buffer used for cell disruption was NaCl/P_i (8 $\text{g}\cdot\text{L}^{-1}$ NaCl, 0.2 $\text{g}\cdot\text{L}^{-1}$ KCl, 1.44 $\text{g}\cdot\text{L}^{-1}$ $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$, 0.24 $\text{g}\cdot\text{L}^{-1}$ KH_2PO_4 , pH 7.4) with 1 mM Tris(2-carboxyethyl)phosphine (TCEP). Cell disruption was performed with a high-pressure homogenizer. The *Strep*-Tactin-bound recombinant protein was washed with the same buffer. Elution of the recombinant protein was performed with NaCl/P_i with 4 mM D-desthiobiotin. The protein purification gave ~3 mg of recombinant SaCCO-*Strep*-tag II fusion protein (Fig. S10). Carotenoid micelles (see above) were resuspended in 100 μL of 2 $\times$ incubation buffer I (200 mM Hepes/NaOH, pH 7.4, 2 mM TCEP, 0.6 mM FeSO_4). The reactions were filled to 200 μL with water and 20 μg of purified SaCCO, and incubated at 30 °C and 500 rpm. The reaction was stopped by addition of 200 μL of acetone. Carotenoids were extracted with small portions of hexane until the hexane phase remained colorless. The solvent was evaporated in the vacuum centrifuge, and the residue was resolved in 300 μL of acetone. Twenty microliters were subjected to HPLC and LC-MS on systems 1 and 2, respectively.

Kinetic analysis and determination of pH and temperature profiles of SaCCO

The kinetic analyses were performed with 20 μg of purified SaCCO and 10, 30, 50 or 80 μM apo-8'-carotenal in incubation buffer I for 20 min at 25 °C and 500 rpm. The experiment was independently repeated four times in duplicate. For determination of K_m and V_{max} , the Michaelis–Menten equation was solved by nonlinear regression with KALEIDAGRAPH 4.1 (Synergy Software, Reading, PA, USA).

Apo-8'-carotenal (80 μM) was applied for the determination of temperature and pH profiles of SaCCO. The reactions were incubated for 1 h at 25 °C (for pH studies) and 500 rpm, and 2 $\times$ incubation buffer I was used for the temperature studies. For the pH studies, Hepes/NaOH stock solutions with different pH values were used for the preparation of 2 $\times$ incubation buffer I. The reactions were stopped, extracted, and dried as described above. The residues were dissolved in acetone, and the apo-8'-carotenal contents were determined by HPLC on system 1 (20 μL sample volume).

Carotenoid cleavage by whole cell catalysis with *E. coli* JM109 pJH66.1

E. coli JM109 pJH66.1 cells were cultivated in LB with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin and 1 mM FeSO_4 at 37 °C and 200 rpm. L-Rhamnose (0.2%, w/v) was added at a $D_{600\text{ nm}}$ of 0.3, and the cultures were further incubated for 5 h at 30 °C and 200 rpm. Cultures without L-rhamnose were used as controls. Cells (1×10^{10} for astaxanthin assay, or 2×10^{10} for all other carotenoids) were harvested by centrifugation (4500 *g* for 7 min) and washed with 1 mL of H_2O . The cells were resuspended in 80 μL (1×10^{10} cells) or 90 μL (2×10^{10} cells) of H_2O . Carotenoid micelles (see above) were resuspended in 100 μL of 2 $\times$ incubation buffer II (200 mM Hepes/NaOH, pH 7.0, 2 mM TCEP, 5 mM FeSO_4). One hundred microliters of cell suspension was added to the carotenoid micelles, and the reactions were incubated on a wheel at room temperature overnight. The reactions were centrifuged (16 000 *g* for 5 min), and the supernatants were collected, mixed with 200 μL of acetone, and extracted with small portions of hexane. The cells were extracted with acetone. Acetone extracts were re-extracted with 1/5 volume water and hexane (see above). Hexane extracts were combined and dried in the vacuum centrifuge. The dried extracts were resolved in 20–50 μL of acetone and used for TLC.

Isolation of carotenoid cleavage products by TLC

The carotenoids of the extracts from whole cell catalysis (see above) were separated on TLC silica gel 60 F_{254} . The mobile phases were hexane/acetone (7 : 3) for canthaxanthin, petroleum/diethyl ether/acetone (40 : 10 : 10) for zeaxanthin, petroleum/acetone/diethyl ether (30 : 20 : 10) for nostoxanthin, and methanol/acetone/water/methyl

tert-butyl ether (30 : 24.6 : 10 : 1) for astaxanthin. The cleavage products were scraped off the plates and eluted with ethanol. The extracts were filtered (0.45 µm) and dried in the vacuum centrifuge. The residues were resolved in acetone and subjected to LC-MS analysis on system 3.

Analytical methods

HPLC and LC-MS analyses were performed on three different systems. System 1 was used for quantification of carotenoids that were extracted from recombinant *E. coli* JM109 strains and for quantification of the apo-8'-carotenal that was extracted from *in vitro* assays with purified SaCCO. HPLC system 1 (Merck-Hitachi, Darmstadt, Germany) consisted of an L7612 degasser, an L6200-A gradient pump, a D-6000A interface module, an L4200 UV-visible detector, a Rheodyne injection valve 7125 with a 100-µL sample loop, and D-7000 HPLC System Manager software. An RP-18 column (250 × 4.6 mm, 5 µm) with a guard column (10 × 4.6 mm, 5 µm) (Trentec, Rutesheim, Germany) was used for separation of the analytes. Solvent A was a mixture of methanol, water, and methyl *tert*-butyl ether (30 : 10 : 1). Solvent B consisted of methanol and methyl *tert*-butyl ether (1 : 1). The flow rate was 1 mL·min⁻¹. The HPLC gradient was as follows (given as percentage of solvent A): 0 min, 100%; 30 min, 0%; 35 min, 0%; 36 min, 100%; and 51 min, 100% [37]. Carotenoid solutions with five different concentrations (0.5, 1, 5, 10, 20 and 30 µg·mL⁻¹) were used for calibration. The absorbance was measured at 450 nm.

System 2 was used for LC-MS analysis of apo-8'-carotenal cleavage products. The HPLC system, column, solvents and elution gradient have been described previously [38]. The system was coupled to an LCMS-2010 mass spectrometer (Shimadzu Deutschland, Dienslaken, Germany). The solvent flow rate was 0.5 mL·min⁻¹. Ionization was performed by ESI and atmospheric pressure ionization in positive mode. The MS parameters were as follows: modus scan; event time, 0.15 s; detector voltage, 1.5 kV; interface voltage, 3.5 kV; CDL voltage, -43 V; CDL temperature, 230 °C; heat block temperature, 200 °C; and detection range, *m/z* 350–1000.

System 3 was used for LC-MS analysis of apocarotenoids produced by PpCCO and for analysis of the carotenoids produced by *S. alaskensis* RB2256 and *E. coli* JM109 pJH88.1. Analyses were performed on a Dionex Ultimate 3000 HPLC system (Dionex Corp., Sunnyvale, CA, USA). Chromatographic separation was performed with a Spherisorb ODS-2 C₁₈ column (250 × 4.6 mm, 5 µm) (VDS Optilab, Berlin, Germany). Solvents A and B were acetone and water, respectively. The flow rate was 1 mL·min⁻¹. The nonlinear (curve mode 5) HPLC gradient was as follows (given as percentage of solvent A): 0 min, 50%; 35 min, 100%; 40 min, 50%; 45 min, 50%. UV-visible detection was performed at 475 nm. MS analyses were

performed with a Dionex MSQ mass spectrometer (Dionex Corp.). The atmospheric pressure ionization ion source was used in positive mode. The MS parameters were as follows: entrance cone, 7.5 µA; gas temperature, 450 °C; cone voltage, 75 V; and detection range, *m/z* 250–1000. Nitrogen was used as the desolvation, cone and collision gas. All data collected in centroid mode were acquired and processed with CHROMELEON 6.0 (Dionex Corp. Sunnyvale, CA, USA).

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Supporting information

The following supplementary material is available:

Fig. S1. Bleaching of carotenoid-producing *E. coli* JM109 strains by PpCCO.

Fig. S2. Time-dependent carotenoid contents of *E. coli* JM109 cultures with and without coexpression of *ppsir1_15490*.

Fig. S3. Time-dependent C₅₀ carotenoid contents of *E. coli* JM109 pJH29.10/pJH62.1 cultures with and without coexpression of *ppsir1_15490*.

Fig. S4. Isolation and LC-MS analysis of zeaxanthin cleavage products synthesized by whole cell catalysis with *E. coli* JM109 pJH66.1.

Fig. S5. Isolation and LC-MS analysis of nostoxanthin cleavage products synthesized by whole cell catalysis with *E. coli* JM109 pJH66.1.

Fig. S6. Isolation and LC-MS analysis of astaxanthin cleavage products synthesized by whole cell catalysis with *E. coli* JM109 pJH66.1.

Fig. S7. Identification of mutated *ppsir1_15490* with enhanced gene expression in *E. coli* by the color screen method.

Fig. S8. LC-MS analysis of an extract from *Sphingopyxis alaskensis* RB2256.

Fig. S9. LC-MS analysis of an extract from *E. coli* JM109 pJH88.1.

Fig. S10. SDS/PAGE analysis of fractions that were collected during purification of the SaCCO-Strep-tag II fusion protein (54.5 kDa) with Strep-Tactin sepharose.

Table S1. Names and properties of the plasmids used in this study.

Table S2. Strains used in this study.

Table S3. Oligonucleotides used for amplification of carotenoid cleavage oxygenase and carotenoid biosynthesis genes.

This supplementary material can be found in the online version of this article.

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