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Isolation and Characterization of Canthaxanthin Biosynthesis Genes from the Photosynthetic Bacterium *Bradyrhizobium* sp. Strain ORS278

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A carotenoid biosynthesis gene cluster involved in canthaxanthin production was isolated from the photosynthetic *Bradyrhizobium* sp. strain ORS278. This cluster includes five genes identified as *crtE*, *crtY*, *crtI*, *crtB*, and *crtW* that are organized in at least two operons. The functional assignment of each open reading frame was confirmed by complementation studies.

Bradyrhizobium strains isolated from *Aeschynomene* stem nodules are photosynthetic (4; see reference 6 for a review), which is a rare trait in *Rhizobium* bacteria. These strains exhibit a photoheterotrophic and strictly aerobic photosynthesis (6; A. Vermeglio, personal communication). In culture, most of these stem isolates show the same pink coloration, while a few strains produce orange pigmentation (12, 16). Pigment analyses showed that bacteriochlorophyll and spirilloxanthin, two pigments of the light harvesting system, are common to all of these photosynthetic *Bradyrhizobium* strains, whereas orange strains produce an additional bicyclic carotenoid, canthaxanthin (4,4′-diketo-β-carotene) (12). This was the first report on the presence of this carotenoid in photosynthetic bacteria. *Bradyrhizobium* sp. strain ORS278 produces the highest quantity of canthaxanthin of all tested photosynthetic bacteria; canthaxanthin represents 85% of its total carotenoid content (12).

Contrary to anaerobic purple phototrophic bacteria, aerobic phototrophic bacteria synthesize an unusually diverse variety of carotenoids, including photosynthetic carotenoids such as spirilloxanthin or spheroidenone, and often a large amount of bicyclic carotenoid (β-carotene and hydroxyl derivatives) (24; see reference 26 for a review). These carotenoids were shown to not be bound to the photosynthetic apparatus of these aerobic bacteria (17, 27) and their function is still unclear—they could have a protective role against photo-oxidative damage, as already observed for several carotenoids (19, 25).

Synthetic canthaxanthin is applied for both direct and indirect food coloring (10, 23). In cosmetology and pharmacology, it is also combined with β-carotene for use as a dermal photoprotector (8). Canthaxanthin is, therefore, a pigment of high economic value, but its level in *Bradyrhizobium* sp. strain ORS278 (1.43 mg/g of dry cell weight) remains insufficient for this organism to be a realistic candidate for natural canthaxanthin production (12). However, it could be possible to enhance the production of canthaxanthin by cloning carotenoid biosynthesis genes of this strain.

In this paper, we describe the cloning and characterization of the canthaxanthin gene cluster of *Bradyrhizobium* sp. strain ORS278.

Isolation of a carotenoid gene cluster. The genes *crtB* and *crtI*, encoding, respectively, phytoene synthase and phytoene desaturase, two enzymes involved in the initial steps of carotenoid biosynthesis (Fig. 1), have been isolated and characterized in various microorganisms (1, 9, 11, 14, 15, 21). In all of these cases, these genes were found to be adjacent and oriented in the same direction. Comparison of the deduced amino

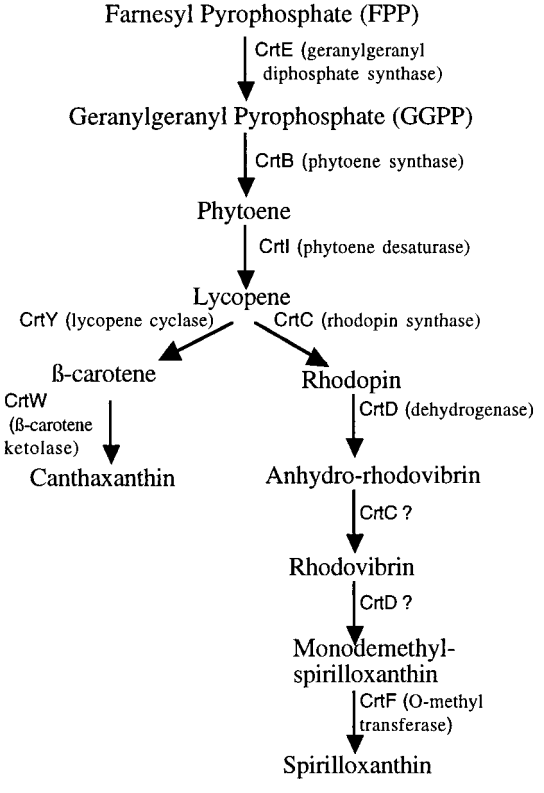

FIG. 1. Scheme of the canthaxanthin (15) and spirilloxanthin (20) biosynthesis pathways.

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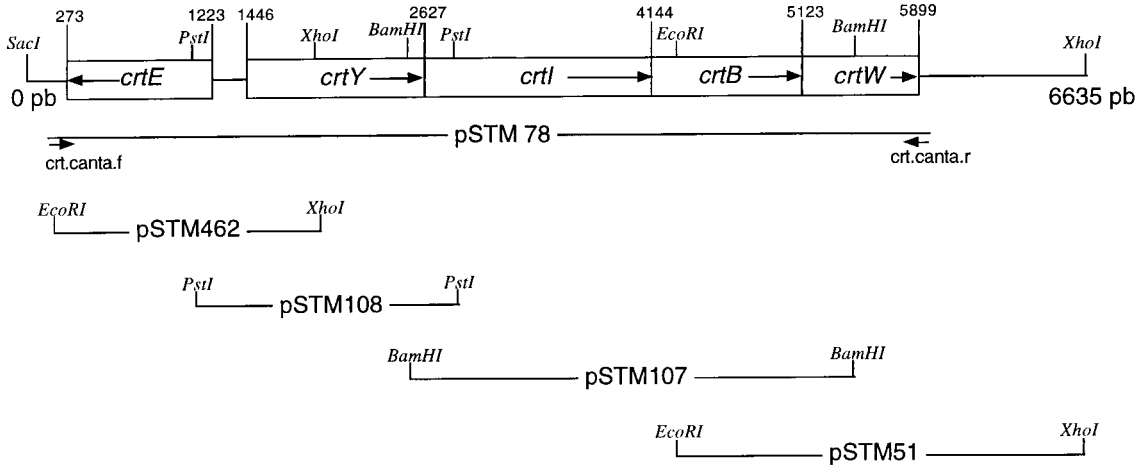

FIG. 2. Organization of the canthaxanthin biosynthesis gene cluster of *Bradyrhizobium* sp. strain ORS278 and locations of various subcloned fragments. The restriction fragments are inserted into pUC18 (pSTM108, pSTM107, and pSTM51) or pUC19 (pSTM462), the *crt* genes are transcribed from the *lac* promoter of the vector. In the plasmid pSTM78, the insert was obtained by Long PCR using the primers Crt.canta.f (5′-GCAACCGGTACCCGAGTTAATTCGCTCGGAATG-3′) and Crt.canta.r (5′-ATGGTGAAGCTTATGCGGCAGCGGGTTTAGTC-3′) and was cloned into pGEM-T (Promega). In pSTM78, the *crtY*, *crtI*, *crtB*, and *crtW* genes are under *lac* promoter control.

acid sequences of the CrtI and CrtB proteins from *Erwinia uredovora*, *Erwinia herbicola*, *Flavobacterium* sp. strain ATCC 21588, *Rhodobacter sphaeroides*, and *Agrobacterium aurantiacum* revealed well-conserved domains at the C-terminal end of CrtI (LVGAGTHPG) and in the central region of CrtB (QLTNIARD). These motifs were chosen for designing the degenerated primers CrtIf (5′-GTNGGNGCRGGCACNCA YCC-3′) and CrtBr (5′-TCGCGRGCRATRTTSGTSARRTG-3′). PCR amplification was performed with a Perkin-Elmer model 2400 thermocycler in a 50-μl (total volume) reaction mixture containing 100 ng of strain ORS278 genomic DNA, each deoxynucleotide triphosphate (200 μM), primers (0.8 μM each), $MgCl_2$ (1.5 mM), 1.25 U of *Taq* DNA polymerase (Promega, Charbonières, France), and the buffer supplied with the enzyme. A touchdown PCR (3) was done as follows: initial denaturation at 94°C for 5 min followed by 20 cycles consisting of a 30-s denaturation at 94°C, 30 s at an annealing temperature of 60 to 50°C, and a 1-min primer extension at 72°C, followed by 15 cycles consisting of a 30-s denaturation at 94°C, 30 s at an annealing temperature at 50°C, and a 1-min primer extension at 72°C. After the final elongation step at 72°C for 7 min, the amplified 620-bp fragment obtained (probe A) was purified by a Wizard procedure and was ligated into a pGEM-T vector (Promega). The ABI Prism BigDye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, Calif.) was used to sequence the cloned PCR product with the universal oligonucleotides M13 forward and M13 reverse. Sequencing reactions were analyzed on an Applied Biosystems model 310 DNA sequencer. The sequence of the amplified 620-bp fragment was highly similar to known CrtB sequences at the amino acid level.

Two specific primers, CrtIBfow.ORS278 (5′-ATTCGCAGC GGCTCGAAGAG-3′) and CrtIBrev.ORS278 (5′-GATCGCC GACATCATCACGC-3′), based on the sequence of the amplified DNA fragment, were designed for PCR screening of a library of the ORS278 strain constructed with the SuperCos I cosmid vector kit (Stratagene, La Jolla, Calif.), as instructed by the manufacturer. Four positive clones were isolated and confirmed by Southern blot analysis by using the 620-bp fragment as a probe. Clone pSTM73, containing an insert of approximately 35 kb, was used to characterize this *crt* gene cluster.

Structure of the canthaxanthin *crt* gene cluster. A 6.5-kb region in the inserted DNA fragment of the pSTM73 cosmid, showing a positive hybridization signal to probe A, was sequenced and analyzed as shown in Fig. 2. This nucleotide sequence had five open reading frames (ORFs) encoding proteins with similarity to known Crt enzymes (Fig. 3). Based on sequence similarity (45% amino acid identity with CrtY of *E. herbicola*), one of these ORFs was assigned to a *crtY* gene which encodes lycopene cyclase, a key enzyme that converts lycopene into the cyclic carotenoid β-carotene. Another ORF was similar in sequence to a *crtW* gene encoding a β-carotene ketolase that synthesizes canthaxanthin from β-carotene via echinenone (15). This indicated that we had isolated a *crt* gene cluster involved in canthaxanthin biosynthesis. Four of the five ORFs, identified as *crtY*, *crtI*, *crtB*, and *crtW*, were found to be clustered in this order in the same orientation, whereas the ORF *crtE* preceded these four but was oriented in the opposite direction (Fig. 2). The *crtY*, *crtI*, and *crtB* genes are closely linked physically; i.e., the stop codons of *crtY* and *crtI* overlap the start codon of the following ORF, suggesting that these

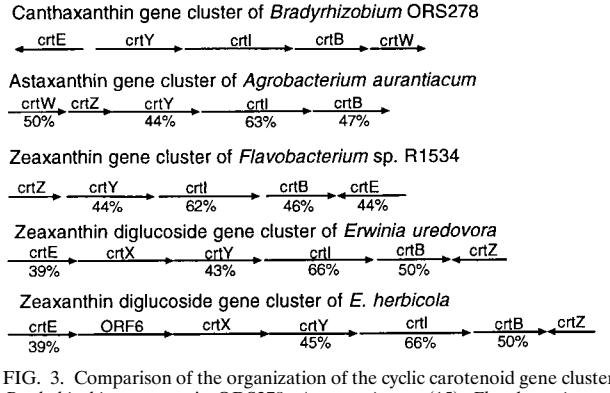

FIG. 3. Comparison of the organization of the cyclic carotenoid gene clusters of *Bradyrhizobium* sp. strain ORS278, *A. aurantiacum* (15), *Flavobacterium* sp. strain R1534 (21), *E. uredovora* (14), and *E. herbicola* (9). Arrows represent the orientations of ORFs. The percentage values below the genes indicate the percentages of amino acid identity compared to *Bradyrhizobium* sp. strain ORS278.

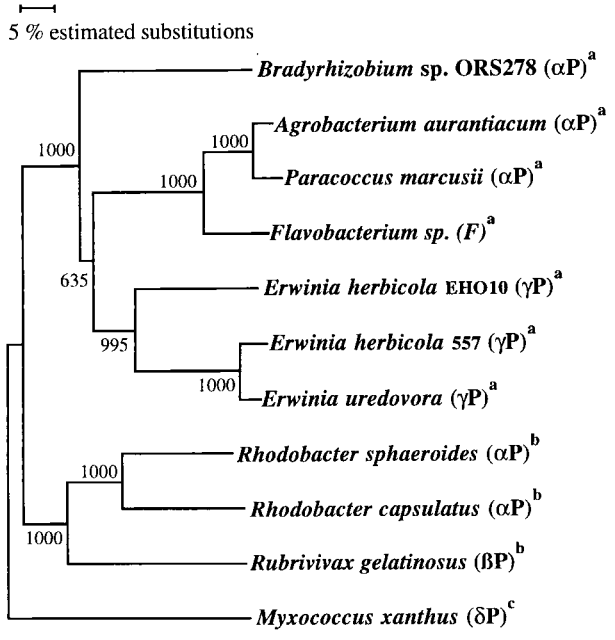

FIG. 4. Phylogenetic tree based on the CrtB sequences and constructed by using the neighbor-joining method (22). Bootstrap values (5), expressed as percentages of 1,000 replications, are given at the branching points. P, *Proteobacteria*; F, *Flavobacteria*; a, bicyclic carotenoid; b, acyclic carotenoid; c, monocyclic carotenoid. GenBank accession numbers are as follows: AF218415, *Bradyrhizobium* sp. strain ORS278; D58420, *A. aurantiacum*; Y15112, *Paracoccus marcusii*; U62808, *Flavobacterium* sp.; M87280, *E. herbicola* EHO10; M90698, *E. herbicola 557*; D90087, *E. uredovora*; AF195122, *R. sphaeroides*; *X52291*, *Rhodobacter capsulatus*; *U87626*, *Rubrivivax gelatinosus*; Z211955, *Myxococcus xanthus*.

genes are translationally coupled (18). Note that the *crtY*, *crtI*, and *crtB* genes always occurred in this order and were oriented in the same direction in the other cyclic carotenoid biosynthesis clusters described previously (see Fig. 3).

Phylogenetic trees were constructed with available CrtI and CrtB sequences. The CrtB proteins (Fig. 4) and the CrtI proteins (data not shown) formed two distinct clusters which do not correlate with the taxonomical position of the strains, but rather with the nature of the carotenoid (cyclic or noncyclic) synthesized under the control of *crtI* and *crtB*. The fact that α- and γ-*Proteobacteria* and *Flavobacteria* group in the same cluster (Fig. 4) suggests that lateral gene transfer has occurred between these phylogenetically unrelated bacteria. Moreover, the fact that strain ORS278 genes are clustered with *crtI* and *crtB* genes from nonphotosynthetic strains producing cyclic carotenoids rather than with photosynthetic strains producing photosynthetic carotenoids raises the question of whether an additional copy of the *crtI* and *crtB* genes involved in the biosynthesis of spirilloxanthin does exist. The *crtC* and *crtD* genes, which were reported to be involved in the biosynthesis of spirilloxanthin from lycopene (20), have just been isolated in another cosmid which did not overlap the pSTM73 cosmid (E. Giraud and B. Dreyfus, unpublished data). We are currently investigating if another copy of the *crtI* and *crtB* genes is physically linked to these *crtC* and *crtD* genes, as has been found in photosynthetic bacteria (1, 11).

Carotenoid production in *Escherichia coli* transformants. *E. coli* transformants carrying the entire *crt* gene cluster of canthaxanthin from strain ORS278, cloned in pGEMT (pSTM78) or SuperCosI (pSTM73), did not produce any carotenoids (Table 1), suggesting that these genes are not expressed or that their products are not functional in *E. coli*. Misawa et al. (15) constructed *E. coli* transformants which accumulate each precursor of the zeaxanthin biosynthesis pathway by introducing various combinations of *E. uredovora crt* genes. To check the functionality of the different ORFs identified in strain ORS278, we complemented several carotenoid-accumulating *E. coli* transformants with plasmids carrying various *crt* genes of strain ORS278 and analyzed carotenoids synthesized by high-pressure liquid chromatography (Table 1). The conditions were as follows: 5-μm Hypersil C_{18} column (250 by 4.6 mm; Alltech, Templemars, France), eluent of acetonitrile-methanol-isopropanol (85/10/5, vol/vol/vol), flow rate of 1 ml/min, and detection at 470 nm (450 nm for β-carotene). Peaks were compared and coeluted with standard compounds then identified by their visible spectra and partition coefficients (12).

When plasmid pSTM78 carrying the complete *crt* cluster

TABLE 1. Analysis of carotenoids accumulated in *E. coli* transformants carrying various combinations of *crt* genes from *E. uredovora* and *Bradyrhizobium* sp. strain ORS278[a]

E. coli host strain characteristics		*E. coli* transformant characteristics after complementation	
Plasmid (*crt* genes of *E. uredovora* carried)	Carotenoid accumulated[c]	Plasmid introduced[d] (*crt* genes of ORS278 carried)	Carotenoid accumulated[e,f]
None	–[g]	pSTM73 (*crtE crtY crtI crtB crtW*)	–
None	–	pSTM78 (*crtE crtY crtI crtB crtW*)	–
pACCRT-E[b] (*crtE*)	GGPP	pSTM78 (*crtE crtY crtI crtB crtW*)	Canthaxanthin (100%) [95.4]
pSTM420 (*crtI crtB crtY*)	–	pSTM462 (*crtE*)	β-Carotene (98%), nic[h] (2%)
pACCRT-E[b] (*crtE*)	GGPP	pSTM107 (*crtI crtB*)	Lycopene (100%)
pACCRT-EB[b] (*crtE crtB*)	Phytoene	pSTM107 (*crtI crtB*)	Lycopene (100%)
pACCRT-EIB[b] (*crtE crtI crtB*)	Lycopene	pSTM108 (*crtY*)	β-Carotene (100%)
pACCRT-EIBY[b] (*crtE crtI crtB crtY*)	β-Carotene	pSTM51 (*crtW*)	Canthaxanthin (90%) [800], echinenone (2%), nic (8%)

[a] Transformants were grown in Luria-Bertani medium for 36 h in the presence of ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), and 0.125 mM isopropyl-1-thio-β-D-galactopyranoside. The carotenoids were extracted according to the method of Lorquin et al. (12).

[b] The plasmids used were described by Misawa et al. (15). Plasmid pSTM420 was obtained after deletion by *Sal*I digestion of the *crtE* gene from the plasmid pACCRT-EIBY.

[c] The carotenoids found in *E. coli* host strains are in accordance with the findings of Misawa et al. (15).

[d] Details on insertion of the various constructed plasmids are presented in Fig. 2.

[e] The percentage of the accumulated carotenoid of the total carotenoid content is indicated in parentheses.

[f] In square brackets, total (*cis* plus *trans*) canthaxanthin level is indicated in micrograms per gram of dry cell weight.

[g] –, carotenoids not detected.

[h] nic, nonidentified compound.

of *Bradyrhizobium* sp. strain ORS278 was introduced into the *E. coli* transformant that had accumulated geranylgeranyl pyrophosphate (GGPP) as a result of the presence of the *crtE* gene of *E. uredovora*, the new transformant obtained was shown to accumulate canthaxanthin. This result indicates that the *crtY*, *crtI*, *crtB*, and *crtW* genes are functional and allow the production of canthaxanthin in *E. coli*. Nevertheless, the amount of canthaxanthin produced remains lower than in the wild-type strain ORS278. When plasmid pSTM462 carrying the *crtE* gene of *Bradyrhizobium* sp. strain ORS278 under the *lac* promoter was introduced into an *E. coli* transformant containing the *crtI*, *crtB*, and *crtY* genes of *E. uredovora*, the new transformant accumulated β-carotene, showing the functionality of the *crtE* gene.

In this study, we cloned and characterized all of the *crt* genes of *Bradyrhizobium* sp. strain ORS278 necessary for canthaxanthin biosynthesis. This is the first report of a cyclic carotenoid biosynthesis gene cluster in a photosynthetic bacterium. It would be interesting to determine the genetic links of this canthaxanthin *crt* gene cluster to the photosynthetic gene cluster. In *Bradyrhizobium* sp. strain ORS278, canthaxanthin production is stimulated by light (13), suggesting that the expression of canthaxanthin biosynthesis genes is regulated by photoinduction, as already reported for other pigments in different organisms (2, 7). Production of this pigment could be optimized by identifying the signal transduction system controlling canthaxanthin biosynthesis. However, characterization of the entire *crt* gene cluster necessary for canthaxanthin biosynthesis already provides a basis for the construction of a recombinant strain that could overproduce this carotenoid.

Nucleotide sequence accession number. The DNA sequence obtained in this study has been deposited in the GenBank database under accession no. AF218415.

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