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Introduction of New Carotenoids into the Bacterial Photosynthetic Apparatus by Combining the Carotenoid Biosynthetic Pathways of *Erwinia herbicola* and *Rhodobacter sphaeroides*

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Carotenoids have two major functions in bacterial photosynthesis, photoprotection and accessory light harvesting. The genes encoding many carotenoid biosynthetic pathways have now been mapped and cloned in several different species, and the availability of cloned genes which encode the biosynthesis of carotenoids not found in the photosynthetic genus *Rhodobacter* opens up the possibility of introducing a wider range of foreign carotenoids into the bacterial photosynthetic apparatus than would normally be available by producing mutants of the native biosynthetic pathway. For example, the *crt* genes from *Erwinia herbicola*, a gram-negative nonphotosynthetic bacterium which produces carotenoids in the sequence of phytoene, lycopene, β-carotene, β-cryptoxanthin, zeaxanthin, and zeaxanthin glucosides, are clustered within a 12.8-kb region and have been mapped and partially sequenced. In this paper, part of the *E. herbicola crt* cluster has been excised and expressed in various *crt* strains of *Rhodobacter sphaeroides*. This has produced light-harvesting complexes with a novel carotenoid composition, in which the foreign carotenoids such as β-carotene function successfully in light harvesting. The outcome of the combination of the *crt* genes in *R. sphaeroides* with those from *E. herbicola* has, in some cases, resulted in an interesting rerouting of the expected biosynthetic sequence, which has also provided insights into how the various enzymes of the carotenoid biosynthetic pathway might interact. Clearly this approach has considerable potential for studies on the control and organization of carotenoid biosynthesis, as well as providing novel pigment-protein complexes for functional studies.

Carotenoids have two major functions in bacterial photosynthesis, photoprotection and accessory light harvesting (6, 25). Spectroscopic studies designed to unravel the molecular details of the photophysical reactions involved in these processes have been greatly aided by the availability of mutants with the same pigment-protein complexes but with altered carotenoid composition (for examples, see references 4, 7, 8, and 24). Initially, these mutants were produced by random chemical mutagenesis or UV treatment (5, 26); however, with the development of molecular genetic methods, these mutations can now be introduced in a more controlled and systematic way, for example, by interposon or transposon mutagenesis (9, 11, 28). In photosynthetic bacteria such as *Rhodobacter capsulatus*, these forms of insertional mutagenesis have produced mutations in *crtI*, *-C*, and *-D*, for example, yielding strains which accumulate phytoene, neurosporene, and neurosporene derivatives, respectively, rather than the normal end products of the biosynthetic pathway, spheroidene and spheroidenone (11).

The genes encoding many carotenoid biosynthetic pathways have now been mapped and cloned in several different species (9, 11, 13, 18, 21, 23, 28), and in the case of *R. capsulatus*, for example, the complete DNA sequence of the *crt* gene cluster is available (2). The availability of cloned genes which encode the biosynthesis of carotenoids not found in *Rhodobacter* spp. opens up the possibility of introducing a wider range of foreign carotenoids into the bacterial photosynthetic apparatus than would normally be made available by producing mutants of the native biosynthetic pathway. For example, the *crt* genes from *Erwinia herbicola*, a gram-negative nonphotosynthetic bacterium which produces carotenoids in the sequence of phytoene, lycopene, β-carotene, β-cryptoxanthin, zeaxanthin, and zeaxanthin glucosides, are now available. These genes are clustered within a 12.8-kb region and have been mapped and partially sequenced (1, 13, 13a, 23; GenBank accession number M87280). This is particularly interesting since it means that some of the carotenoids found in higher-plant photosynthesis are now available from a convenient bacterial source.

In this paper, part of the *E. herbicola crt* cluster has been excised and expressed in various *crt* strains of *Rhodobacter sphaeroides*. This has produced light-harvesting complexes with a novel carotenoid composition, in which the foreign carotenoids function successfully in light harvesting. The outcome of the combination of the *crt* genes in *R. sphaeroides* with those from *E. herbicola* has, in some cases, resulted in an interesting rerouting of the expected biosynthetic sequence, which has also provided insights into how the various enzymes of the carotenoid biosynthetic pathways might interact. Clearly this approach has considerable potential for studies on the control and organization of carotenoid biosynthesis, as well as providing novel pigment-protein complexes for functional studies.

MATERIALS AND METHODS

R. sphaeroides strains were grown semiaerobically in the dark as described previously (14), with 20 μg of neomycin per ml to select for the presence of transposon Tn*5*. One microgram of tetracycline per ml was used to maintain selection for

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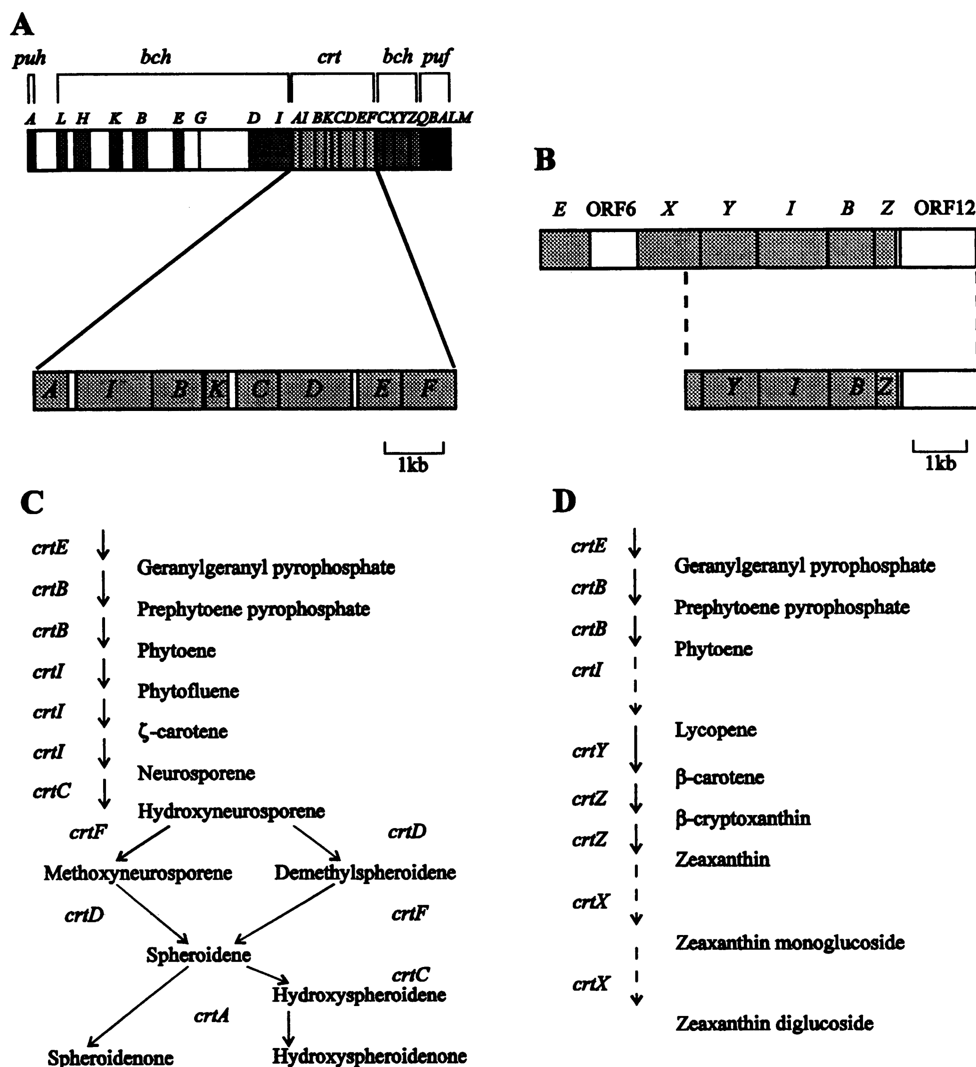

FIG. 1. (A) Map of the photosynthetic gene cluster of *R. sphaeroides* together with the carotenoid gene cluster. (B) Carotenoid gene cluster of *E. herbicola*. The 5,999-bp *Sal*I-*Eco*RI fragment of the *E. herbicola* cluster used for expression in *R. sphaeroides* is indicated below, with the *Sal*I site located within *crtX* and the *Eco*RI site at the end of ORF12. Beneath each cluster is the relevant biosynthetic pathway for the carotenoids in each organism plus the carotenoid gene (*crt*) assignments: *R. sphaeroides* pathway (C) or *E. herbicola* pathway (D).

the expression vector pRKSK1 and its derivatives. pRKSK1 was constructed from the broad-host-range vector pRK415 (16). The *puc* promoter was subcloned from pRKSK1 (19) as a 320-bp *Stu*I-*Kpn*I fragment, the latter restriction site having been introduced just upstream of the ribosome binding sequence by directed mutagenesis (15); the *Stu*I site of the 320-bp fragment and the *Eco*RI site of pRK415 were filled in and ligated, which regenerated an *Eco*RI site.

The carotenoid composition of whole cells was determined as described in Takaichi and Shimada (27). The pigments were extracted with chloroform-methanol (3:1, vol/vol), and the solution was filtered through a Hewlett-Packard membrane filter. They were then analyzed with a Hewlett-Packard high-pressure liquid chromatography system equipped with a Hewlett-Packard 1040A photodiode array detector and a Waters μBondapak G8 column. The elution was performed with a combination of gradient and isocratic elution with water in methanol. The pigments were identified by their retention times and absorption spectra.

The B800-850 antenna complex from strain TC40(pRER1A) was prepared as described by Hawthornthwaite and Cogdell (12). The absorption spectrum of the complex was recorded by a Shimadzu UV 160-A spectrophotometer, and the fluorescence excitation and emission spectra were recorded by a Perkin-Elmer LS50 luminescence spectrophotometer.

RESULTS

Analysis of the carotenoid composition of *R. sphaeroides* strains containing *Erwinia crt* genes. Figure 1 shows the two relevant biosynthetic pathways together with the gene assignments and physical maps of the clusters. The *R. sphaeroides* cluster was characterized as a result of transposon Tn*5* insertions, and although the sequence of this region has been completed (17a), the map is largely based on the data of Coomber et al. (9). The part of the *Erwinia* cluster cloned that was used was the 5,999-bp *Sal*I-*Eco*RI fragment carrying the genes *crtY*, *-I*, *-B*, and *-Z*. The omission of a 4,196-bp *Hin*dIII-

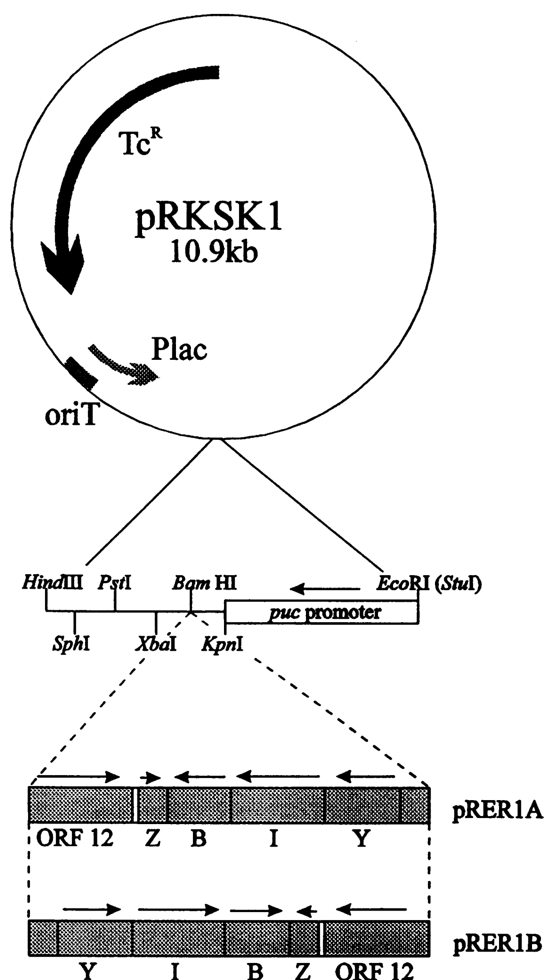

FIG. 2. Map of the plasmid pRKSK1 used for expression of *E. herbicola crt* genes in *R. sphaeroides*. The orientations of the *E. herbicola crt* insert which give rise to pRER1A and pRER1B are shown.

*Sal*I fragment carrying *crtE* and part of *crtX* was convenient since *R. sphaeroides* already possesses *crtE* and *crtX* encodes the enzyme catalyzing glycosylation reactions, which are not relevant to this study. Thus, the *Sal*I-*Eco*RI *E. herbicola crt* fragment has the potential to direct the incorporation of zeaxanthin into *R. sphaeroides*.

This *crt* fragment was cloned in both orientations into the broad-host-range vector pRKSK1, which was constructed from pRK415 (16) and the *Stu*I-*Kpn*I fragment of the strong *puc* promoter (20) which normally drives the transcription of the *pucB* and *pucA* genes, especially under low-oxygen or anaerobic conditions. The *Sal*I-*Eco*RI fragment was blunt ended by a filling-in reaction, and the resulting fragment was ligated into *Bam*HI-cut pRKSK1, which also had blunted ends as a consequence of the same filling-in procedure. The map of pRKSK1 is shown in Fig. 2 together with the resulting *crt* plasmids pRER1A and pRER1B, which were transferred into several *R. sphaeroides* carotenoid mutants. The results are summarized in Table 1; in each case, the plasmid pRKSK1 was also mobilized as a control. In pRER1A, the direction of transcription of *crtY*, *-I*, and *-B* is matched to the orientation of the *puc* promoter; the situation is reversed in pRER1B. The strains harboring pRKSK1 produced carotenoids consistent with the genotypes and phenotypes already published (9) and will not be discussed further. TC70, which has transposon Tn*5* inserted into *crtB* (9), synthesizes carotenoids characteristic of the wild type, such as spheroidenone, when pRER1A is introduced. It appears that the function of the disrupted *crtB* gene in the host is replaced by the plasmid-borne sequence but that the normal pathway of carotenoid synthesis in *R. sphaeroides* is rejoined despite the probable presence of enzymes capable of synthesizing lycopene and β-carotene. In the *crtI* strain TC72, pRER1A directs the synthesis of a mixture of carotenoids characteristic of both *R. sphaeroides* (spheroidenones) and *Erwinia* spp. (lycopene). In addition, it appears that some desaturation of demethylated spheroidene is occurring, forming small amounts of dehydrorhodopin. The mutations in the *crtC* and *crtD* strains TC40 and TC18, respectively, prevent the *R. sphaeroides* pathway from completing the synthesis of spheroidenone, so they were used as recipients to see if this increased the proportion of *Erwinia* carotenoids synthesized. In these cases, both orientations of the insert were tested to see if the level of transcription through the *Erwinia* cluster had any quantitative effects on carotenoid composition. Data in Table 1 show that the amounts of *Erwinia* carotenoids present in TC40 and TC18 recipients increased dramatically, with the dominant carotenoid in each case being β-carotene. Comparison of the two orientations (pRER1A and pRER1B) for each recipient shows that some differences in carotenoid composition do occur; for example, TC40(pRER1A) synthesizes only carotenoids specific for the *Erwinia* pathway (β-carotene, zeaxanthin, and β-cryptoxanthin), whereas TC40(pRER1B) synthesizes small amounts of neurosporene and phytoene. There are differences between TC40 and TC18 as recipients of these plasmids, but in each case, the main feature is the extremely small amount of neurosporene, despite the fact that the appropriate genes, if not the enzymes, are present for the desaturation of phytoene to form neurosporene. This will be commented on in the Discussion.

Analysis of the interaction between *Erwinia* carotenoids and the bacterial photosynthetic apparatus. It was important to find out whether or not these foreign carotenoids could carry out a light-harvesting function. Moreover, the introduction of novel carotenoids into the well-characterized bacterial photosynthetic unit has implications for the assembly of the unit, as well as for the specificity and efficiency of interaction between the carotenoids and bacteriochlorophyll molecules. For this reason, some measurements of the absorbance and fluorescence properties of the transconjugant strain TC40(pRER1A) were carried out. This strain was selected since it contained only *Erwinia* carotenoids, mainly β-carotene (see Table 1).

Figure 3 shows the absorbance spectra of membranes isolated from TC40(pRER1A) which were used as a starting point for the purification of the LH2 complex; it is clear that there might be some shift in the LH1 and LH2 absorbance peaks due to the presence of different carotenoids when the inset in Fig. 3 is inspected. This point will have to be examined in more detail. In addition, there could be a slight impairment of LH2 assembly, since it is known that this process is particularly susceptible to the type(s) and amount(s) of carotenoids present (17). Nevertheless, this demonstrates that LH assembly is possible in the presence of largely β-carotene.

The next experiments were carried out to see if the *Erwinia* carotenoids could function in the bacterial photosynthetic unit by transferring energy to the bacteriochlorophylls within the light-harvesting complexes. Replacement of neurosporene by β-carotene would not necessarily preserve the efficiency of this process, so fluorescence excitation spectra were measured with

TABLE 1. Carotenoids present in *R. sphaeroides* strains carrying *Erwinia crt* genes

Recipient strain and chromosomal genotype	Plasmid	Carotenoid(s) present (%)
TC70 (*crtB*)	pRKSK1	None
	pRER1A	Spheroidenone (70), zeaxanthin (9), phytoene (8), neurosporene (4), dehydrorhodopin (3), and OH-spheroidenone (2)
TC72 (*crtI*)	pRKSK1	Phytoene (100)
	pRER1A	Spheroidenone (37), OH-spheroidenone (20), dehydrorhodopin (18), lycopene (17), and phytoene (8)
TC40 (*crtC*)	pRKSK1	Neurosporene (95) and phytoene (5)
	pRER1A	β-Carotene (84), zeaxanthin (12), and β-cryptoxanthin (3)
	pRER1B	β-Carotene (51), dehydrorhodopin (14), neurosporene (9), phytoene (8), lycopene (8), β-cryptoxanthin (7), and zeaxanthin (3)
TC18 (*crtD*)	pRKSK1	Neurosporene (73), chloroxanthin (14), and dihydrospheroidenone (13)
	pRER1A	β-Carotene (39), zeaxanthin (22), dehydrorhodopin (14), phytoene (12), neurosporene (4), and β-cryptoxanthin (3)
	pRER1B	β-Carotene (88), zeaxanthin (8), and β-cryptoxanthin (4)

membranes prepared from TC40(pRER1A). It is already known that in green strains of *R. sphaeroides* such as the control strain TC40(pRKSKI), there is efficient energy transfer from the native carotenoid, neurosporene, to the bacteriochlorophylls (7). The accompanying fluorescence excitation spectrum shows that those wavelengths that elicit fluorescence from the light-harvesting complexes coincide with the carotenoid absorbance profile, making it likely that the "new" carotenoids transfer energy and function correctly. In order to examine this point further, the absorbance spectrum of the purified LH2 complex, together with the fluorescence excitation spectrum, was measured (Fig. 4); the appearance of peaks characteristic of carotenoids in the excitation spectrum would demonstrate energy transfer from the foreign carotenoid(s) to the bacteriochlorophylls. The wavelength maxima of the peaks in the visible region at 458, 489, and 518 nm compare well with the peaks in the visible region of Fig. 4 at 458, 482, and 518 nm, so we conclude that β-carotene, and presumably the minor carotenoids zeaxanthin and β-cryptoxanthin, transfer energy to bacteriochlorophylls within the antenna complexes. Although the efficiency of the process has not been measured, these data show that β-carotene can functionally replace neurosporene in the bacterial photosynthetic unit.

Reaction center photooxidation was also assayed, and it was found that these complexes are active: a photosynthetic unit of 67 bacteriochlorophylls per reaction center was calculated, which is consistent with approximately equal proportions of LH2 and LH1 complexes and, therefore, with the absorbance spectra in Fig. 3. Thus, both light-harvesting and photochemical reactions can be measured in TC40(pRER1A), which indicates that the presence of β-carotene in particular has no damaging consequences for photosynthetic complexes. However, when attempts were made to measure photosynthetic

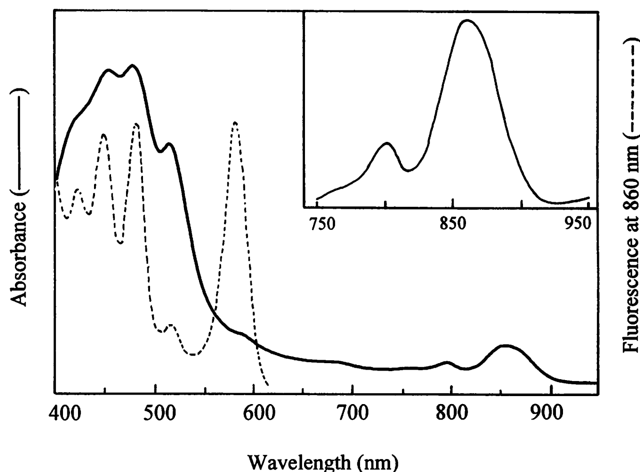

FIG. 3. Absorbance and fluorescence excitation spectra of membranes from TC40(pRERIA). The inset shows the absorbance of bacteriochlorophylls in the near infrared region which arise from light-harvesting complexes of the photosynthetic apparatus. The fluorescence excitation spectrum measures the ability of a series of wavelengths in the carotenoid region to elicit fluorescence from the light-harvesting bacteriochlorophylls at 860 nm.

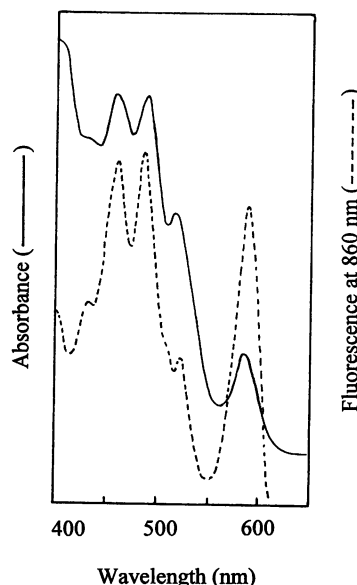

FIG. 4. Absorbance spectrum of the LH2 light-harvesting complex purified from TC40(pRERIA) in the visible region together with the fluorescence excitation spectrum, as for Fig. 3, with fluorescence from the complex detected at 860 nm.

growth rates, the transconjugant strain TC40(pRER1A) lost the capacity to synthesize *Erwinia*-specific carotenoids. This arose because the host strain TC40 exhibits photosynthetic growth at wild-type rates, and therefore, it has an advantage over the transconjugant.

DISCUSSION

The introduction of *Erwinia* carotenoids into bacterial photosynthetic complexes raises some interesting questions about the carotenoid biosynthetic pathways involved, as well as the assembly and function of photosynthetic complexes. In the first instance, however, it is clear that transcription through the *Erwinia crtY*, *-I*, and *-B* genes has occurred, since there is a large quantity of β-carotene synthesized. The DNA sequence of this fragment indicates that there are two other open reading frames present, ORF12 of unknown function and ORF11, which is designated *crtZ*. Both of these should be transcribed antagonistically to *crtY*, *-I*, and *-B*, and so *crtZ* in particular will not benefit from the presence of the *puc* promoter. This might account for the relatively low levels of zeaxanthin obtained, since the *crtZ* gene product is needed to hydroxylate β-carotene to β-cryptoxanthin and then zeaxanthin. In anticipation of this difficulty, both orientations of the *Erwinia crt* insert were cloned, but the data in Table 1 show that this had only a small impact on the amount of zeaxanthin produced. Transcription through *crtZ* is presumably driven by an endogenous promoter or more likely by readthrough from the *lac* promoter in pRKSK1.

Analysis of the carotenoids produced by TC70(pRER1A) shows that the lost function in the recipient (*crtB*) is replaced, but the carotenoids synthesized are mostly those characteristic of *R. sphaeroides*. It appears likely that the *Erwinia* phytoene synthase simply substitutes for the *R. sphaeroides* enzyme. The lesion in TC72 (*crtI*) ensures that the *Erwinia* phytoene desaturase must be functioning in the transconjugant strain TC72(pRERIA) since there is a large proportion of spheroidenone and derivatives (57%) present (Table 1). If the *Erwinia* phytoene desaturase was fundamentally different from the *R. sphaeroides* enzyme, then lycopene would be the major product instead or perhaps there would be major proportions of the subsequent products of the *Erwinia crtY* and *crtZ* genes, β-carotene and zeaxanthin. Neurosporene precedes lycopene in the series of desaturations, so although there is a significant amount of lycopene produced (17% of the carotenoids), the *Erwinia* phytoene desaturase must be able to provide the *R. sphaeroides* CrtC enzyme with neurosporene.

A significant change occurs in transconjugants arising from the *crtC* strain TC40; in this case, the expression of the *Erwinia* genes completely reroutes carotenoid biosynthesis to *Erwinia*-specific carotenoids and there are no detectable levels of the native carotenoid neurosporene. There are several possible explanations for this. One might be that any neurosporene produced by the *R. sphaeroides* desaturase can be converted to lycopene by the *Erwinia* desaturase, but this would not be compatible with neurosporene being a bound or sequestered intermediate in the desaturation series. A second possibility is that there are carotenoid biosynthetic supercomplexes in which the specificity or type of phytoene desaturation to form either neurosporene or lycopene could be superimposed on the desaturase by the presence of other enzymes, in this case, the *crtC* or *crtY* gene products. Removal of CrtC would then allow one or both of the existing desaturases [both the *R. sphaeroides* and *Erwinia* enzymes are presumably present in TC40(pRER1A)] to reroute the pathway to zeaxanthin because of an effect exerted by the *crtY* gene product. We also note that there have been several reports of the negative inhibition of phytoene desaturase by, for example, neurosporene, lycopene, and β-carotene (3, 18, 22). Carotenoid overproduction resulting from the plasmid-based expression employed here might, therefore, alter the relative composition of the carotenoids produced by either or both desaturases. Once lycopene has been synthesized, the CrtY and CrtZ proteins are able to convert this to β-carotene and zeaxanthin, and the association between the *Erwinia* CrtI and CrtY proteins is sufficient to ensure that no free lycopene is detected.

After the new carotenoids, mainly β-carotene, have been synthesized, there are no major obstacles to incorporating them into the assembly pathway for the photosynthetic unit, although it should be pointed out that on a cell-for-cell basis TC40(pRER1A) only synthesizes complexes at approximately 20% the level of TC70(pRER1A). Thus, the presence of these carotenoids may slow down assembly or promote faster turnover of the complexes in the cell, particularly LH2. This lowered cellular level of complexes, albeit active ones, results in an inability to measure photosynthetic growth rates. However, this does not diminish the value of this heterologous expression system, since it is well able to produce new photosynthetic complexes which can be subjected to a variety of spectroscopic techniques in vitro. The aerobic mode of growth was therefore used to avoid any stress imposed by photosynthetic growth. The availability of these new antenna complexes will provide the opportunity to measure the energy transfer properties of β-carotene and perhaps zeaxanthin in vivo by ultrafast time-resolved techniques, particularly since the latter pigment has been implicated in a mechanism of energy dissipation within the LHCII light-harvesting complex of plants (10). The availability of the relatively simple bacterial antenna containing zeaxanthin might be a useful model system in this respect.

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