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Random mutagenesis and overexpression of rhodopin-3,4-desaturase allows the production of highly conjugated carotenoids in *Rhodospirillum rubrum*

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ABSTRACT

The *crtD* gene of the purple bacterium *Rhodospirillum rubrum*, encoding rhodopin desaturase, was cloned into a broad-host range expression plasmid (pRKCAG53) and transferred to the *R. rubrum crtD*$^-$ mutant, ST4, which restored the wild-type phenotype and produced the carotenoid spirilloxanthin. pRKCAG53 was randomly mutated in an *Escherichia coli* mutator strain and then transferred to ST4 for selection of non-wild-type phenotypes. Strains containing the mutated expression plasmid exhibited two coloured phenotypes: a "brown" phenotype, corresponding to 3,4,3′,4′-tetrahydrospirilloxanthin, arising from plasmids containing an inactivated *crtD* gene, and secondly, a "dark pink" phenotype. Absorption and mass spectra and HPLC analysis obtained from hexane extracts of brown mutants, confirmed the carotenoid assignment above. DNA sequence analysis of the *crtD* genes from the brown transconjugants showed frameshifts at the extreme C-terminus, suggesting that this domain forms part of the active site. Spectral analysis of the dark pink strains showed an additional, non-natural double bond formed at the carotenoid end, yielding the asymmetric carotenoids, 3,4,3′,4′-tetradehydrorhodopin – and 3′,4′-didehydroanhydrorhodovibrin, each containing 14 conjugated double bonds. For only two dark pink strains, was a mutation in *crtD* detected, in both cases at the N-terminus of CrtD. Otherwise, the higher conjugation was ascribed to an elevated plasmid copy number.

Introduction

The production of carotenoids in purple bacteria is an interesting novel strategy for the biotechnological realisation of this class of substances [8,22]. Purple bacteria naturally produce carotenoids in high amounts and incorporate them into their photosynthetic apparatus, which consists of a light-harvesting complex (LH1)[1] and a reaction centre [19]. The pathway of carotenoid biosynthesis in purple bacteria has recently been divided into two main classes: the "normal" spirilloxanthin-type pathway and the "unusual" spirilloxanthin pathway (reviewed in [21]). In the "normal" spirilloxanthin pathway, which occurs in the organism employed in this study, *Rhodospirillum rubrum* [3], the central 24 carbons of phytoene are sequentially desaturated, by a single enzyme, phytoene desaturase, to yield the carotenoid, lycopene, where the 3,4- and 3′4′-carbons remain saturated, followed by hydration, desaturation, and methylation at each end, to yield the symmetric carotenoid, spirilloxanthin (Fig. 1, central column). The 3,4- and 3′,4′-end desaturation steps are performed by a single gene product, which encodes the enzyme rhodopin desaturase (CrtD). It has been shown previously, both by PCR-mediated gene shuffling, followed by expression in *Escherichia coli* [16], and high-level coexpression of different enzyme combinations in *E. coli* [1], that phytoene desaturase can also perform both 3,4- and 3′,4′-desaturation. Recently, we have shown, using an *R. rubrum crtD*$^-$ mutant, ST4 [11], that phytoene desaturase, even at naturally occurring expression levels, can in fact perform both 3,4- and 3′,4′-desaturation under conditions of limiting oxygen [2]. However, in all of these studies, the levels of carotenoids corresponding to these reactions are only minor, and the major carotenoid in the *crtD*-mutant ST4 at the end of the growth phase is 3,4,3′,4′-tetrahydrospirilloxanthin [11,2], which is the product of two sequential hydration and methylation reactions in the absence of 3,4,3′,4′-desaturation. The study of ST4 has confirmed that the carotenoid biosynthesis enzymes CrtC (lycopene hydratase), CrtD, and CrtF (3,4-didehydrorhodopin methylase) have only a low end group specificity and are quite capable of acting combinatorially on a wide variety of carotenoid substrates. Thus, the normal "linear" spirilloxanthin

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[1] *Abbreviations used:* LH1, light-harvesting complex 1; PK, "pink" strain; DP, "dark pink" strain; B, "brown" strain.

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pathway observed in *R. rubrum* is primarily a consequence of kinetic matching of sequential steps, and not due to a fixed order of reaction. This may be different for the corresponding enzymes of the related bacteria *Rhodobacter capsulatus* or *Rhodobacter sphaeroides*, where end-modification apparently only occurs at one end of the molecule (see [21]. This is particularly striking for the enzyme CrtD, which, at least chemically speaking, should be able to perform 3′,4′-desaturation in the absence of hydration (see below).

We hypothesised that in *R. rubrum*, the symmetric 3,4,3′,4′-end saturation of the carotenoid molecule may be due to the particular domain structure of the *R. rubrum* CrtD enzyme. Consequently, using random mutagenesis, it may be possible to arrest the enzyme after the first end-desaturation, thereby leading to the accumulation of an asymmetric carotenoid during growth. This hypothesis was supported by the observation that despite the clear homology amongst CrtDs from different purple bacteria, the putative topology of the *R. rubrum* enzyme, as predicted from its Kyte and Doolittle hydropathy profile [12] is significantly different from those of CrtDs from *Rb. sphaeroides* and *Rb. capsulatus* (data not shown).

In our preliminary study, we decided to adopt a novel mutagenic strategy. We have reported previously [22] that the *R. rubrum* *crtD*$^{-}$ mutant ST4 can be complemented by a DNA fragment of the *R. rubrum* chromosome containing several genes of carotenoid biosynthesis (Fig. 2). The transconjugant produces spirilloxanthin exclusively in wild-type quantities. Here, we show that random mutation of the *crtD* gene, followed by expression in ST4, allows the isolation of brown mutants containing a single frameshift within the open reading frame encoding the active site domain. In addition, we have isolated and characterised several "dark pink" strains which produce the new unnatural carotenoids, 3,4,3′,4′-tetradehydrorhodopin, and 3′,4′-didehydroanhydrorhodovibrin, which contain 14 conjugated double bonds, due to overexpression of the unmodified *crtD* gene in the ST4 background.

Materials and methods

Growth of bacteria

E. coli cultures were grown in Luria–Bertani medium [15] at 37 °C with the following antibiotics concentrations added as required: ampicillin (Na^+-salt), 100 μg/ml (amp_{100}); kanamycin sulphate, 50 μg/ml (kan_{50}); tetracycline-HCl, 10 μg/ml (tet_{10}). *R. rubrum* was cultivated photoheterotrophically, anaerobically in Sistrom minimal medium A (M medium) [18] in closed bottles on a magnetic stirrer at 30 °C or on M medium agar-plates in an anaerobic jar (Oxoid) under a CO_2/H_2 atmosphere (Gas Generating Kit, Anaerobic System (BR0038B) from Oxoid). 20 μg/ml kanamycin sulphate and 4 μg/ml tetracycline-HCl were added as required.

Construction and random mutagenesis of the plasmid pRKCAG53

Plasmid DNA was isolated by using kits obtained from QIAGEN, Roche Diagnostics or Eppendorf and was cloned by standard procedures [15]. Restriction and other standard enzymes for molecular biology were from New England Biolabs.

The *crtD* expression plasmid, pRKCAG53 was created as follows. First, the plasmid pBsSGE5 [22], which contains a 6.5 kb EcoRV fragment of *R. rubrum* chromosomal DNA (Fig. 2), ligated into the SmaI

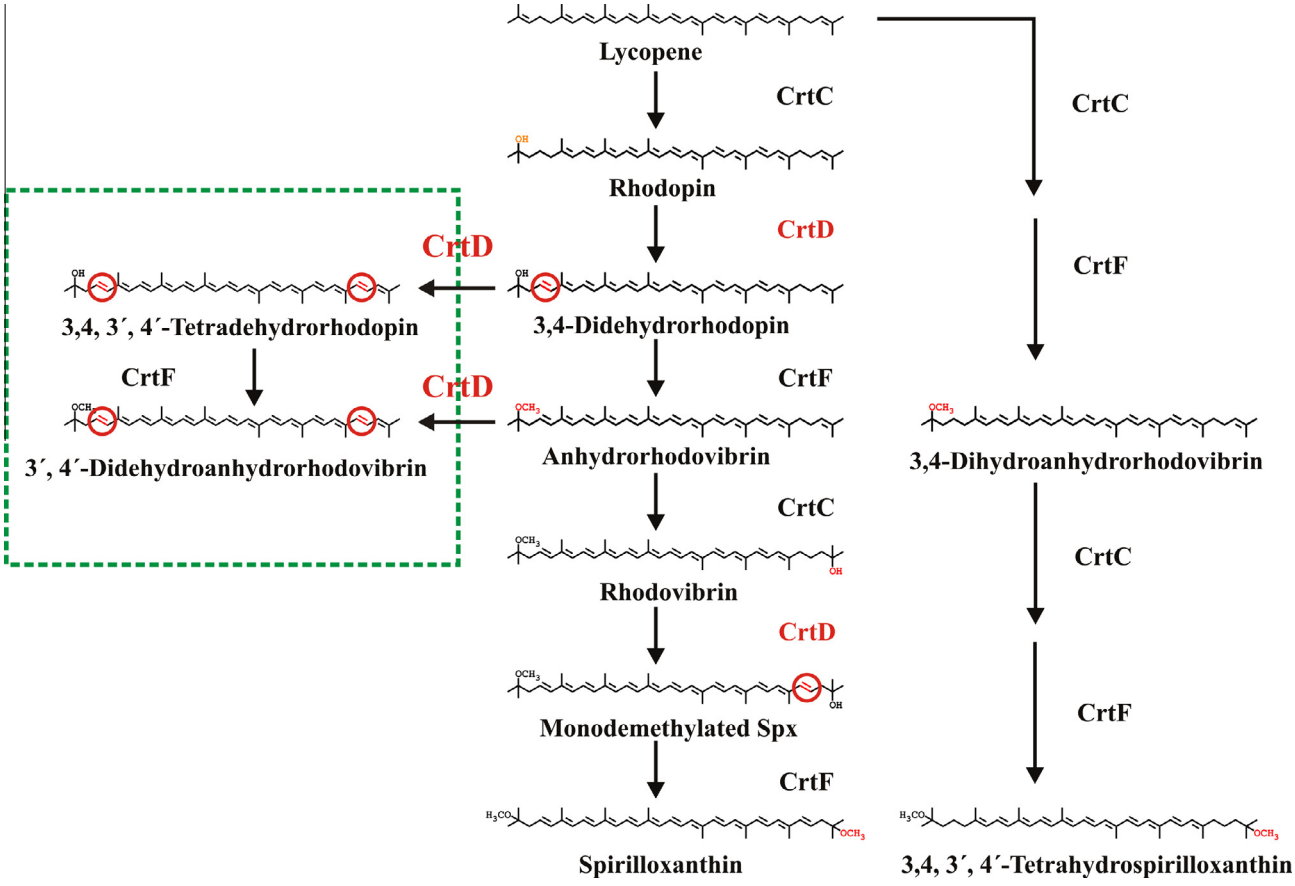

Fig. 1. Summary of the carotenoid pathways proposed in this study. The "normal" spirilloxanthin pathway, leading to the formation of spirilloxanthin in wild-type *R. rubrum* [3], and the "unusual" spirilloxanthin pathway, resulting in 3,4,3′,4′-tetrahydrospirilloxanthin in the mutant ST4 [11], are shown in the central and right columns, respectively. The putative CrtD-mediated pathways (indicated by red circles) found in this study, leading to the formation of 3,4,3′,4′-tetradehydrorhodopin and 3′,4′-didehydroanhydrorhodovibrin are shown in the green dotted box.

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