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Functional assembly of the foreign carotenoid lycopene into the photosynthetic apparatus of *Rhodobacter sphaeroides*, achieved by replacement of the native 3-step phytoene desaturase with its 4-step counterpart from *Erwinia herbicola*

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Summary

Photosynthetic organisms synthesize a diverse range of carotenoids. These pigments are important for the assembly, function and stability of photosynthetic pigment–protein complexes, and they are used to quench harmful radicals. The photosynthetic bacterium *Rhodobacter sphaeroides* was used as a model system to explore the origin of carotenoid diversity. Replacing the native 3-step phytoene desaturase (CrtI) with the 4-step enzyme from *Erwinia herbicola* results in significant flux down the spirilloxanthin pathway for the first time in *Rb. sphaeroides*. In *Rb. sphaeroides*, the completion of four desaturations to lycopene by the *Erwinia* CrtI appears to require the absence of CrtC and, in a *crtC* background, even the native 3-step enzyme can synthesize a significant amount (13%) of lycopene, in addition to the expected neurosporene. We suggest that the CrtC hydroxylase can intervene in the sequence of reactions catalyzed by phytoene desaturase. We investigated the properties of the lycopene-synthesizing strain of *Rb. sphaeroides*. In the LH2 light-harvesting complex, lycopene transfers absorbed light energy to the bacteriochlorophylls with an efficiency of 54%, which compares favourably with other LH2 complexes that contain carotenoids with 11 conjugated double bonds. Thus, lycopene can join the assembly pathway for photosynthetic complexes in *Rb. sphaeroides*, and can perform its role as an energy donor to bacteriochlorophylls.

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Introduction

Carotenoids are among the most widespread of all natural pigments and have recently been the focus of intense interest in both plant biology and medicine. They are important in photosynthesis because they carry out three major functions. First, they absorb solar energy which is transferred to chlorophylls; second, they are important for the assembly and stability of some photosynthetic pigment–protein complexes (Zurdo *et al*., 1993; Lang and Hunter, 1994); and third, they act as photoprotectors by preventing the formation of singlet oxygen and by directly quenching this and other harmful radicals (Foote, 1976).

The synthesis of phytoene, which has three conjugated double bonds, is followed by subsequent dehydrogenations of the molecule catalysed by phytoene desaturase. This produces neurosporene (three desaturations, nine conjugated double bonds) in *Rhodobacter* (Giuliano, Pollock and Scolnik, 1986), or lycopene (four desaturations, 11 conjugated double bonds) in *Erwinia* (Fraser *et al*., 1992), plants and other photosynthetic bacteria such as *Rhodopseudomonas acidophila* and *Rhodospirillum rubrum*. Further modifications catalysed by other enzymes include the introduction of double bonds, hydroxy and methoxy groups and, in *Erwinia* and plants, cyclization of the ends of the molecule to produce a diverse range of final products. The photochemical properties of carotenoids, such as the energy levels of their excited singlet states, change in a graded way as the number of conjugated double bonds increases, and so we have suggested that phytoene desaturase occupies a pivotal role in determining carotenoid diversity and function (Garcia-Asua *et al*., 1998).

It is now possible to examine the basis for this diversity in a controlled way *in vivo*, by creating new carotenoid pathways in the photosynthetic bacterium, *Rhodobacter sphaeroides.* In this bacterium, and in the closely related *Rhodobacter capsulatus*, the genes encoding the carotenoid biosynthetic pathway enzymes are clustered within a region of the chromosome which is contained within a larger 45 kb cluster of photosynthesis-related genes (Coomber *et al*. 1990; Alberti, Burke and Hearst,

1995; Lang *et al*., 1995; Naylor *et al*., 1999). In the non-photosynthetic bacterium *Erwinia herbicola*, which produces plant-type carotenoids, genes for carotenoid biosynthesis genes are also clustered (Hundle *et al*., 1994). This information allows us to reprogramme the carotenoid biosynthetic machinery of *Rb. sphaeroides*; for example, the *E. herbicola crtYIBZ* genes have been used to direct the synthesis of the cyclic carotenoid β-carotene in *Rb. sphaeroides* (Hunter *et al*., 1994). In this paper, we report the consequences of a single gene replacement by introducing the 4-step phytoene desaturase from *E. herbicola* into a *crtIC* double mutant of *Rb. sphaeroides*. This manipulation replaces the native 3-step desaturase with the 4-step enzyme and, at the same time, blocks the subsequent biosynthetic step (CrtC) in *Rb. sphaeroides*, thereby preventing subsequent modifications of lycopene (see Fig. 1). As a result, lycopene is produced from phytoene, instead of neurosporene, and accumulates in the cell.

However, it is important to investigate whether this new carotenoid can play a functional role. There is a wealth of structural and spectroscopic data on carotenoids on which to base this assessment. Recent structural data show that the functions of carotenoids in photosynthesis depend on specific, short-range (<5 Å) contacts between carotenoid and chlorophyll pigments. Such contacts can now be observed in atomic detail and have been elucidated by studies on the structure of the light harvesting LH2 complexes from the photosynthetic bacteria *R. acidophila* (McDermott *et al*., 1995) and *Rhodospirillum molischianum* (Koepke *et al*., 1996). Lycopene is the carotenoid present in the latter, and this provides a rationale for choosing lycopene as a replacement carotenoid in the LH2 complex of *Rb. sphaeroides*, which is structurally similar to the LH2 from *R. acidophila* (Walz *et al*., 1998). Thus, there is an opportunity to engineer the carotenoid biosynthetic pathway of *Rb. sphaeroides*, and to put data obtained on one of the final products of membrane protein assembly, the LH2 complex, in a structural context. From the viewpoint of assembly, it has been observed that coloured carotenoids such as neurosporene are essential structural elements during the biogenesis of the peripheral antenna complex, LH2 (Lang *et al*., 1995). Interestingly, however, both the reaction centre and the core antenna complex (LH1) do not have this requirement, and can be successfully assembled in the absence of carotenoids (Lang and Hunter, 1994). The LH2 complex containing lycopene has been solubilized and purified; we demonstrate that lycopene has been functionally assembled into this membrane protein complex, by measuring the transfer of light energy absorbed by lycopene to the bacteriochlorophylls of the LH2 complex. Our results are discussed in relation to the branching of the carotenoid pathway and the assembly and function of bacterial light-harvesting complexes.

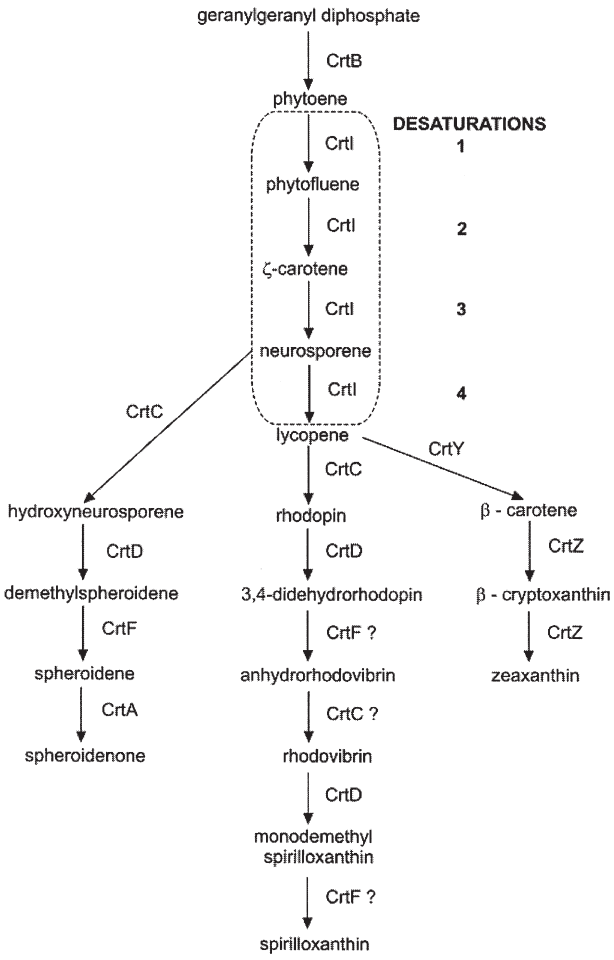

Fig. 1. Carotenoid biosynthesis pathways leading to spheroidene/spheroidenone (*Rhodobacter sphaeroides*), spirilloxanthin (putatively *Rhodospirillum rubrum*) and zeaxanthin (*Erwinia herbicola*). The reactions catalysed by phytoene desaturase are contained within the dashed outline. The four desaturations catalysed by the CrtI from *E. herbicola* produce lycopene. The *Rb. sphaeroides* CrtI catalyses three desaturations, producing neurosporene. The *R. rubrum* pathway has not been subjected to detailed genetic or biochemical analyses, and the steps catalysed by CrtC and F in particular await further studies. However, recent work shows the presence of a CrtD in *R. rubrum* (Komori *et al*., 1998). In *Rubrivivax gelatinosus*, CrtC and CrtD have been identified and shown to be involved in both the spheroidene and the spirilloxanthin pathways (Ouchane *et al*., 1997).

Results

To engineer the production of lycopene in *Rb. sphaeroides*, it was necessary to replace the native 3-step phytoene desaturase with the 4-step enzyme, and to ensure that the lycopene produced was not metabolized further by the CrtC/D/F/A enzymes (see Fig. 1). This

requires the disruption of both the native *crtI* gene and the subsequent biosynthetic step (encoded by *crtC*), thereby preventing subsequent modifications of lycopene (see Fig. 1). The following experiments were necessary at the outset to examine the consequences of separately complementing *crtI* and *crtC* mutants of *Rb. sphaeroides* with the *crtI* gene from *E. herbicola*.

Expression of the E. herbicola crtI *gene encoding a 4-step desaturase in a* crtI *mutant of* Rb. sphaeroides *produces a new carotenoid pathway*

Complementation of a *crtI* mutant TC71 (Lang *et al*., 1994) with the *crtI* from *E. herbicola,* which encodes the 4-step desaturase, would be expected to re-route the carotenoid pathway of *Rb. sphaeroides*, which uses the 3-step enzyme, down the spirilloxanthin pathway (see Fig. 1). To test this, pERWI2, containing the *Erwinia crtI* gene in the broad host range vector pRKSK1, was introduced using conjugative transfer into TC71. The control transcongugant strain, TC71[pHLKPD1], contained the native *crtI* encoding the 3-step desaturase. In each case, expression of plasmid-borne *crtI* genes arose from the use of low oxygen conditions, which are known to activate the *puc* promoter used in pRKSK1 (Hunter *et al*., 1994).

The recipient strain TC71 contains normal cellular levels of phytoene on a per cell basis (Table 1). The Tn*5* insertion into *crtI* in mutant TC71 could, in theory, cause polar effects on *crtB* in the event of strong transcriptional coupling between *crtI* and *B*. As *crtB* encodes phytoene synthase, the enzyme that precedes phytoene desaturase in the carotenoid biosynthetic pathway, such a polar effect could limit phytoene production. However, our data suggest that if there is transcriptional coupling between *crt I* and *B*, it must be weak, given the levels of phytoene measured. This makes it all the more difficult to explain the relatively low amounts of carotenoid in TC71[pHLKPD1] and TC71[pERW12], at approximately 45% of that found in the wild type (Table 1). Other factors might come into play in these transconjugant strains, for example inhibition of phytoene desaturation by an end-product of the pathway such as spheroidenone. There are several examples in the literature of such inhibition; other phytoene desaturases are inhibited by carotenoids such as neurosporene, lycopene and β-carotene (Bramley and Davies, 1976; Sandman and Kowalczyk, 1989; Martinez-Laborda *et al*., 1990). It is interesting to note that there is no such limitation on carotenoid levels in G1c[pERW12], in which spheroidenone is absent (Table 1).

The transconjugant strain TC71[pERWI2] synthesized a mixture of carotenoids from both the spheroidenone and spirilloxanthin series (see Table 1), amongst them spheroidenone, neurosporene, lycopene and a ketospirilloxanthin-like carotenoid later identified as 2,2′-diketospirilloxanthin. The broadly similar amounts of carotenoid synthesized per cell for both TC71 transconjugants indicate that in relative terms there is significant flux down the spirilloxanthin pathway in TC71[pERWI2], amounting to approximately half the total carotenoid. However, the 4-step desaturase could have directed all of the flux down this pathway in principle, so clearly there must be another factor influencing the number of desaturations catalysed by the *Erwinia* enzyme. This will be commented on in the *Discussion*.

Expression of the E. herbicola crtI *gene in a* crtC *mutant of* Rb. sphaeroides

To prevent further modification of lycopene produced by the *Erwinia* phytoene desaturase, it was necessary to stop the first hydroxylation step, assumed to be catalysed by the *Rb. sphaeroides* CrtC enzyme. Mutant G1c of *Rb. sphaeroides*, which contains a point mutation in *crtC* and, therefore, accumulates mainly neurosporene (Holmes and Crofts, 1977), formed the basis for this and subsequent work. When the *E. herbicola crtI* was introduced into the *crtC* mutant G1c, lycopene (83%) becomes the dominant carotenoid, although a substantial amount of neurosporene is also found (17%; Table 1). It is clear that the disruption of *crtC* is sufficient to arrest metabolism of neurosporene in G1c and of lycopene in G1c[pERW12]. Thus, the CrtD enzyme requires a hydroxylated substrate, and that the pathway is obliged to follow the sequence CrtC-CrtD. This is consistent with data on the purified CrtD enzyme from *Rb. sphaeroides* obtained by (Albrecht *et al*., 1997), and with recent experiments by Steiger and colleagues (Steiger *et al*., 2000) on the *Rubrivivax gelatinosus* CrtD.

In G1c[pERW12], the desired blockage of the pathway has been achieved at the CrtC stage, and the plasmid-encoded *Erwinia* desaturase sucessfully competes with the native desaturase for the phytoene substrate, resulting in lycopene as the major carotenoid. This shift in favour of the 4-step desaturase is probably a consequence of the plasmid copy number (4–6 per cell; Davis *et al*., 1988), and of the strong *puc* promoter used to drive transcription of the plasmid-encoded desaturase.

Strain G1c[pERW12] overproduces carotenoid (lycopene) on a cellular basis, in contrast with the lowered carotenoid production in TC71[pHLKPD1] and TC71[pERW12] in the previous section. Limitations such as a weak polar effect of the *crtI* mutation in TC71 on *crtB,* or inhibtion of phytoene desaturase activity by spheroidenone are absent from G1c[pERW12]. The overproduction of carotenoid in G1c[pERW12] is in contrast to the lowered levels of lycopene-associated photosynthetic complexes, manifested as bacteriochlorophyll per cell

Table 1. HPLC analysis of the carotenoids of *Rhodobacter sphaeroides crtI*, *crtC* and *crtIC* mutants complemented with *crtI* genes encoding native and *Erwinia herbicola* phytoene desaturases.

Mutant/strain	Description	Phytoene	ζ-Carotene	Neurosporene	Lycopene	Spheroidene /one	2,2′-diketo spirilloxanthin	Bacteriochloro -phyll/cell	Carotenoid /cell	Carotenoid /bacteriochlorophyll
TC71	*crtI*::Tn*5* mutant	**100**						45 ± 9	110 ± 21	2.5
TC71[pHLKPD1]	TC71 complemented *in trans* with *crtI* from *Rb. sphaeroides*	5	2	30		**63**		72 ± 13	44 ± 9	0.6
TC71[pERWI2]	TC71 complemented *in trans* with *crtI* from *E. herbicola*	10	8	10	5	20	**48**	43 ± 12	46 ± 4	1.1
G1c	*crtC* point mutant of *Rb. sphaeroides*			**98**	2			117 ± 3	128 ± 22	1.1
G1c[pERWI2]	G1c complemented *in trans* with *crtI* from *E. herbicola*			17	**83**			60 ± 10	180 ± 32	3.0
DM2	*crtIC* double mutant: point mutation in *crtC*; *crtI*::Tn*5*	**100**						55 ± 6	114 ± 33	2.2
DM2[pHLKPD1]	DM2 complemented *in trans* with *crtI* from *Rb. sphaeroides*			**87**	13			127 ± 15	135 ± 18	1.1
DM2[pERWI2]	DM2 complemented *in trans* with *crtI* from *E. herbicola*	**4**		3	**93**			66 ± 4	60 ± 7	0.9
NCIB 8253	Wild type					**100**		100	100	1.0

The main carotenoid accumulated for each strain/sample is highlighted in bold. The amount of carotenoid is expressed as a percentage of the total. Pigment content per cell is expressed in arbitrary units. Between four and eight data sets were collected, from two independent cultures. The cell pigment data were compared by first normalizing to the absorbance at 680 nm, which gives an estimate of cell density, and then assigning the values for the wild type as 100.

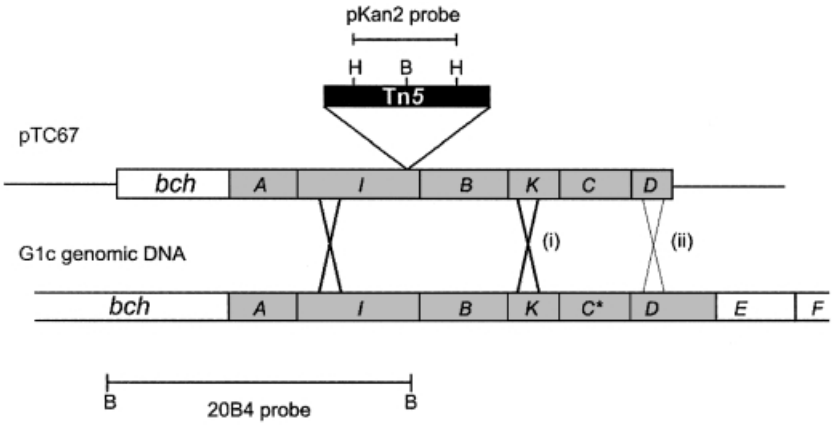

Fig. 2. Schematic representation of the construction of a double crtIC mutant by directed transposon insertion. The desired recombination process is highlighted with two bold crosses. If recombination occurs in *crtD* (ii), the defective *crtC* gene (*crtC**) will be replaced by the functional gene and a *crtI* single mutant will be obtained. If recombination (i) occurs the *crtC** point mutation is maintained in the genome and the native functional *crtI* is replaced by the Tn*5*-mutated *crtI*. 20B4 and pKan2 were two of the probes used for verification of the correct double mutant by Southern blot hybridization. *bch*, bacteriochlorophyll genes; *AIBKCD* in the grey box, carotenoid biosynthesis genes with counterparts in pTC67; *E, F* other *crt* biosynthesis genes; H, *Hin*dIII; B, *Bam*HI.

(Table 1). This shows that compared with the biosynthesis of bacteriochlorophyll, carotenoid biosynthesis tends to be less tightly coupled to photosystem assembly.

Construction and properties of a crtIC *double mutant of* Rb.sphaeroides*, and its complementation with* crtI *from* E. herbicola

To increase the percentage of lycopene, inactivation of the host phytoene desaturase in a *crtC* background was required. This necessitated the construction of a *crtIC* double mutant of *Rb. sphaeroides*, providing a decisive block in the native pathway, and a background in which to examine a 4-step desaturase in the absence of a competing native 3-step desaturase and the CrtC enzyme.

Mutant G1c formed the basis for the double mutant. G1c was the recipient for pTC67, a derivative of the pSUP202 suicide vector containing part of the *Rb. sphaeroides* carotenoid biosynthesis gene cluster with a Tn*5* transposon insertion located in the 3′-region of *crtI* (see Fig. 2). This insertion exerts a weaker polar effect on *crtB*, in comparison with that in TC71 (Lang *et al*., 1994). pTC67 was introduced into G1c by conjugative transfer to force recombination and replacement of the functional *crtI* with the disrupted one. Resulting putative mutants were coloured blue as a result of the synthesis of bacteriochlorophyll and of phytoene (colourless) and, after verification of the correct strain (see *Experimental procedures*), the chosen mutant was designated DM2. High-performance liquid chromatography (HPLC) analysis of DM2 (Fig. 3) demonstrated that phytoene was the sole carotenoid present, and that complementation with the native *crtI* gene resulted in the synthesis of mainly neurosporene (Table 1). Cellular levels of carotenoid and bacteriochlorophyll are restored to this transconjugant, in contrast to TC71[pHLKPD1] (see earlier). Thus, this mutant had been successfully engineered to avoid further modification of the product of the native 3-step phytoene desaturase, and was capable of wild-type levels of carotenoid production.

The absorbance spectrum of mutant DM2 indicated the lack of the 800 and 850 nm bands characteristic of the light-harvesting LH2 complex, as seen for the *crtI* mutant TC72 (Lang *et al*., 1994). Accordingly, the cellular level of bacteriochlorophyll was approximately half that of the wild type in DM2, although phytoene levels were appreciable

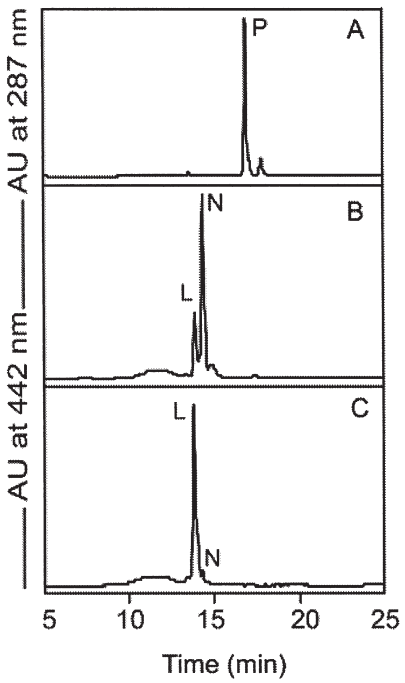

Fig. 3. HPLC traces of the double *crtIC* mutant DM2 and its transconjugant strains:
A. DM2.
B. DM2[pHLKPD1].
C. DM2[pERWI2].
DM2[pHLKPD1] and DM2[pERWI2] contain the native (3-step) and *E. herbicola* (4-step) phytoene desaturases respectively. In all cases, samples were analysed using a PDA detector in the range 270–650 nm. L, lycopene; N, neurosporene; P, phytoene; AU, absorbance units.

(Table 1). However, when DM2 was complemented with the native *crtI* gene, the characteristic LH2 bands were restored (results not shown), as were bacteriochlorophyll levels. This is consistent with the observations of Lang and Hunter (1994) in which LH2 formation was abolished in phytoene-containing mutants, and only occurred when neurosporene and subsequent carotenoids were synthesized.

The next experiment was designed to reveal some of the inherent catalytic abilities of the desaturases of *Rb. sphaeroides* and *E. herbicola*, by transferring pERWI2, which contains the *E. herbicola crtI* gene in a broad host-range plasmid (see *Experimental procedures*), into the *crtIC* mutant DM2. This results in replacement of the native phytoene desaturase with the *E. herbicola* counterpart. HPLC analysis showed that DM2[pERWI2] synthesized 93% lycopene, 3% neurosporene and 4% phytoene (Fig. 3, and Table 1), and the cellular level of carotenoid was approximately half that found in the wild type. The percentage of lycopene has been raised by 10% in DM2[pERWI2] in comparison with G1c[pERW12], although the cellular level of carotenoid is significantly higher in G1c[pERW12], which contains two competing desaturases. Analysis of the control transconjugant strain DM2[pHLKPD1] showed that the synthesis of neurosporene was restored and, unexpectedly, a significant amount (13%) of lycopene (see Table 1). It can be seen that a small amount of lycopene is synthesized even in strain G1c, but this tendency has been increased in DM2[pHLKPD1]. The native 3-step phytoene desaturase must therefore be capable of a further step, in which some of the neurosporene produced is converted to lycopene.

Functional assembly of lycopene into the photosynthetic apparatus

It was important to find out whether or not lycopene could carry out a light-harvesting function, which would have implications for the assembly of the LH complexes and for the specificity and efficiency of interaction between the carotenoids and bacteriochlorophyll molecules. The results in Table 1 show that DM2[pERWI2] synthesizes approximately half the cellular levels of bacteriochlorophyll found in the wild type, so there is some impairment of photosystem assembly in the presence of lycopene. This lowered level of photosynthetic complexes is accompanied by a similar decrease in cellular carotenoid content. Membranes were prepared from DM2[pERWI2], and from the *crtC* mutant G1c as a control. The absorbance spectra of these membranes (Fig. 4A) show peaks at approximately 800 and 850 nm in both samples arising from the LH2 complex; the LH1 complex is also present, judging by the 875 nm shoulder on the 850 nm band. The fluorescence excitation spectra showed a good

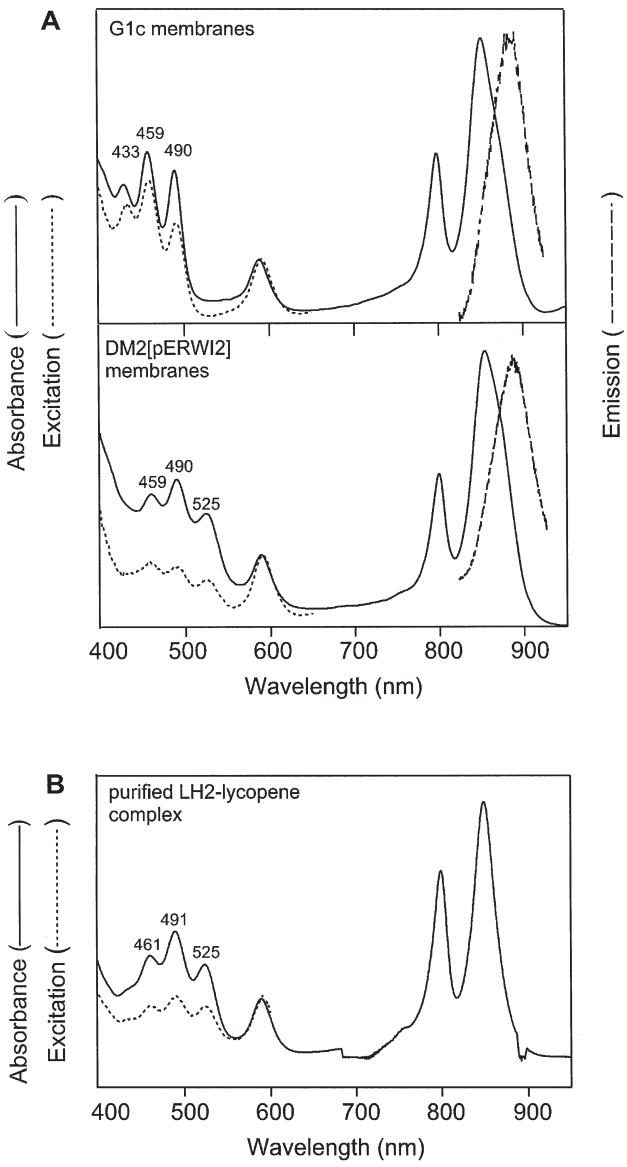

Fig. 4. Absorbance and fluorescence spectroscopy of photosynthetic membranes and complexes containing lycopene.
A. Absorbance, and fluorescence excitation and emission spectra of G1c and DM2[pERWI2] membranes. The absorbance and excitation spectra were recorded between 400 and 600 nm, and were normalized at the Q_x band of Bchl at approximately 590 nm. For the excitation spectra, detection was at 890 nm. In the emission spectra, LH2 was selectively excited at 800 nm. The DM2[pERWI2] membranes contain mainly lycopene (Table 1); the G1c membranes are included as a control containing a carotenoid, neurosporene, which is native to *Rb. sphaeroides*. The absorbance maxima in the carotenoid region are indicated.
B. Absorbance and fluorescence excitation spectra of LH2 complexes purified from detergent-solubilized membranes from DM2[pERWI2]. The absorbance and excitation spectra have been normalized at the Q_x band of the Bchl at approximately 590 nm. Fluorescence detection was at 890 nm. The absorbance maxima in the carotenoid region are indicated.

match with the absorbance spectra in the 400–500 nm region, which qualitatively demonstrates that carotenoid to bacteriochlorophyll energy transfer occurs whether neurosporene (G1c) or lycopene (DM2[pERWI2]) is present. It has already been demonstrated that efficient energy transfer from neurosporene occurs in green strains (Cogdell *et al*., 1981). The relative peak heights of the excitation spectra in Fig. 4A show that energy transfer from neurosporene to bacteriochlorophyll is more efficient than from lycopene. However, this does not necessarily imply inefficient assembly of lycopene into LH complexes (see next section).

The absorbance maxima of neurosporene in membrane-bound complexes in strain G1c are shifted to the red by about 20–25 nm when compared with free neurosporene (data not shown). Similar red shifts are also seen in DM2[pERWI2], in which the spectrum is shifted about 20–25 nm with respect to the spectrum of free lycopene. Such red shifts, of approximately 20 nm, are characteristic of antenna-bound carotenoids (Andersson *et al*., 1991). The three peaks seen at 459, 490 and 525 nm case in the fluorescence excitation spectrum of DM2[pERWI2] correspond to lycopene. The fluorescence emission spectra, in which LH2 is selectively excited at 800 nm, show that the majority of emission is from the LH1 complex at approximately 900 nm in both the neurosporene-containing control (G1c), and in DM2[pERW12]. Thus, the introduction of lycopene has not impaired the normal process of energy transfer from the LH2 complex to LH1.

The incorporation of lycopene into the photosynthetic apparatus of this mutant shows that this carotenoid can perform its roles in assembly of the photosynthetic membrane, energy donation to bacteriochlorophylls and in intercomplex energy transfer. However, previous work has shown that in terms of assembly, the carotenoid requirements for LH2 and LH1-RC complexes differ substantially (Lang *et al*., 1994), with LH2 being the more selective. Therefore, it was necessary to examine the lycopene-containing LH2 complex in isolation.

Investigation into the functional incorporation of lycopene into LH2: solubilization, purification and spectroscopic analysis of the complex

The demonstration that the carotenoid biosynthetic pathway could be re-routed to produce lycopene, as well as the availability of high-resolution structural data for LH2, provided further impetus to carry out experiments on this complex. The complex was solubilized and purified from membranes of DM2[pERWI2] (see *Experimental procedures*). The absorbance spectrum of the purified lycopene–LH2 complex is displayed in Fig. 4B, and it shows the characteristic near-infrared maxima at approximately 800 and 850 nm. The relative peak heights are partly a consequence of the 1:2 ratio of 800:850 bacteriochlorophylls, and are those expected for the wild-type complex. Therefore, the assembly of lycopene into LH2 has not diminished binding of B800 bacteriochlorophylls within the complex, despite the close association of carotenoid and the B800 pigments apparent in the LH2 structure (McDermott *et al*., 1995; Fowler *et al*., 1997). The absorbance maxima at 461, 490 and 524 nm arise from the lycopene present, and can be compared with maxima of free lycopene in hexane at 444, 470 and 502 nm (not shown). Such red shifts, of approximately 20 nm, are characteristic of antenna-bound carotenoids, as noted in the previous section and in the work of Andersson and colleagues (Andersson *et al*., 1991). The fluorescence excitation spectrum in Fig. 4B measures the ability of light absorbed by carotenoid to elicit fluorescence from the LH2 bacteriochlorophylls. The excitation peaks, at 461, 491 and 525 nm, closely correspond to the absorbance maxima, and demonstrate that lycopene does transfer energy to the bacteriochlorophylls present. The excitation peak at 591 nm arises from bacteriochlorophyll and, as bacteriochlorophyll fluorescence is being measured, can be taken as the peak height corresponding to fully efficient transfer. Normalization of the 590 nm excitation and absorbance peaks allows us to calculate the efficiency of carotenoid to bacteriochlorophyll transfer by comparing the relative excitation and absorbance peak heights for the lycopene in LH2. We estimate that the efficiency of this transfer is 54%.

Discussion

A new carotenoid pathway in Rb. sphaeroides*: production of 2,2′-diketospirilloxanthin*

Expression of the *E. herbicola crtI* encoding a 4-step desaturase in a *crtI* mutant of *Rb. sphaeroides* redirects the native carotenoid pathway, which normally employs the 3-step enzyme. The result is now significant flux down the spirilloxanthin pathway (see Fig. 1) for the first time in *Rb. sphaeroides*, on the basis of both cellular levels and percentage of the total carotenoid (Table 1). Production of 2,2′-diketospirilloxanthin shows that the main barrier to the production of such carotenoids in *Rb. sphaeroides* is the nature of the phytoene desaturase; all of the other enzymes needed are present, and are able to metabolize lycopene as well as neurosporene. Thus, the homologues of CrtC, D and F must also be present in bacteria such as *R. rubrum*, which synthesizes spirilloxanthin. Similar conclusions were reached by Ouchane *et al*. (1997) in their mutagenesis study of carotenoid biosynthesis in *R. gelatinosus*, which synthesizes carotenoids of both the spirilloxanthin and spheroidenone pathways. The present work shows that the combination of a 4-step desaturase

and *R. sphaeroides* CrtC is sufficient to produce a carotenoid phenotype resembling that of *R. gelatinosus,* and that the CrtC enzyme of *Rb. sphaeroides* can hydroxylate both neurosporene and lycopene. The synthesis of 2,2′-diketospirilloxanthin in TC71[pERW12], rather than the spirilloxanthin found in *R. rubrum*, can be attributed to the introduction of keto groups by the CrtA enzyme, which normally converts spheroidene to spheroidenone. This suggests a lack of such an enzyme activity in *R. rubrum*.

The interaction of CrtC with phytoene desaturase

Despite the synthesis of 2,2′-diketospirilloxanthin in TC71[pERW12], it is evident that there still some flux down the native pathway (20% spheroidenone; Table 1), even though a 4-step CrtI is the only phytoene desaturase present. This enzyme is fully capable of catalysing four desaturations of phytoene to lycopene, as seen, for example, in strain DM2[pHLKPD1] of *Rb. sphaeroides*. However, we note that in this case CrtC is absent. We suggest that the native CrtC enzyme can intervene in the sequence of desaturations carried out by the 'foreign' 4-step enzyme, so that some of the catalytic cycles only complete three desaturations. In TC71[pERW12], which does contain a functional CrtC, this intervention directs 20% of the carotenoid down the spheroidenone pathway. Could CrtC exert a similar influence on the native 3-step enzyme? In the absence of CrtC, in the *crtIC* mutant DM2, the native desaturase produces a significant amount (13%) of lycopene, in addition to the expected neurosporene (see DM2[pHLKPD1] in Table 1). Lycopene is also synthesized in small amounts (2%) by the *crtC* mutant G1c. The native 3-step phytoene desaturase therefore appears to have a limited capability to catalyse a further step, and this tendency is normally curtailed when CrtC is present.

The idea that the catalytic properties of phytoene desaturases can be influenced by interactions with the ensuing enzyme in the biosynthetic pathway is consistent with conclusions from a previous study. This examined the consequences of expressing *Erwinia crtBIYZ* genes in *crtI* and *crtC* mutants of *Rb. sphaeroides* (Hunter *et al*., 1994). In the *crtI* (but functional CrtC) background, carotenoids of both the spheroidenone and spirilloxanthin pathways were obtained; again, the native CrtC appeared to be able to hydroxylate the product of three out of the four possible desaturations catalysed by the *Erwinia* CrtI. In the *crtC* mutant, which possesses the native 3-step phytoene desaturase as well as a 4-step *Erwinia* desaturase, conversion to non-*Rb. sphaeroides* carotenoids (84% β-carotene, 12% zeaxanthin and 3% β-cryptoxanthin; see Fig. 1 for this pathway) was complete. The absence of CrtC could have allowed the 'foreign' 4-step enzyme, and possibly the native enzyme as well, to carry out their full repertoire of desaturations. It was speculated in the work of Hunter and colleagues (Hunter *et al*., 1994) that the CrtY enzyme, which efficiently cyclizes the lycopene produced by the *Erwinia* desaturase, could also have exerted an influence by increasing the tendency of phytoene desaturase to carry out one further step.

The data presented here blur the apparent differences between the 3- and 4-step desaturases. Although the *Rb. sphaeroides* and *E. herbicola* desaturases are sufficiently different to produce widely varying proportions of lycopene and neurosporene, the differences between them are not absolute. This is also borne out in sequence alignments, which show 41.7% identity between the CrtI enzymes of *Rhodobacter* and *E. herbicola* (Armstrong *et al*., 1990). The capacity of phytoene desaturases to carry out three or four desaturations was addressed recently in a study by Wang and Liao (2001), who succeeded in using directed evolution to greatly enhance the capacity of the *R. sphaeroides* CrtI to carry out four desaturations. The eventual outcome of their mutagenesis experiments catalysed the production of 90% lycopene. This work shows that the major determinant for the number of desaturations is the CrtI sequence. However, when carotenoid biosynthesis pathways are formed from series of enzymes *in vivo*, other factors come into play, which can influence catalysis by phytoene desaturases.

The production of lycopene in Rb. sphaeroides*, and its functional assembly into the photosynthetic apparatus*

Construction of the *crtIC* mutant DM2 blocks the native pathway and provides a background for expression of the 4-step desaturase from *Erwinia*. The transconjugant strain DM2[pERW12] synthesizes 93% lycopene. This is the first time that it has been possible to engineer lycopene as the major carotenoid in *R. sphaeroides*, and it provides a new opportunity to investigate light harvesting complexes containing this carotenoid.

The spectroscopic studies in Fig. 4 show that this new carotenoid is assembled into light-harvesting complexes, and that it can transfer absorbed light energy to the bacteriochlorophylls, albeit less efficiently than neurosporene. We have also demonstrated that the introduction of lycopene has not impaired the normal process of energy transfer from the LH2 complex to LH1.

Thus, this carotenoid can perform its roles in assembly of the photosynthetic membrane, energy donation to bacteriochlorophylls and in intercomplex energy transfer. The membranes prepared from DM2[pERW12] contained intact photosynthetic units, so to facilitate a closer examination of the lycopene-containing LH2 complex, membranes were solubilized in detergent and the LH2 complex was purified. From the absorbance spectra, it was clear

that lycopene is correctly assembled into the LH2 complex in *Rb. sphaeroides*, as its absorbance peaks (which are sensitive to their environment) are at wavelengths characteristic for carotenoids with 11 conjugated double bonds. It is interesting to note the the absorption maxima of lycopene when bound to LH2 from *Rb. sphaeroides* are exactly the same as for rhodopin-glucoside (also 11 conjugated double bonds) bound to the native LH2 from *R. acidophila* (MacPherson *et al*., 2001).

The fluorescence excitation spectra allowed us to quantify the efficiency of energy transfer from lycopene to bacteriochlorophylls within LH2. The figure obtained, 54%, compares favourably with other LH2 complexes that contain carotenoids with 11 conjugated double bonds. In LH2 from *Rps. acidophila*, for example, the efficiency of carotenoid to bacteriochlorophyll energy transfer is 56% (MacPherson *et al*., 2001). In both cases, the efficiency of singlet–singlet energy transfer to bacteriochlorophyll (also very sensitive to correct occupancy of its binding site) is close to the maximum for carotenoids with 11 conjugated double bonds (Desamero *et al*., 1998). The replacement of carotenoids that are native to *Rb. sphaeroides,* such as neurosporene and spheroidenone by lycopene, creates new energy transfer pathways from lycopene to bacteriochlorophyll in the LH2 complex. These pathways can now be examined in detail using ultra-fast methods to provide new insights into the role of carotenoids in energy transfer dynamics of LH complexes.

Experimental procedures

Construction of a double crtI-crtC *mutant of* Rhodobacter sphaeroides *and complementation analysis with the native* crtI

Conjugative transfer between G1c and the S17-1 *Escherichia coli* strain containing pTC67 was carried out as described previously by Hunter and Turner (1988). As a result of this process, 180 blue colonies (accumulating phytoene) were isolated. It was necessary to investigate whether homologous single or double recombination had occurred; in the first case involving integration of the suicide vector in the chromosome, and in the second involving replacement of the native *crtI* with the *crtI* bearing the Tn*5* insertion (Fig. 2). Southern blot analysis was used to distinguish between these possibilities, and to confirm the correct insertion of Tn*5* in the targeted gene (not shown; details in following section). Complementation analysis was performed *in trans* on the 25 blue mutants that contained the *crtI*::Tn*5* genomic insertion, using a functional, native *crtI* borne on pHLKPD1, which replicates in *Rb. sphaeroides*. A total of 18 out of the transconjugants were red (containing spheroidenone) and seven were green (neurosporene). In the red ones, *crtI*::Tn*5* must have originally exchanged with the genomic copy of *crtI* through double homologous recombination as intended, but the point mutation in *crtC* had also been replaced with the wild-type gene borne on pTC67. In the green ones, recombination must have occurred in which *crtI* was inactivated, but the point mutation in *crtC* had been retained.

Verification of the presence of single homologous recombination process by Southern blot hybridization

Five blue colonies (three positives and two negatives), which turned green and red, respectively, after conjugative transfer of pHLKPD1, were randomly chosen for Southern blot hybridization analysis. Genomic DNA of G1c (*crtC* mutant), TC67 (*crtI* mutant) and each of these five mutants was prepared, digested overnight with *Bam*HI and electrophoresed on 0.8% agarose gels. Included in this analysis was *Bam*HI-cut pSUP202 and its derivative, pTC67, used in this experiment. Each Southern blot from the three gels was hybridized with a different probe, namely 20B4, pSUP202 and pKan2.

20B4 is a *Bam*HI fragment containing most of *crtI*, the whole of *crtA* and part of the bacteriochlorophyll biosynthetic cluster (Fig. 2). This probe should give rise to two bands, the larger one corresponding to *crtA*, part of the bacteriochlorophyll biosynthetic cluster and one half of Tn*5*, and the smaller one corresponding to a very short *crtI* fragment and the other half of Tn*5*. It should be noted that there is a single *Bam*HI site in the middle of Tn*5* (see Fig. 2). In all cases, only the signal corresponding to the larger fragment could be seen as a result of the fact that the transposon had inserted about 30 bp upstream of the *crtI* stop codon. However, this analysis demonstrated that there was an insertion into *crtI*.

pKan2 corresponds to the 3.5 kb *Hin*dIII Tn*5* fragment, which spans the central *Bam*HI site in Tn*5* (see Fig. 2); this was used to confirm the insertion of Tn*5*. When the *Bam*HI-digested genomic DNA samples were probed with this fragment, two bands were observed, as expected, which proved that the transposon had been integrated in the chromosome. No bands were detected in the case of G1c, the negative control.

Finally, it was necessary to check that the whole pTC67 plasmid had not integrated in the chromosome as a result of single homologous recombination. This was demonstrated by probing the blot with pSUP202, the plasmid vector that forms the basis of pTC67. Bands were only detected in the positive controls pTC67 and pSUP202, confirming the absence of single recombination events that would have led to integration of the vector into the genome.

Construction of plasmids pHLKPD1 and pERWI2 for the overexpression of the host and foreign CrtI enzymes

The *Erwinia herbicola crtI* gene was amplified by polymerase cahin reaction (PCR) using the construct pRER1B (Hunter et al., 1994) as a template, together with the crtI forward 5′-CGTCTAGAATTCGGAGAGATAAAGGATGAAAAAAAC CGTTG-3′ and reverse 3′-TTTCGGTGGCGGTCGGACTAC TTAAGTTCGAACGG-5′ primers. To ensure an optimum level of gene expression a Shine–Dalgarno sequence had been introduced in the forward primer. The PCR product of about 1.5 kb was then digested with *Xba*I and *Hin*dIII (sites engi-

neereed in the primers) and cloned into pRKSK1 to give pERWI2.

Complementation analysis of DM2 with the native phytoene desaturase was performed using pHLKPD1, a pRKSK1 derivative containing the *Rb. sphaeroides crtI* gene (Lang et al., 1994)

Growth conditions for Rb. sphaeroides

M22+ medium (Hunter and Turner, 1988), supplemented with vitamins, was used for growth of all strains and liquid cultures were supplemented with 0.1% casamino acids. Antibiotics were used at the following concentrations (in $\mu g\ ml^{-1}$): 1 (tetracycline); 20 (neomycin); 5 (streptomycin). By varying the growth conditions of *Rb. sphaeroides*, two states could be achieved: (i) chemoheterotrophic growth (dark semi-aerobic) for maximum pigment production; and (ii) photosynthetic growth (anaerobic). When dark semi-aerobic growth was required, 70 ml of M22+ (see below) medium were inoculated in a 100 ml conical flask and incubated at 180 r.p.m and 34°C until the cells became pigmented. Illuminated anaerobic growth was obtained in 150 ml bottles or 30 ml universal bottles filled with M22+ medium, which were capped tightly and placed under low light intensity ($3\ W\ m^{-2}$) at room temperature. Growth on solid medium under photosynthetic conditions was achieved by use of anaerobic jars. This involved the use of Oxoid Gas Generation kits, which generate hydrogen and carbon dioxide gases.

Pigment handling, identification and characterization

Carotenoid pigments were extracted using a slightly modified version of the method of Britton and Riesen (1995), in which cell pellets are dissolved in acetone/methanol 2:1 (v/v) and subsequently transferred into ether. If solvent partition was not achieved, water was added to aid the formation of two layers: an upper hydrophobic layer containing the pigments and a lower polar layer with the cell debris. In the few cases when good partition was still not obtained, a few crystals of NaCl were added. The hydrophobic layer was then separated and dried up under oxygen-free N_2. Contact of carotenoids with O_2 was avoided at all stages, and their extraction and general handling was carried out in the dark. Carotenoid fractions were either stored in sealed glass vials or used immediately.

Dried carotenoid extracts were dissolved in solvents A and B in the ratio 80:20 (v/v) [A being acetonitrile/water 9:1 (v/v) and B, ethyl acetate], which was the starting equilibration buffer. All high-performance liquid chromatography (HPLC) solvents were rigorously filtered and degassed. Carotenoids were separated on a Spherisorb ODS2 column (250 × 4.6 mm, i.d.) using a modified protocol provided by Professor A. Young (Liverpool John Moores University, UK). The flow rate was constant ($1\ ml\ min^{-1}$) and the elution gradient was the following: 0–6 min, 20% to 60% B; 6–15 min, 60% B isocratic step; 15–20 min, 60–100% B; 20–20.5 min, 100% to 20% B; 20.5–30 min, 20% B isocratic step. Most carotenoids were eluted during the isocratic 6–15 min step. Elution was monitored using a Waters 996 photodiode array detector, scanning from 270 to 600 nm every 2 s. Chromatograms at 442 nm were derived from the accumulated absorbance scans using the Millenium software (Waters).

Integration of the carotenoid peaks in the chromatogram was performed at the wavelength corresponding to the absorption maximum (λ_{max}) of each carotenoid and results are always given as percentages of the total sum of the areas. For the purpose of this work, accurate quantitative determination was not required as our main interest was to compare carotenoid accumulation between various strains.

The $A^{1\%}/_{1\,cm}$ or specific absorption coefficient is the absorbance of a 1% (w/v) solution in a 1 cm path cuvette at a defined wavelength. An arbitrary value of 2500 is usually used when no specific values are available for an individual carotenoid or for a mixture of pigments (Britton and Young, 1993). This value was therefore assumed for all carotenoids, except in the case of phytoene, which has an extremely low specific absorption coefficient (1250). The integrated areas for the phytoene peaks were therefore multiplied by two.

Purified carotenoids were dried and resuspended in two or three different solvents (typically acetone, ethanol/methanol and CS_2) for preliminary identification purposes. Spectra were taken in the 270–600 nm range, and peak values were compared with published data (Davies, 1976). When possible, co-elution of the unknown carotenoid with standards in HPLC was demonstrated. In the case of 2,2′-diketospirilloxanthin, this carotenoid was further identified by mass spectrometry, for which we are grateful to Dr Shinichi Takaichi in Kawasaki, Japan.

Determination of cellular levels of carotenoid and bacteriochlorophyll

Whole cells were extracted twice in the dark with nine volumes of ethanol prewarmed to 50°C, and the carotenoids in the pooled ethanol extracts were then partitioned into hexane for subsequent quantification using absorbance spectroscopy. In a separate series of experiments, cells were sonicated before extraction, to try to improve the amount of pigment extracted, although this proved to be unnecessary. Bacteriochlorophyll levels were measured following extraction of one volume of whole cells or cell sonicate with nine volumes of acetone:methanol (7:2 v/v) in the dark, and subsequent quantification by absorbance spectroscopy, using an EmM of 75 at 772 nm. The experiments were repeated so that between four and eight data sets were collected from two independent cultures. The data were compared on a per cell basis by normalizing to the absorbance at 680 nm. For the purposes of comparing between different mutant strains, the data were normalized so that the wild-type strain was assigned an arbitrary value of 100.

Preparation of membranes from Rb. sphaeroides

Cell-free intracytoplasmic membrane (ICM) fractions for use in absorbance spectroscopy were isolated from semi-aerobic cultures of *Rb. sphaeroides* strains using the method of Olsen and colleagues (Olsen et al., 1994). All steps were carried out, as far as possible, at 4°C. Harvested cells from a 1.5 l culture were resuspended in 20 ml of 10 mM Tris pH 7.5, 1 mM EDTA; a few crystals of DNase I were added, and the cells disrupted

in a French pressure cell at 18 000 psi. The broken cells were layered onto a discontinuous (15–40% w/w sucrose gradient, made up in 1 mM Tris, 1 mM EDTA, pH 7.5), and the gradient centrifuged in a Beckmann Ti45 rotor at 27 000 r.p.m. (57 000 *g*) for 4 h. The ICM fraction formed a band at the 15–40% interface, and was collected with a micropipette. Isolated fractions were stored at –20°C.

Purification of the LH2 complex from strain DM2[pERW12]

Membranes from strain DM2[pERW12] were prepared as described above, and resuspended in 10 mM Tris-HCl, pH 8.0, to an absorbance of 50 at 850 nm. The membranes were then solubilized by adding lauryl dimethyl amine oxide (LDAO) to 1% v/v. LH2 complexes were then purified by ion exchange chromatography on Whatman DE52 DEAE cellulose. The good fractions as judged spectrophotometrically (no LH1 and A850:280 greater than 2.5:1) were used to record the absorption spectra, and the fluorescence emission and excitation spectra. For the emission spectrum, the A850 was 0.15.

Spectroscopy

Absorbance spectra were recorded on a Beckman DU640 spectrophotometer. Fluorescence excitation and emission spectra were recorded on a Spex FluoroLOG spectrofluorimeter (Jobin Yvon). For excitation spectra, the excitation slits were 5 nm and the emission slits were 10 nm; for emission spectra the excitation slits were 10 nm and the emission slits were 5 nm.

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Note added in proof

The energy transfer properties of lycopene–LH2 complexes have now been studied in more detail using fluorescence excitation and ultrafast transient absorption spectroscopies [Hörvin Billsten, H., Herek, J.L., Garcia-Asua, G., Hashøj, L., Polívka, T., Hunter, C.N. and Sundström, V. (2002) Dynamics of energy transfer from lycopene to bacteriochlorophyll in genetically-modified LH2 complexes of *Rhodobacter sphaeroides*. *Biochemistry* (in press)]

References

Alberti, M., Burke, D.H., and Hearst, J.E. (1995) Structure and sequence of the photosynthesis gene cluster. In *Anoxygenic Photosynthetic Bacteria*. Blankenship, R.E., Madigan, M.T., and Bauer, C.E. (eds). The Netherlands: Kluwer Academic Publishers, pp. 1083–1106.

Albrecht, M., Ruther, A., and Sandmann, G. (1997) Purification and biochemical characterization of a hydroxyneurosporene desaturase involved in the biosynthetic pathway of the carotenoid spheroidene in *Rhodobacter sphaeroides*. *J Bacteriol* **179:** 7462–7467.

Andersson, P.O., Gillbro, T., Ferguson, L., and Cogdell, R.J. (1991) Absorption spectral shifts of carotenoids related to medium polarizability. *J Photochem Photobiol* **54:** 353–360.

Armstrong, G.A., Alberti, M., and Hearst, J.E. (1990) Conserved enzymes mediate the early reactions of carotenoid biosynthesis in non-photosynthetic and photosynthetic prokaryotes. *Proc Natl Acad Sci USA* **87:** 9975–9979.

Bramley, P.M., and Davies, B.H. (1976) ξ-carotene biosynthesis by extracts of the C115 mutant of *Phycomyces blakesleeanus*. *Phytochemistry* **15:** 1913–1916.

Britton, G., and Riesen, R. (1995) Worked examples of isolation and analysis, example 3: bacteria. In *Carotenoids, Vol. 1A: Isolation and Analysis.* Britton, G., Liaaen-Jensen, S., and Pfander, H. (eds). Basel: Birkhäuser, pp. 227–238.

Britton, G., and Young, A.J. (1993) *Carotenoids in Photosynthesis.* Britton, G., and Young, A.J. (eds). London: Chapman & Hall, pp. 409–457.

Cogdell, R.J., Hipkins, M.F., Macdonald, W., and Truscott, T.G. (1981) Energy-transfer between the carotenoid and the bacteriochlorophyll within the B800–850 light-harvesting pigment-protein complex of *Rhodopseudomonas-Sphaeroides. Biochim Biophys Acta* **634:** 191–202.

Coomber, S.A., Chaudhri, M., Connor, A., Britton, G., and Hunter, C.N. (1990) Localized transposon Tn*5* mutagenesis of the photosynthetic gene cluster of *Rhodobacter sphaeroides*. *Mol Microbiol* **4:** 977–989.

Davies, B.H. (1976) Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*. Goodwin, T.W. (ed.). London: Academic Press, pp. 38–165.

Davis, J., Donohue, T.J., and Kaplan, S. (1988) Construction, characterisation and complementation of a Puf$^-$ mutant of *Rhodobacter sphaeroides*. *J Bacteriol* **170:** 320–329.

Desamero, R.Z.B., Chynwat, V., van der Hoef, I., Jansen, F.J., Lugtenburg, J., Gosztola, D., *et al.* (1998) Mechanism of energy transfer from carotenoids to bacteriochlorophyll: light harvesting by carotenoids having different extents of pi electron conjugation incorporated into the B850 antenna complex from the carotenoidless bacterium *Rhodobacter sphaeroides* R26–1. *J Phys Chem B* **102:** 8151–8162.

Foote, C.S. (1976) Photosensitized oxidation and singlet oxygen: consequences in biological systems. In *Free Radicals and Biological Systems.* Pryer, W.A. (ed.). New York: Academic Press, pp. 85–133.

Fowler, G.J.S., Hess, S., Sundström, V., and Hunter, C.N. (1997) The role of $\beta Arg._{10}$ in the B800 bacteriochlorophyll and carotenoid pigment environment within the light harvesting LH2 complex of *Rhodobacter sphaeroides. Biochemistry* **36:** 11282–11291.

Fraser, P.D., Misawa, N., Linden, H., Yamano, S., Kobayashi, K., and Sandmann, G. (1992) Expression in *Escherichia coli*, purification, and reactivation of the recombinant *Erwinia uredovora* phytoene desaturase. *J Biol Chem* **270:** 19891–19895.

Garcia-Asua, G., Lang, H.P., Cogdell, R.J., and Hunter, C.N. (1998) Carotenoid diversity: a modular role for

the phytoene desaturase step. *Trends Plant Sci* **3:** 445–449.

Giuliano, G., Pollock, D., and Scolnik, P.A. (1986) The gene *crtI* mediates the conversion of phytoene into colored carotenoids in *Rhodopseudomonas capsulata*. *J Biol Chem* **261:** 12925–12929.

Holmes, N.G., and Crofts, A.R. (1977) The carotenoid shift in *Rhodopseudomonas sphaeroides*. Change induced under continuous illumination. *Biochim Biophys Acta* **461:** 141–150.

Hundle, B., Alberti, M., Nievelstein, V., Beyer, P., Kleinig, H., Armstrong, G.A., Burke, D.H., and Hearst, J.E. (1994) Functional assignment of *Erwinia herbicola* Eho10 carotenoid genes expressed in *Escherichia coli*. *Mol General Genet* **245:** 406–416.

Hunter, C.N., and Turner, G. (1988) Transfer of genes coding for apoproteins of reaction center and light-harvesting LH1 complexes to *Rhodobacter sphaeroides*. *J Gen Microbiol* **134:** 1471–1480.

Hunter, C.N., Hundle, B.S., Hearst, J.E., Lang, H.P., Gardiner, A.T., Takaichi, S., and Cogdell, R.J. (1994) Introduction of new carotenoids into the bacterial photosynthetic apparatus by combining the carotenoid biosynthetic pathways of *Erwinia herbicola* and *Rhodobacter sphaeroides*. *J Bacteriol* **176:** 3692–3697.

Koepke, J., Hu, X.C., Muenke, C., Schulten, K., and Michel, H. (1996) The crystal structure of the light-harvesting complex II (B800–B850) from *Rhodospirillum molischanum. Structure* **4:** 581–597.

Komori, M., Ghosh, R., Takaichi, S., Hu, Y., Mizoguchi, T., Koyama, Y., and Kuki, M. (1998) A null lesion in the rhodopin 3,4-desaturase of *Rhodospirillum rubrum* unmasks a cryptic branch of the carotenoid biosynthetic pathway. *Biochemistry* **37:** 8987–8994.

Lang, H.P., and Hunter, C.N. (1994) The relationship between carotenoid biosynthesis and the assembly of the light-harvesting LH2 complex in *Rhodobacter sphaeroides*. *Biochem J* **298:** 197–205.

Lang, H.P., Cogdell, R.J., and Hunter, C.N. (1994) Early steps in carotenoid biosynthesis: sequence and transcriptional analysis of the *crtI* and *crtB* genes of *Rhodobacter sphaeroides*. Overexpression and reactivation of *crtI. Escherichia coli* and *Rhodobacter sphaeroides*. *J Bacteriol* **176:** 3859–3869.

Lang, H.P., Cogdell, R.J., Takaichi, S., and Hunter, C.N. (1995) Complete DNA sequence, specific Tn*5* insertion map, and gene assignment of the carotenoid biosynthesis pathway of *Rhodobacter sphaeroides*. *J Bacteriol* **177:** 2064–2073.

MacPherson, A.N., Arellano, J.B., Fraser, N.J., Cogdell, R.J., and Gillbro, T. (2001) Efficient energy transfer from carotenoid S2 state in a photosynthetic light-harvesting complex. *Biophys J* **80:** 923–930.

Martinez-Laborda, A., Balsalobre, J.M., Fontes, M., and Murillo, F.J. (1990) Accumulation of carotenoids in structural and regulatory mutants of the bacterium *Myxococcus xanthus*. *Mol Gen Genet* **223:** 205–210.

McDermott, G., Prince, S.M., Freer, A.A., Hawthornthwaite-Lawless, A.M., Papiz, M.Z., Cogdell, R.J., and Isaacs, N.W. (1995) Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria. *Nature* **374:** 517–521.

Naylor, G.W., Addlesee, H.A., Gibson, L.C.D., and Hunter, C.N. (1999) The photosynthesis gene cluster of *Rhodobacter sphaeroides*. *Photosyn Res* **62:** 121–139.

Olsen, J.D., Sockalingum, G.D., Robert, B., and Hunter, C.N. (1994) Modification of a hydrogen bond between a bound chromophore and the α subunit of the light-harvesting LH1 antenna of *Rhodobacter sphaeroides*. *Proc Natl Acad Sci USA* **91:** 7124–7128.

Ouchane, S., Picaud, M., Vernotte, C., ReissHusson, F., and Astier, C. (1997) Pleiotropic effects of *puf* interposon mutagenesis on carotenoid biosynthesis in *Rubrivivax* gelatinosus: a new gene organization in purple bacteria. *J Biol Chem* **272:** 1670–1676.

Sandmann, G., and Kowalczyk, S. (1989) *In vitro* carotenogenesis and characterisation of the phytoene desaturase reaction in *Anacystis*. *Biochem Biophys Res Commun* **163:** 916–921.

Steiger, S., Astier, C., and Sandmann, G. (2000) Substrate specificity of the expressed carotenoid 3,4 desaturase from *Rubrivivax gelatinosus* reveals the detailed reaction sequence to spheroidene and spirilloxanthin. *Biochem J* **349:** 635–640.

Walz, T., Jamieson, S.J., Bowers, C.M., Bullough, P.A., and Hunter, C.N. (1998) Projection structures of three photosynthetic complexes from *Rhodobacter sphaeroides*: LH2 at 6 Å, LH1 and RC-LH1 at 25 Å. *J Mol Biol* **282:** 833–845.

Wang, C., and Liao, J.C. (2001) Alteration of product specificity of *Rhodobacter sphaeroides* phytoene desaturase by directed evolution. *J Biol Chem* **276:** 41161–41164.

Zurdo, J., Fernandez, C.C., and Ramirez, J.M. (1993) A structural role of the carotenoid in the light-harvesting II protein of *Rhodobacter capsulatus*. *Biochem J* **290:** 531–537.