Biosynthesis of soluble carotenoid holoproteins in *Escherichia coli*

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Carotenoids are widely distributed natural pigments that are excellent antioxidants acting in photoprotection. They are typically solubilized in membranes or attached to proteins. In cyanobacteria, the photoactive soluble Orange Carotenoid Protein (OCP) is involved in photoprotective mechanisms as a highly active singlet oxygen and excitation energy quencher. Here we describe a method for producing large amounts of holo-OCP in *E. coli*. The six different genes involved in the synthesis of holo-OCP were introduced into *E. coli* using three different plasmids. The choice of promoters and the order of gene induction were important: the induction of genes involved in carotenoid synthesis must precede the induction of the *ocp* gene in order to obtain holo-OCPs. Active holo-OCPs with primary structures derived from several cyanobacterial strains and containing different carotenoids were isolated. This approach for rapid heterologous synthesis of large quantities of carotenoproteins is a fundamental advance in the production of antioxidants of great interest to the pharmaceutical and cosmetic industries.

The human body is constantly exposed to external (ultraviolet radiation, pollution, cigarette smoke, toxic chemicals) and internal (side reactions of respiration, oxidation of nutrients) factors which induce the formation of Reactive Oxygen Species (ROS). Due to the harmful effects of ROS, the pharmaceutical and cosmetic industries have a significant interest in the production of new antioxidant molecules. For many applications, the medium in which the anti-oxidant effect is desired is water-based and requires a hydrophilic antioxidant. Water soluble carotenoid proteins fit these requirements. Carotenoids are widely distributed natural pigments which play important roles in photosynthesis, nutrition and illness prevention. They have a protective role in photosynthetic and non-photosynthetic organisms including humans by serving as protective colorants or by quenching singlet oxygen (\(\cdot O_2\)) and free radicals induced by exogenous sensitizers or produced by metabolic processes (reviews\(^1\)–\(^4\)). Carotenoids which are relatively hydrophobic molecules, typically occur solubilized in membranes or non-covalently attached to membrane or soluble proteins. In photosynthetic organisms, they are mainly found in the membrane-embedded, chlorophyll-containing antennae where they have the dual activities of harvesting solar energy and quenching excess energy and \(\cdot O_2\) (see reviews\(^1\)–\(^3\)). A number of water soluble carotenoid proteins from photosynthetic organisms have also been characterized\(^6\)–\(^8\). The cyanobacterial Orange Carotenoid Protein (OCP) is one of the best characterized soluble carotenoid proteins. We have recently demonstrated that the OCP is an excellent antioxidant—better than vitamin C, trolox, tocopherol and isolated carotenoids\(^9\). The OCP protects cyanobacteria by quenching the \(\cdot O_2\) formed in reaction centers and antennae\(^6\). It was first described by Holt and Krogmann in 1981\(^10\) and is present in the majority of cyanobacteria containing phycobilisomes (PBs), the large extra-membrane antenna formed by blue and red phycobiliproteins\(^11\).

The OCP is a photoactive protein\(^12\); it is essential for a photoprotective mechanism that decreases the excitation energy arriving at photochemical reaction centers\(^13\). The OCP has an \(\alpha\)-helical N-terminal domain (residues 15–165) and an \(\alpha/\beta\) C-terminal domain (residues 190–317) (Fig. 1A)\(^14\). The carotenoid, 3'-hydroxyechinenone (hECN), spans both domains which are joined by a flexible linker. The presence of a ketocarotenoid is essential for OCP photoactivity\(^15\). Light absorption by the carotenoid induces conformational changes in the carotenoid and in the protein that are essential for its photoprotective function\(^12\) (Fig. 1B). In darkness, the OCP is orange (OCP\(^o\)); upon illumination, it becomes red. The red form (OCP\(^r\)) is the active form of the protein\(^12,15\). Only OCP\(^r\) is able to bind the PBs. Once the OCP\(^r\) is bound, the carotenoid interacts with a chromophore of the PB core and quenches the excitation energy\(^16\)–\(^18\). This photoprotective mechanism is activated by blue-green light but not by...
Here we describe the construction of \emph{E.coli} strains that are able to synthesize large amounts of OCP homologs from different cyanobacterial strains incorporating various carotenoids \textit{in vivo}. This fast holo-OCP production has already enabled us to further understand the function of different carotenoids in OCPs, for example that canthaxanthin-OCPs are very good energy and \textit{O}_2 quenchers. The work described here is important not only to accelerate the elucidation of the OCP photoprotective mechanism by rapid synthesis of variant OCPs, but promises to enable the isolation and characterization of other carotenoid proteins with potential applications for promoting human health.

**Results**

**Biosynthesis of His-tagged holo-OCPs in \emph{E.coli} cells.** The aim of our work was to synthesize holo-OCPs (OCPs attaching one carotenoid molecule) from \textit{Synechocystis}, \textit{Arthrospira} and \textit{Anabaena} strains in \emph{E.coli} cells. For this purpose, the genes coding for enzymes involved in the synthesis of the desired carotenoids (supplementary Fig. 1) and the \textit{ocp} gene must be expressed in the same cell. It is known that in \textit{Synechocystis} and \textit{Arthrospira OCP} binds hECN\textsuperscript{10,14}. Previous work showed that it is difficult to obtain large quantities of this carotenoid in \emph{E.coli} cells\textsuperscript{29,30}. We decided to express the \textit{ocp} gene in the presence of two other ketocarotenoids: echinenone (ECN) and canthaxanthin (CAN). \textit{Synechocystis} OCP is able to bind ECN and the ECN-OCP is photoactive and induces PB fluorescence quenching\textsuperscript{27}. In contrast, \textit{Arthrospira OCP} weakly binds ECN\textsuperscript{31}. Prior to this study, nothing was known about the \textit{Anabaena} OCP. Although we did not know if CAN-OCP would be active, we hypothesized that the carbonyl groups present in CAN rings could allow photoactivity and stabilization of the carotenoid binding.

The \emph{E.coli} cells producing holo-OCPs carried three plasmids. The first plasmid, pAC-BETA (or pACCAR\textsubscript{16}D) contained the \textit{Erwinia herbicola} (or \textit{Erwinia uredovora}) operon carrying the four genes (\textit{crtB, crtE, crtI, crtY}), which are necessary to synthesize \textit{β}-carotene. In the second plasmid the \textit{crtO} gene of \textit{Synechocystis} or the \textit{crtW} gene from \textit{Anabaena PCC 7210} was introduced. While \textit{CrtO} is a monoketolase synthesizing mostly echinenone from \textit{β}-carotene\textsuperscript{32}, \textit{CrtW} is a diketolase that catalyses the formation of canthaxanthin\textsuperscript{33} (supplementary Fig. 1). The \textit{ocp} genes were cloned in a third plasmid (pCDFDuet-1). In order to maintain the three plasmids within the same \emph{E.coli} cell, the use of three different and compatible replication origins and three different antibiotic resistances was required (Fig. 2).

The operon containing the \textit{crtB, crtE, crtI} and \textit{crtY} genes was under the control of the constitutive \textit{ctE} promoter. Thus, the \textit{β}-carotene was constitutively synthesized in the \emph{E.coli} cells. The \textit{crtO} and \textit{crtW} genes were under the control of the arabinose inducible promoter araBAD. The transcription of \textit{ocp} genes was controlled by the IPTG-inducible T7lac promoter. A sequential induction of these genes was controlled by the IPTG-inducible T7lac promoter. A sequential induction of these genes was essential to isolate high quantities of holo-OCP. The expression of \textit{ocp} genes in \emph{E.coli} containing a relatively high concentration of \textit{β}-carotene. Subsequently the expression of \textit{ocp} genes was induced in \emph{E.coli} cells containing high concentrations of ECN or CAN in their membranes.

The \emph{E.coli} cells carrying the \textit{crtO} gene contained 15–25% of \textit{β}-carotene, 70–80% ECN and 4–6% CAN. When the \textit{ocp} genes were expressed in the presence of ECN, a mixture of apo- and holo-OCPs was obtained in all cases, but the proportion of holo-OCP varied (Table 1). The presence of photoactive holo-OCPs was already detected \textit{in vivo}. Orange cells containing holo-OCPs became red when they were illuminated with high intensities of white light (Fig. 3A). In contrast, yellow and orange \emph{E.coli} cultures containing orange or red light that are not absorbed by the carotenoid. However, the OCP photoprotects cyanobacteria from strong orange-red light; this protection is related to the \textit{O}_2 quenching activity of the OCP.

Due to the outstanding antioxidant properties of carotenoids and their role in human health, substantial effort has been devoted to the engineering of noncarotenogenic bacteria to produce high quantities of their role in human health, substantial effort has been devoted to the engineering of noncarotenogenic bacteria to produce high quantities of carotenoids, for example that canthaxanthin-OCPs are very good energy and \textit{O}_2 quenchers. The work described here is important not only to accelerate the elucidation of the OCP photoprotective mechanism by rapid synthesis of variant OCPs, but promises to enable the isolation and characterization of other carotenoid proteins with potential applications for promoting human health.

**Figure 1** | The OCP and photoprotection. (A) Structure of the OCP from \textit{Synechocystis} PCC 6803 (Protein Data Bank ID: 3MG1) The OCP monomer in the OCP\textsuperscript{0} state. The N-terminal arm (residues 1–22) (green) interacts with the C-terminal domain (residues 196–315) (blue). The C-term is colored in rose. The N-terminal domain (residues 22–165) is orange. The hydroxy-echinenone (hECN) spans both domains of the protein. Tyr201 and Trp288 of the C-terminal domain hydrogen bond to the carbonyl group of hECN. Tyr44 and Trp110 of the N-terminal domain interact with the hydroxyl ring of hECN. The OCP was modified adding amino acids in the N-terminus (green) and/or the C-terminus (rose). (B) Model of the OCP-related photoprotective mechanism. 1) Upon light absorption the orange OCP\textsuperscript{0} is converted into the active open red OCP\textsuperscript{0}. 2) OCP\textsuperscript{0} binds to the PB core and induces fluorescence and excitation energy quenching.


only genes involved in β-carotene and ECN (without the ocp gene) did not change colour upon illumination (Fig. 3A). Once the cells were broken, a notable difference in the colour of the supernatants (soluble fraction) was observed. The supernatant derived from E. coli cells synthesizing only carotenoids, because the ocp gene was not induced, was clear (Fig. 3Bb) and the orange colour was concentrated in the membranes (Fig. 3Ba). The slight colour observed in the supernatant is attributable to a leak of the T7 promoter and the presence of a small concentration of OCP. In contrast, in the cells synthesizing only carotenoids, because the ocp gene under the control of the ara promoter (arabinose inducible); CrtO enzyme converts β-carotene in echinenone; pCDF-OCP carries the ocp gene under the control of an IPTG inducible promoter (red).

The first Synechocystis ocp overexpressed genes contained an addition of 18 nucleotides coding for six His just before the stop codon (Syn-Ctag) or an extension of 45 nucleotides after the first ATG (Syn-pDuet) (supplementary Fig. 2 and Table 1). This extension which includes the sequence coding for six-His is already included in the commercial pCDFDuet-1 plasmid. Analysis of the resulting two isolated Synechocystis OCPs revealed that the addition of the N-terminal extension of 45 nucleotides increased the total amount of OCP present in the cells (from 4–6 mg/L to 18–22 mg/L) and the yield of holo-OCP (from 25–40% (Syn-Ctag-ECN) to 35–45% (Syn-pDuet-ECN) (Table 1).

Since a modification on the N-terminus seemed to increase the yield of holoOCPs in E.coli cells, other ocp modifications were tested. In all cases (with the exception of one), nine to 45 nucleotides coding for a series of non-charged or charged amino acids were added just after the first ATG in the ocp gene containing a sequence coding for six-His in its 3’ end (supplementary Fig. 2 and Table 1). In the construction lacking the C-terminal His-tag, 27 nucleotides including the sequence coding for six-His were added to the 5’ end (Syn-3aaNtag) (supplementary Fig. 2 and Table 1). Analysis of the isolated Synechocystis OCPs showed that addition of 8 to 10 amino acids largely increased the yield of holo-OCP. More than 95% of isolated Synechocystis OCP contained a carotenoid molecule. When only 3 to 6 amino acids were

| Table 1 | Percentage and amount of holo-OCP obtained from 1 L culture of E.coli. The Sequences of amino acids added to the N- or C-termnus are given |
|---|---|---|---|
| Name of OCP | Added Amino acid sequence | Total OCP (mg/L) | Holo-OCP % of total OCP | Holo-OCP (mg/L) |
| ECN – Synechocystis OCPs | | | | |
| Syn-CtagECN | -HHHHHStopCodon | 4–6 | 25–40 | 1–5 |
| Syn-pDuetECN | MGSSHHHHHHH-5QDP- | 18–22 | 35–45 | 5–10 |
| Syn-MIX1aaCtag-ECN | MGSSRLNDPEKTDPQ | 20–22 | 30–40 | 5–10 |
| Syn-NC15aCtag-ECN | MGSSSANQAVTNQPQV- | 18–21 | 45–55 | 8–10 |
| Syn-NC10aCtag-ECN | MGSSSANQAVTV | 9–11 | >95 | 10 |
| Syn-NCBaaCtag-ECN | MGSSSANQANQ | 15–17 | >95 | 15 |
| Syn-NCaCtag-ECN | MGSSSANQA | 19–21 | 78–85 | 15–20 |
| Syn-NC3aCtag-ECN | MGSS- | 8–10 | 70–80 | 5–10 |
| Syn-CBaaCtag-ECN | MGSSKARRA | 20–22 | 78–85 | 15–20 |
| Syn-3aaNtag-ECN | MGSSHHHHHHH- | 30–35 | ≈95 | 30–35 |
| CAN – Anabaena and Arthrospira OCPs | | | | |
| Ana-3aaNtag-ECN | MGSSHHHHHHH | 50–60 | 50–60 | 25–35 |
| Ana-Ctag-ECN | -HHHHHStopCodon | 50–60 | 40–45 | 25–25 |
| Arthro-3aaNtag-ECN | MGSSHHHHHHH | 25–30 | 30–40 | 5–15 |
| Arthro-Ctag-ECN | -HHHHHStopCodon | 15–20 | 10–25 | 1–5 |
| CAN – OCPs | | | | |
| Arthro-3aaNtag-CAN | MGSSHHHHHHH | 25–30 | 60–65 | 15–20 |
| Arthro-Ctag-CAN | -HHHHHStopCodon | 30–35 | 50–60 | 15–20 |
| Ana-3aaNtag-CAN | MGSSHHHHHHH | 8–10 | 75–85 | 5–10 |
| Ana-Ctag-CAN | -HHHHHStopCodon | 60–70 | 40–45 | 25–30 |

added, the yield of holoprotein also increased but slightly less (Table 1). Finally, addition of 9 amino acids, including 6 His, in the N-terminus, in the absence of C-terminal His-tag (Syn-3aaNtag) allowed the isolation of the largest quantity of holo-OCP containing almost no apo-protein: 30–35 mg holo-OCP (Table 1). All of the isolated Synechocystis holo-OCPs contained more than 95% ECN with only traces of CAN (supplementary Fig. 3). These results suggested that a slight destabilisation of the OCP N-terminal arm is necessary to increase and/or to stabilise OCP carotenoid binding. In the OCP, this arm interacts with the C-terminal domain and seems to stabilize the closed structure of the orange form1,4,27 (Fig. 1).

Arthrospira and Anabaena ocp genes, containing sequences coding for an His-tag in the N- or the C-terminus, were also expressed in E.coli cells synthesising ECN. Although the His-tag in the N-terminus increased the yield of holo Anabaena and Arthrospira OCPs (to 60 and 40%, respectively), still a large amount of apo-OCP was present (Table 1). Since the membranes remained coloured indicating the presence of ECN, the low concentration of holo-OCPs was not related to insufficient carotenoid production. The holo-Anabaena and holo-Arthrospira OCPs contained mostly ECN with traces of CAN (supplementary Fig. 3).

In an attempt to increase the yield of Arthrospira and Anabaena holo-OCPs, the ocp genes were induced in E.coli cultures synthesising ECN. This strain, carrying the crw gene, contained 15–25% of β-carotene, 50–60% CAN, 7–9% ECN and 5–7% of an unknown carotenoid. Indeed, the presence of CAN increased the yield of holo-protein to 60% in the case of Arthrospira OCP. In contrast, the presence of CAN decreased the yield of Synechocystis-OCP (Syn-3aaNtag-CAN) to 75–85% and of holo Anabaena-OCP (Ana-3aaNtag-CAN) to 40–45% (Table 1). While the holo-Anabaena- CAN-OCP contained mostly CAN with only traces of ECN, holo-Synechocystis-CAN-OCP contained 50–70% CAN and holo-Arthrospira-CAN-OCP contained only 50–55% CAN (supplementary Fig. 3). Our results indicated that the binding and/or the stability of carotenoids in the protein differs between Synechocystis and Arthrospira or Anabaena OCPs. Most probably only the presence of hECN will allow the isolation of more than 95% of holo Arthrospira or Anabaena OCPs.

Characteristics of the isolated OCPs. The isolated proteins are orange in darkness and red in strong light (Fig. 4A). Orange ECN-OCPs (OCP) absorbance spectra show maxima at 472 and 496 nm and a shoulder at 450 nm, comparable to the native cyanobacteria OCPs (Fig. 4A and supplementary Fig. 4A). The absorbance spectra of CAN-OCPs were slightly red shifted compared to ECN-OCPs with maxima at 475 and 500 nm (Fig. 4A and supplementary Fig. 4B). The maximum of OCP absorbance spectra was at 510 nm for ECN-OCPs and 525 nm for CAN-OCPs.

All three CAN-OCPs and Synechocystis and Arthrospira ECN-OCPs completely converted to their red form (OCP) under illumination (Fig. 4B). The kinetics of OCP to OCP photoconversion of the three CAN-OCPs and ECN-Arthrospira-OCP were similar (t1/2 = 4–7 sec) and faster than that of the ECN-Synechocystis OCP (t1/2 = 18 sec) (Fig. 4B). The slower photoconversion of Synechocystis OCP compared to Arthrospira OCP was previously observed when the proteins were overexpressed in Synechocystis cells11. Anabaena ECN-OCP only partially converted to OCP, suggesting slight differences in the carotenoid-protein interaction in this protein (Fig. 4B). N- and C-terminal His-tagged Synechocystis OCPs presented similar conversion kinetics from OCP to OCP (Fig. 4C). When both termini of the protein were modified, an acceleration of OCP accumulation was observed, suggesting a destabilization of the closed OCP (Fig. 4C). Only one exception was observed: the addition of 8 charged amino acids hindered the conversion OCP to OCP (Fig. 4C).

The capacity of N-terminal His-tagged ECN- and CAN-OCPs to quench O2 was studied. Electron paramagnetic resonance (EPR) spin trapping was applied for O2 detection using TEMPD-HCl (2,2,6,6-tetramethyl-4-piperidone). When this nitrone reacts with O2, it is converted into the stable nitroxide radical, which is paramagnetic and detectable by EPR spectroscopy. The production of O2 was induced by illumination of the photosensitizer methylene blue. Figure 5A shows the typical EPR signal of the nitroxide radical obtained after 3 min illumination (1000 μmol photons m−2 s−1) in a solution containing methylene blue and TEMPD-HCl in the absence or presence of the OCP. The presence of only 1.5 μM holo-OCP decreased the EPR signal between 65 and 85% and 4 μM OCP quenched nearly the entire EPR signal. These results indicated that all of the E. coli-derived OCPs are very good O2 quenchers (Fig. 5B). The slight differences in the efficiency of O2 quenching are likely due to the presence of higher concentrations of apo-protein for the same concentration of holo-OCP (see supplementary Fig. 5).
Nevertheless, our results suggested that *Arthrospira* OCP has a slightly better activity as $^1$O$_2$ quencher than *Synechocystis* OCP. In contrast, *Anabaena*-ECN-OCP had a slightly lower activity as $^1$O$_2$ quencher. The nature of the bound carotenoid seemed not to influence the activity, as previously suggested.

Finally, the ability of the OCPs to quench PB fluorescence was tested. *Arthrospira* and *Synechocystis* OCPs isolated from *E. coli* were able to induce a large PB fluorescence quenching (Fig. 6A). The OCPs induced the fastest kinetics of fluorescence quenching and *Anabaena* OCPs were able to induce large fluorescence quenching (Fig. 6A). However, a fast fluorescence recovery was observed only with *Synechocystis* N-terminal His-tagged OCPs (Fig. 6C). Thus, the behavior of OCPs is affected by the location of the His-tag.

**Discussion**

The use of soluble carotenoproteins as antioxidants to promote human health is an area of active research and, consequently, methods to produce them in high yields are important. The aim of our work was to synthesize holo-OCPs (OCPs attaching the carotenoid) in *E. coli* to develop a method for obtaining high quantities of carotenoproteins. Using the method described here, we obtained 200 times more holo-OCP in 20% of the time of previously established purifications involving overexpression in cyanobacterial cells (C Bourier de Carbon, A Wilson and D Kirilovsky, unpublished data). In only four days more than 30 mg holo-*Synechocystis* OCP can be obtained from 1 L of *E. coli* cells using the construction Syn-3aaNtag-ECN.

The key elements of this production method are the choice of promoters and the sequential induction of genes. $\beta$-carotene must be present in the membrane before induction of *ctrO* or *ctrW* genes leading to the synthesis of ECN or CAN, respectively. More importantly, ECN and CAN have to be synthesized in advance and present in the membrane before induction of the ocp gene. The presence of IPTG in the growth medium inhibits cell growth even at low concentrations. In addition, the T7lac promoter cannot be induced at temperatures higher than 30°C. In contrast, arabinose enhances cell growth and the araBAD promoter allows induction at 37°C. Thus, the carotenoid genes must be induced first with arabinose at 37°C to obtain a high concentration of carotenoid-containing cells and then, the carotenoprotein gene could be induced by IPTG addition at lower temperatures (20 to 28°C) to slow down protein synthesis, allowing protein folding and carotenoid binding.

The possibility of isolate holo-OCPs from *E. coli* cells constitutes a major advance for the investigation of the molecular mechanism of OCP since it facilitates rapid isolation of mutant proteins with new characteristics. The method has already permitted us to further characterize OCPs revealing different phenotypes related to specificity and strength of carotenoid binding. We demonstrate here that all OCPs are able to bind CAN and that CAN-OCPs are photoactive and able to induce large PB fluorescence quenching, like the native hECN-OCPs. This demonstration was not previously possible when OCPs were isolated by overexpression in *Synechocystis* cells since they contain only traces of CAN.

We also show here that *Arthrospira*, *Anabaena* and *Synechocystis* OCPs are characterized by different phenotypes in terms of specificity and strength of carotenoid binding. *Synechocystis* OCP binds and stabilizes ECN better than CAN. In contrast, *Arthrospira* and *Anabaena* OCPs preferentially bind CAN over ECN and have a low affinity for both carotenoids. It is difficult to explain these differences based in the comparison of *Arthrospira* and *Synechocystis* OCP structures due to the high sequence identity among amino acids forming the carotenoid-binding pocket and the similar carotenoid orientation in the proteins (supplementary Fig 9A). Nothing is known about how the carotenoid is introduced in the apo-OCP. We can hypothesize that the OCP is synthesized by membrane bound ribosomes and that the carotenoid is introduced during the synthesis of the N-terminal domain. It could be possible that the amino acids involved in this initial binding differ from those in
the carotenoid-binding pocket of mature OCP. Many of the amino acid substitutions among the three OCPs are localized on the outer surface of the N-terminal domain and could have a role in primary binding (supplementary Fig 9B and 9C).

The most unexpected result was the different behavior in PBs binding of Synechocystis OCPs with a His-Tag on the N-terminus compared to those with the tag on the C-terminus. In the past, all OCP characterizations and the construction of stable OCP-PBs complexes were made with isolated C-terminal His-tagged OCPs. These OCPs, once bound to PBs at 0.5 or 0.8 M phosphate, remain almost permanently attached and the PBs remain quenched. This characteristic allowed the isolation of quenched OCP-PB complexes. Here, we show that the absence of the C-terminal His-tag largely accelerates the dark recovery of PB fluorescence, suggesting a decreased stability of bound OCP. In contrast, OCP binding kinetics were only slightly affected. In addition, a longer N-terminus in C-terminal His-tagged OCPs hinders the OCP binding and destabilizes the strong OCP attachment to PBs. Our results strongly suggest that while the C-terminal His-tag increases the stability of OCP-PB complexes, a longer N-terminus destabilizes this attachment.

Production of antioxidant molecules and proteins is a topic of considerable general interest to plant and human biologists since oxidative stress is involved in many processes leading to cell death or tissue damage. Here we show that all three recombinant OCPs show excellent activity as O2 quencher. Other soluble carotenoproteins also display good antioxidant properties like Astaxanthin and could be used in nutraceutics, cosmetics, etc. The possibility of engineering noncarotenogenic bacteria to produce carotenoproteins, like the OCP, which are present at relative low concentrations in the native organisms, constitutes a major breakthrough in efforts to obtain large quantities of carotenoid molecules as antioxidants.

In addition, the ability to synthesize holo-OCPs in E. coli is an important step in the construction of a biofuel (biomass) producing microorganism using sunlight as a natural source of energy. In order to create these minimal entities new synthetic reaction centers containing the minimal number of components needed for electron transport are being constructed and antenna molecules are being attached to them to expand the spectral range for light absorption (for example\cite{39-43}). Presently, nothing is done to protect these systems. The OCP, a good quencher of excitation energy and singlet oxygen, is an excellent candidate for this function. It can regulate the excitation energy arriving to the reaction centers and quench the singlet oxygen formed by the inevitable secondary, dangerous reactions.

**Methods**

**Amplification and cloning of Crt genes encoding enzymes involved in carotenoid synthesis and of ocp gene.** The plasmids pAC-BETA (gift of Prof F. Cunningham) and pACCA16\(\Delta\)ctxT (gift of Prof G. Sandmann), which contain a P15A origin of replication and the crtB, crtE, crtL and crtV genes under the control of the promoter of \(\text{Erwinia herbicola}\) and \(\text{Erwinia amylovora}\) respectively, were used\cite{44-45}. All results presented in this article were obtained with pAC-BETA.

The cto and ctw genes were cloned into a modified Plasmid pBAD/gII A (from Invitrogen) which contains a PB32 origin of replication, an arabinose inducible promoter (araBAD) and an ampicillin resistance marker. The Plasmid pBAD/gII A was first modified to avoid the export of the recombinant protein into the periplasmic space of the cells. For this purpose, the region encoding the “gene III signal sequence” was deleted. Primers used for the PCR mutagenesis were pBAD/gII Atn (F and R) (supplementary Fig. 7). The modified plasmid pBAD/gII A was named pBAD. The Plasmid pBAD was digested with BglII and EcoRI restriction enzymes to clone the cto gene (\(\text{CrtO}\) gene) of Synechocystis PCC6803 or with NcoI and EcoRI restriction enzymes to clone the ctw gene (\(\text{CrtW}\) gene) of \(\text{Erwinia herbicola}\) and \(\text{Erwinia amylovora}\) respectively, used\cite{44-45}. All results presented in this article were obtained with pAC-BETA.

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The cto gene was cloned in the plasmid pCDFDuet-1 (from Novagen). The plasmid pCDFDuet-1 contains a CDF origin of replication, T7lac promoter and Streptomycin/Spectinomycin resistance. The sequences of synthetic oligonucleotides (primers) used in the amplification and modification of all the genes are described in supplementary Fig. 7.

**N-terminal His-tagged OCP.** The pCDFDuet-1 plasmid was digested with EcoRI and NcoI to clone the different ocp genes (\(\text{CrtO}\) gene) of Synechocystis PCC6803, \(\text{CrtW}\) gene) of \(\text{Erwinia herbicola}\) and \(\text{Erwinia amylovora}\) respectively, used\cite{44-45}. All results presented in this article were obtained with pAC-BETA.

The plasmid pCDFDuet-1 was digested with EcoRI and NcoI to clone the different ocp genes (\(\text{CrtO}\) gene) of Synechocystis PCC6803, \(\text{CrtW}\) gene) of \(\text{Erwinia herbicola}\) and \(\text{Erwinia amylovora}\) respectively, used\cite{44-45}. All results presented in this article were obtained with pAC-BETA.
C. terhisTagHisTag

PCC 7120 were cloned in the plasmid. The primers OCPsyn-Ctag (F and R) were used to amplify the cDNA of the OCP from the plasmid p2A734SH51. The primers OCPpAna-Ctag (F and R) were used to amplify the cDNA of the OCP from genome DNAs of Anabaena PCC 7120, the C-terminal His-tag was then added by PCR mutagenesis. The resulting PCR products were introduced into pCDF-duet1 to create the pCDO-OCPSyn-Ctag, pCDO-OCParthro-Ctag and pCDO-OCpAna-Ctag plasmids.

Modifications in His-tagged OCPs

The modifications in His-tagged OCPs were performed as described in Supplementary Table 1. The modification 3aaNtag was also created in the pCDO-OCPSyn-Ctag plasmid. The modification 3aaNtag from Arthrospira and Anabaena was created using the pCDO-OCParthro-pDuet and pCDO-OCpAna-pDuet plasmids as templates and the oligonucleotides described in Supplementary Fig. 7.

Transformation of E. coli cells and induction of genes.

E. coli BL21 Gold (DE3) cells from Agilent Technologies (F- ompT hsdSB (r m b) dcm + Tet r (DE3) endA A Hsr) were used for OCP production. BL21 cells were transformed simultaneously with three plasmids: pAC-BETA, pBAD-CrtO (or pBAD-CrtW) and pCDO-OCP. The pCDO-OCP plasmid contains WT or modified sequences of OCPs.

Measurements of OCP fluorescence quenching activity.

Isolated Synecystis PCC 6803, Arthrospira Platensis PCC7345 and Anabaena PCC 7120 were cloned in the plasmid. The primers OCPsyn-Ctag (F and R) were used to amplify the cDNA of OCP from the plasmid with OCPsyn-CterHisTagHisTag. The primers OCPpAna-Ctag (F and R) were used to amplify the cDNA of the OCP from genome DNAs of Anabaena PCC 7120, the C-terminal His-tag was then added by PCR mutagenesis. The resulting PCR products were introduced into pCDF-duet1 to create the pCDO-OCPSyn-Ctag, pCDO-OCParthro-Ctag and pCDO-OCpAna-Ctag plasmids.

Measurement of carotenoid content in OCPs.

The carotenoid content of E. coli cells and the isolated OCPs was analysed by High-Performance Liquid Chromatography (HPLC) and Mass spectrometry as described in.

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Author contributions
C.B.C. and D.K. designed research; C.B.C., A.T., A.W. and F.P. performed research; C.B.C., A.T. and D.K. analyzed data; D.K. wrote the paper with inputs of C.B.C. and A.T.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: Phycosource (that paid the salary of CBC) has deposited a patent describing the method to obtain holo-OCP in E.coli. However, the value of the patent will not be affected by this article.

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