THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 15, Issue of April 9, pp. 15076–15083, 2004

Printed in U.S.A.

Two Distinct *crt* Gene Clusters for Two Different Functional Classes of Carotenoid in *Bradyrhizobium**

Received for publication, November 5, 2003, and in revised form, January 20, 2004
Published, JBC Papers in Press, January 20, 2004, DOI 10.1074/jbc.M312113200

Eric Giraud‡§, Laure Hannibal‡, Joël Fardoux‡, Marianne Jaubert‡, Philippe Jourand‡, Bernard Dreyfus‡, James N. Sturgis¶, and Andre Verméglio‖

From the ‡Laboratoire des Symbioses Tropicales et Méditerranéennes, Institut de Recherche pour le Développement, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Agro-Montpellier, Institut National de la Recherche Agronomique, Université de Montpellier 2 TA 10/J, Campus de Baillarguet, 34398 Montpellier Cedex 5, France, the ¶Laboratoire d'Ingénierie de Systèmes Macromoléculaires (UPR9027), Institut de Biologie Structurale et Microbiologie, CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France, and the ‖Commissariat à l'Energie Atomique (CEA)/Cadarache Département d'Ecophysiologie Végétale et de Microbiologie-Laboratoire de Bioénergétique Cellulaire, UMR 163-CNRS-CEA, Univ-Méditerranée CEA 1000, 13108 Saint Paul lez Durance Cedex, France

Aerobic photosynthetic bacteria possess the unusual characteristic of producing different classes of carotenoids. In this study, we demonstrate the presence of two distinct *crt* gene clusters involved in the synthesis of spirilloxanthin and canthaxanthin in a *Bradyrhizobium* strain. Each cluster contains the genes *crtE*, *crtB*, and *crtI* leading to the common precursor lycopene. We show that spirilloxanthin is associated with the photosynthetic complexes, while canthaxanthin protects the bacteria from oxidative stress. Only the spirilloxanthin *crt* genes are regulated by light via the control of a bacteriophytochrome. Despite this difference in regulation, the biosyntheses of both carotenoids are strongly interconnected at the level of the common precursors. Phylogenetic analysis suggests that the canthaxanthin *crt* gene cluster has been acquired by a lateral gene transfer. This acquisition may constitute a major selective advantage for this class of bacteria, which photosynthesize only under conditions where harmful reactive oxygen species are generated.

Carotenoids comprise a large class of pigments that are widely distributed in living organisms. They are synthesized by all photosynthetic organisms from bacteria to plants where they play at least three essential functions (1). First, they act as accessory light-harvesting pigments by absorbing light in the 450–570 nm region. Second, they are important for the assembly and stability of some of these light-harvesting complexes. Finally they operate as photoprotectors by directly quenching both triplet excited (bacterio)chlorophylls and singlet oxygen. Carotenoids are also synthesized by a wide variety of non-photosynthetic bacteria (2). Less is known about their precise function in these bacteria, but it is well accepted that their strong antioxidant character may protect the organisms against (photo)oxidative damage.

A remarkable feature of aerobic phototrophic bacteria, besides their ability to photosynthesize only under aerobic condition, is their carotenoid composition. Indeed most strains synthesize, in addition to the carotenoids involved in photosynthesis such as spirilloxanthin, a large amount of unusual carotenoid molecules (3). The most striking complexity is observed for *Erythrobacter* species such as *Erythrobacter longus* or *Erythrobacter ramosum*, which have been reported to produce about 20 different carotenoids (4, 5). Another example comes from various strains of photosynthetic *Bradyrhizobium*, symbionts of *Aeschynomene* (6), which synthesize, in addition to spirilloxanthin, large amounts of canthaxanthin of unknown function (7, 8).

All carotenoids are synthesized from geranylgeranyl pyrophosphate. This compound is formed by the enzyme geranylgeranyl pyrophosphate synthase (CrtE), which catalyzes the condensation of farnesyl pyrophosphate with an isopentyl pyrophosphate moiety. The second step catalyzed by phytoene synthase (CrtB) is the formation of phytoene from the head-to-head condensation of two molecules of geranylgeranyl pyrophosphate. Subsequent dehydrogenations catalyzed by the phytoene desaturase (CrtI) convert the phytoene to neurosporene in three desaturation steps or to lycopene in four steps. After the action of these three enzymes (CrtE, CrtB, and CrtI), the biosynthetic pathways diverge depending on the species leading to the accumulation of various different carotenoids. The synthesis of canthaxanthin from lycopene necessitates two enzymes: CrtY, which catalyzes cyclization of lycopene leading to β-carotene, and CrtW, which oxygenates β-carotene to form canthaxanthin (see Fig. 1*A*) (9). The sequence of the reactions from lycopene to spirilloxanthin includes the successive reactions of hydration, desaturation, and methylation catalyzed, respectively, by the three enzymes CrtC, CrtD, and CrtF (see Fig. 1*A*) (10, 11). These reactions are performed first on one-half of the molecule and then on the other half.

The genes encoding many carotenoid biosynthetic enzymes (*crt* genes) have been characterized in plants and in various bacteria (12, 13). In bacteria, they are always found clustered, except in the Cyanobacteria. In purple photosynthetic bacteria, the genes involved in carotenoid biosynthesis are localized within the photosynthesis gene cluster, a 45-kb DNA region that contains the essential genes involved in the synthesis of the photosynthetic apparatus (14–17). In aerobic photosynthetic bacteria, a *crt* gene cluster has been characterized for the *Bradyrhizobium* ORS278 strain (18). This cluster contains the five *crt* genes, *crtE*, *crtY*, *crtI*, *crtB*, and *crtW*, necessary for the canthaxanthin biosynthesis. Neither photosynthesis genes

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF182374.

§ To whom correspondence should be addressed: Inst. de Recherche pour le Développement, LSTM TA 10/J, Campus de Baillarguet, 34398 Montpellier Cedex 1, France. Tel.: 33-467593783; Fax: 33-467593802; E-mail: Giraud@mpl.ird.fr.

nor specific genes of spirilloxanthin biosynthesis from lycopene (*crtC*, *crtD*, or *crtF*) have been identified in this cluster.

Light stimulation of carotenoid biosynthesis has been reported in numerous organisms including plants, fungi, and bacteria (19). In higher plants, regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression (20). This expression is controlled by a phytochrome, a plant photoreceptor that mediates response to red and far-red light through photoconversion between two stable conformations, a red-absorbing form (Pr) and a far-red-absorbing form (Pfr). Biochemical and genetic studies have recently demonstrated the occurrence of phytochrome-like proteins in photosynthetic and non-photosynthetic bacteria (21, 22). Such a (bacterio)phytochrome appears to control the synthesis of the carotenoid deinoxanthin in the non-photosynthetic bacteria *Deinococcus radiodurans* (23).

In this report, we describe the characterization of a second *crt* gene cluster, in the *Bradyrhizobium* ORS278 strain, coding the enzymes of spirilloxanthin synthesis. This second *crt* gene cluster contains all the genes necessary for the synthesis of spirilloxanthin from farnesyl pyrophosphate. Biochemical analysis and phenotypes of mutants deleted in specific genes of canthaxanthin and spirilloxanthin synthesis allow us to establish the involvement of spirilloxanthin in the photosynthesis activity and the protective role of canthaxanthin in response of the bacteria to oxidative stress. We also demonstrate that the spirilloxanthin *crt* genes are specifically regulated by light via the control of a bacteriophytochrome. These results provide the first demonstration of two independent and differently regulated *crt* gene clusters in a living organism.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bradyrhizobium sp. strain ORS278 (wild-type strain) and isogenic mutants were grown in a modified yeast extract mannitol agar medium with addition of the appropriate antibiotic when required (24). All the strains were cultured for 7 days in sealed Petri dishes at 35 °C in either complete darkness or different continuous illumination conditions provided by light-emitting diodes of different wavelengths between 590 and 870 nm with an irradiance of 6.6 μmol of photons/m^2/s. *Escherichia coli* was grown in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics.

Pigment Analysis—Cells grown at the surface of the Petri dishes in the dark or under different light conditions were resuspended in 6 ml of water + 9 g/liter NaCl and centrifuged for 10 min at 4,000 × *g*. The pellets were extracted three times in the dark with 1 ml of cold acetone/methanol (7:2, v/v). The carotenoids in the pooled extracts were analysis by HPLC[1] using a Waters Alliance 2690 system. The conditions were: 5-μm Hypersil C_{18} column (250 × 4.6 mm, Alltech), acetonitrile/methanol/isopropanol (40:50:10, v/v/v) as eluent, 0.8 ml/min flow rate. The eluted fractions were monitored using a Waters 996 photodiode array detector scanning from 270 to 600 nm every 2 s. Carotenoids were identified by their retention times and by comparison of the spectral features with those of pure compounds or with reported data. The amount of canthaxanthin was determined from the area of the peak detected at 480 nm using a calibration curve obtained with a canthaxanthin standard kindly provided by Aventis. The amount of spirilloxanthin was estimated from the area of the peak detected at 494 nm using the canthaxanthin correlation coefficient due to the lack of spirilloxanthin standard. The data represent the mean of three independent cultures.

Light Action Spectrum of crt Gene Expression—The mutants harboring the various *lacZ-crt* fusions were grown under continuous illumination with low irradiance of different wavelengths as described previously (25). After growth, the cells under the illuminated area were resuspended in 3 ml of water, and β-galactosidase activity was measured as described previously (24).

Construction of crt Mutant Strains—Constructions of mutants deleted in the bacteriophytochrome (278Δ*BrbphP*) and in the transcriptional factor PpsR (278Δ*ppsR*) have been described previously (25). For the construction of a *crtCD* mutant (278Δ*crtCD*), a region of about 3.5 kb containing *crtC* and *crtD* genes of ORS278 was amplified by PCR using the primers 5′-TAGTCGACGCAATGGCGCGCCACGATCTATC-3′ and 5′-ACAGTCGACCGGTCTTGGAGCGGTGATAATG-3′ and subsequently cloned in the pGEM-T vector (Promega, Madison, WI). A 2-kb region containing part of the *crtC* and *crtD* genes was deleted by XhoI digestion and replaced by the 4.7-kb SalI *LacZ*-Km^r cassette of pKOK5 (26). The resulting 6.2-kb SalI insert containing the mutated *crtC* and *crtD* genes was cloned into the pJQ200mp18 suicide vector (27). To mutate the *crtE.c* gene of the canthaxanthin *crt* gene cluster, a region of about 1.2 kb containing *crtE.c* gene was amplified by PCR using the primers 5′-GGTAGATCTGGTCTGCATGCGCGGATGAAACAG-3′ and 5′-GGAAGATCTCGAAGGCAGGTTCAGAGTATG-3′, digested by BglII, and cloned into the BamHI sites of pJQ200mp18. The 4.7-kb BamHI *LacZ*-Km^r cassette of pKOK5 was then inserted into the unique BamHI site of *crtE.c*. To mutate the *crtY* gene, a 1.8-kb PstI fragment of pSTM78 (18) containing *crtY* gene was cloned into the PstI sites of pJQ200mp18. A 0.4-kb XhoI fragment containing part of *crtY* was then deleted and replaced by the 4.7 SalI *LacZ*-Km^r cassette of pKOK5. To mutate the *crtI.s* gene of the spirilloxanthin *crt* gene cluster, a region of 1.4 kb was amplified by PCR using the primers 5′-CGGGATCCTTGGCTGGCGAAAGCGTCAATTTC-3′ and 5′-CGGGATCCAGGACGACAGGCGCTGCTCGAAATC-3′, digested by BamHI, and cloned into the BamHI sites of pJQ200mp18. The 4.7-kb SalI *LacZ*-Km^r cassette of pKOK5 was then inserted into the unique XhoI site of *crtI.s*. For each construction, we verified by PCR and sequencing that the *lacZ* reporter gene was in the correct orientation. The pJQ200 derivatives obtained, which encoded a counterselective *sacB* marker, were transformed into *E. coli* S17-1 for mobilization into ORS278 as described previously (24). Double recombinants were selected on sucrose, and the insertion was confirmed by PCR.

Preparation of Membranes and RC-LH1 Complexes—A 200-ml culture of *Bradyrhizobium* (wild type (WT) or mutant) was collected and resuspended in 10 ml of Tris-HCl buffer (50 mM, pH 8). The cells were disrupted by three passages through a French press at 50 megapascals. The suspension was centrifuged for 10 min at 4,000 × *g* to remove the unbroken cells and cells debris. The supernatant was loaded on a discontinuous sucrose gradient (0.6–1.2 M sucrose, 50 mM Tris-HCl buffer, pH 8) and centrifuged at 255,000 × *g* for 90 min. The membranes localized at the interface of the two sucrose layers constitute the chromatophore fraction, while the pellet contains the cytoplasmic membranes. Each fraction was diluted in 25 ml of Tris-HCl buffer (50 mM, pH 8), centrifuged at 255,000 × *g* for 90 min to removed the sucrose, and then resuspended in Tris-HCl buffer (10 mM, pH 8).

RC-LH1 complexes were isolated by addition of 1.5% LDAO to purified chromatophores or cytoplasmic membranes whose optical density was adjusted to 5 OD/cm at 870 nm. After an incubation of 15 min at room temperature in the dark, the membrane suspension was loaded on a discontinuous sucrose gradient (0.1, 0.2, 0.3, 0.6 M sucrose, 50 mM Tris-HCl, pH 8, 0.02% LDAO) and centrifuged at 255,000 × *g* for 90 min. All canthaxanthin molecules do not enter the sucrose gradient. The RC-LH1 particles, collected at the interface between the 0.2 and 0.3 M sucrose layers, were diluted in Tris-HCl (50 mM, pH 8, 0.02% LDAO) and centrifuged at 255,000 × *g* for 180 min. They were resuspended in Tris-HCl (10 mM, pH 8, 0.02% LDAO). Further purification of the canthaxanthin-containing fraction and of the RC-LH1 particles was performed using a mono-Q column coupled to an FPLC (Amersham Biosciences) and submitted to a NaCl gradient.

Absorption and Fluorescence Spectroscopy—Absorption spectra and light-induced absorption changes in intact cells were measured as described previously (24). Fluorescence excitation and emission spectra were recorded on a Spex Fluorolog 3 spectrofluorometer (Jobin Yvon). For excitation spectra, the excitation slits were 5 nm, and the emission was measured at 870 nm (on the blue side of the emission spectrum where the instrument sensitivity was highest) with 15-nm slits. For emission spectra excitation was through 10-nm slits, and emission was measured through 7-nm slits. Emission spectra were corrected for the wavelength dependence of the instrument response, and excitation spectra were corrected for variations in excitation intensity. For all fluorescence spectra the detector was protected from scattered excitation by a Wratten 88A gelatin filter.

RESULTS

Isolation and Characterization of the Spirilloxanthin Biosynthesis Genes—In all photosynthetic bacteria studied so far, the

[1] The abbreviations used are: HPLC, high pressure liquid chromatography; RC, reaction center; LH, light harvesting; WT, wild type; LDAO, lauryldimethylamine oxide.

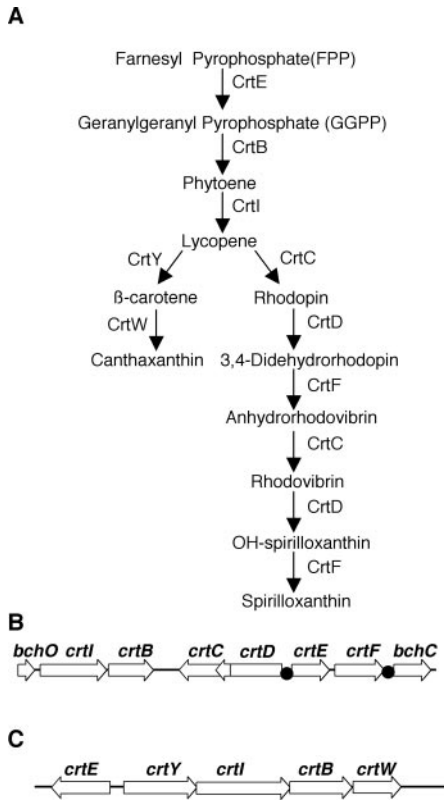

FIG. 1. **Biosynthesis of canthaxanthin and spirilloxanthin.** *A*, carotenoid biosynthesis pathways leading to canthaxanthin and spirilloxanthin. *B* and *C*, organizations of the canthaxanthin (*B*) and spirilloxanthin (*C*) gene clusters of *Bradyrhizobium* sp. strain ORS278. *Filled circles* indicate the positions of PpsR binding sites.

carotenoid biosynthesis genes have been found linked to the photosynthesis gene cluster. We have previously isolated, from a genomic DNA library of the ORS278 strain, a cosmid (pSTM1) with an insert of ~35 kb that contains some photosynthesis genes (24). Its partial sequencing reveals a gene arrangement close to that of purple photosynthetic bacteria with the superoperonal structure *bchCXYZpufBALM* conserved. This prompted us to check whether the upstream region of *bchC* gene contains some of the carotenoid synthesis genes as observed in other photosynthetic bacteria. The sequencing of this region reveals the presence of six open reading frames encoding proteins with similarities to known Crt enzymes assigned to *crtE*, *crtF*, *crtC*, *crtD*, *crtI*, and *crtB* genes (Fig. 1*B*). The orientation of these open reading frames suggests the existence of a minimum of three operons (*crtEF*, *crtCD*, and *crtIB*) as usually observed in purple photosynthetic bacteria (2). The overlap observed between *crtD* and *crtC* (348 bp) was also reported in *Rubrivivax gelatinosus* (28). The canthaxanthin *crt* gene cluster (Fig. 1*C*) was previously isolated from the cosmid pSTM73 (18). We showed by PCR that there is no overlap between this cosmid and the cosmid pSTM1 containing the spirilloxanthin gene cluster. Together these results demonstrate the presence of two distinct *crt* gene clusters in *Bradyrhizobium* ORS278, a spirilloxanthin gene cluster localized in the photosynthesis gene cluster region and a canthaxanthin gene cluster localized in a different part of the genome. Interestingly the genes *crtE*, *crtI*, and *crtB* that encode enzymes of lycopene biosynthesis, a common precursor of canthaxanthin and spirilloxanthin, are found in both clusters. However, they are obviously different and show little similarity (38% identity between the two CrtE proteins and 43 and 51.6% for the CrtB and CrtI pairs, respectively). To distinguish these genes, we call the genes from the spirilloxanthin *crt* cluster

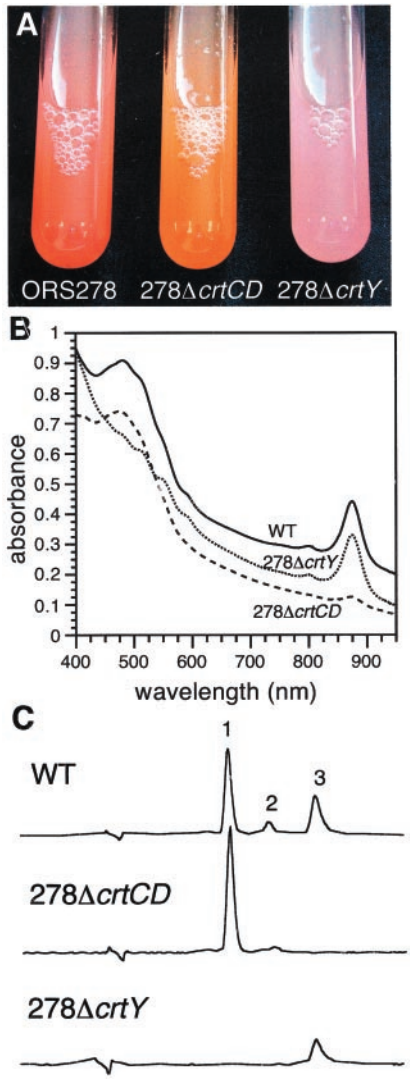

FIG. 2. **Carotenoid analyses of *Bradyrhizobium* ORS278 wild-type strain and the mutants 278Δ*crtCD* and 278Δ*crtY*.** *A*, pigmentation of WT strain and the mutants 278Δ*crtCD* and 278Δ*crtY*. *B*, absorption spectra of intact cells for the WT strain (*continuous line*) and the mutants 278Δ*crtCD* (*dashed line*) and 278Δ*crtY* (*dotted line*). The absorbency level at 950 nm has been arbitrarily adjusted to avoid overlapping spectra. *C*, HPLC separation of the carotenoids of WT strain and the mutants 278Δ*crtCD* and 278Δ*crtY*. Peaks correspond to the following: *1*, *trans*-canthaxanthin; *2*, *cis*-canthaxanthin isomers; *3*, spirilloxanthin.

crtE.s, *crtI.s*, and *crtB.s* and those from the canthaxanthin *crt* cluster *crtE.c*, *crtI.c*, and *crtB.c*.

To demonstrate that the canthaxanthin and the spirilloxanthin *crt* gene clusters we have identified are each responsible for the biosynthesis of the appropriate one of these two carotenoids, we constructed a mutant deleted in *crtY* gene (278Δ*crtY* strain, canthaxanthin *minus*) and a mutant deleted in the *crtCD* genes (278Δ*crtCD* strain, spirilloxanthin *minus*). The pigmentation of both mutants is clearly different from the WT strain (Fig. 2*A*). The 278Δ*crtCD* mutant presents an orange color characteristic of canthaxanthin, while the 278Δ*crtY* mutant deleted in a canthaxanthin gene is pink as is typical for spirilloxanthin. These variations in color are essentially due to the difference in absorption profiles of the carotenoids in the 400–600 nm region of the three strains (Fig. 2*B*). HPLC analysis of the carotenoids extracted from each mutant (Fig. 2*C*) confirmed the absence of spirilloxanthin in the 278Δ*crtCD* mutant as well as the absence of canthaxanthin in the 278Δ*crtY* mutant.

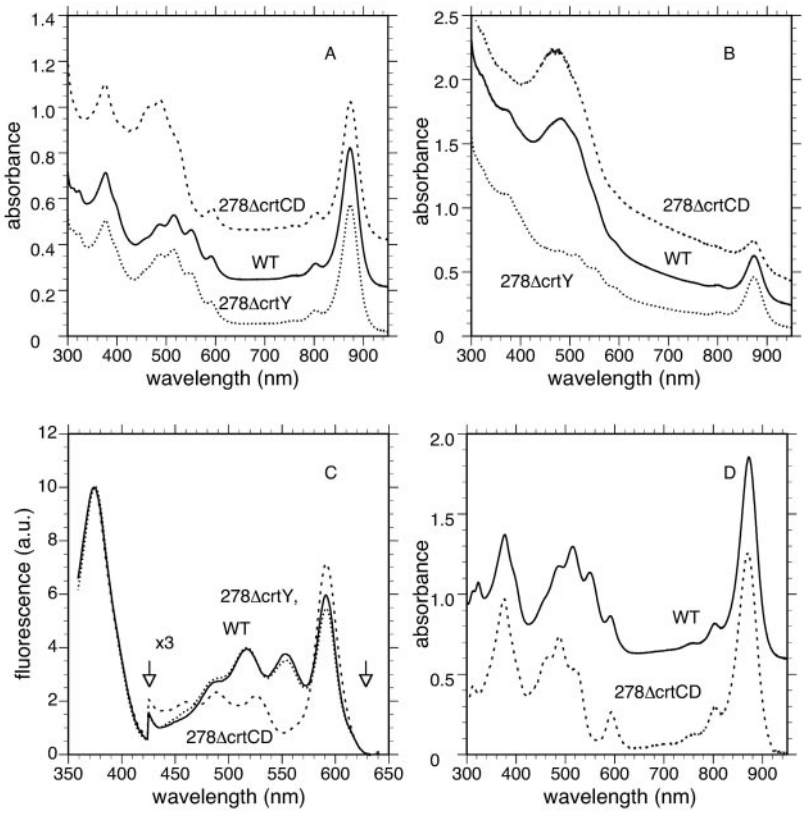

FIG. 3. **Absorption and fluorescence properties of *Bradyrhizobium* ORS278 wild-type strain and the mutants 278Δ*crtCD* and 278Δ*crtY*.** Absorption spectra of the intracytoplasmic (*A*) and cytoplasmic membranes (*B*) isolated from the WT strain (*continuous line*) and the mutants 278Δ*crtCD* (*dashed line*) and 278Δ*crtY* (*dotted line*). *C*, fluorescence excitation spectra for intact cells of the WT (*continuous line*) and the mutants 278Δ*crtCD* (*dashed line*) and 278Δ*crtY* (*dotted line*). The fluorescence emission is measured around 990 nm; between 425 and 630 nm the fluorescence spectra have been multiplied by a factor 3. For *A*, *B*, and *D*, the absorbency level at 950 nm has been arbitrarily adjusted to avoid overlapping spectra. *D*, absorption spectra of the purified RC-LH1 particles isolated from the WT strain (*continuous line*) and the mutant 278Δ*crtCD* (*dashed line*). *a.u.*, arbitrary units.

Membrane Localization and Function of Canthaxanthin and Spirilloxanthin—Aerobic photosynthetic bacteria are known to possess a small amount of photosynthetic apparatus correlated with few invaginations of the cytoplasmic membrane (5). In a first attempt to clarify the function of the two carotenoids present in *Bradyrhizobium* ORS278, cytoplasmic and intracytoplasmic fractions of the membrane were separated on a sucrose gradient after breakage of the WT cells. The two types of membranes could be separated as a function of their density (see "Experimental Procedures"). The cytoplasmic membranes form a pellet, while the intracytoplasmic membrane fragments (chromatophores) sediment at the interface between 0.6 and 1.2 M sucrose layers. The absorption spectrum of the chromatophores reveals the presence of bacteriochlorophyll (873, 590, and 375 nm) and spirilloxanthin (548, 515, and 485 nm) molecules but no detectable canthaxanthin (Fig. 3*A*). In contrast the cytoplasmic membrane contains a large amount of canthaxanthin in addition to the photosynthetic pigments (Fig. 3*B*). From the relative amounts of bacteriochlorophyll molecules present in these two fractions, we deduce that about 40% of the photosynthetic units are present in the chromatophores, while the rest is localized in the cytoplasmic membrane. This experiment clearly shows that spirilloxanthin molecules are associated with the photosynthetic apparatus in agreement with previous observations on several other species (10). Another proof that only spirilloxanthin molecules are associated with the photosynthetic apparatus was obtained by the measurement of the excitation spectrum of intact cells of the bacteriochlorophyll fluorescence around 890 nm (Fig. 3*C*). Comparison between the absorption spectrum of intact cells (Fig. 2*B*) and the excitation spectrum associated with the fluorescence emission of the LH1 complexes (Fig. 3*C*) shows that only the spirilloxanthin (at 550, 515, and 485 nm) molecules are able to transfer energy to the photosynthetic units. There is no evidence of energy transfer from canthaxanthin. In addition, identical excitation spectra are measured for both the WT and the 278Δ*crtY* mutant (canthaxanthin *minus*) (Fig. 3*C*). These results clearly demonstrate that spirilloxanthin, but not canthaxanthin, transfers light energy to the photosynthetic apparatus. An additional proof comes from the characteristics of the absorption spectrum of purified RC-LH1 complexes. These complexes can be easily purified from the cytoplasmic or the chromatophore membranes after addition of 1.5% LDAO (see "Experimental Procedures"). These particles do not contain any canthaxanthin as shown by both their absorption spectrum (Fig. 3*D*) and analysis of their carotenoid content (data not shown). The phenotypes of mutants deleted in one of the genes of the canthaxanthin (278Δ*crtY*) or the spirilloxanthin (278Δ*crtCD*) pathways also demonstrate that spirilloxanthin and not canthaxanthin is associated with the photosynthetic apparatus. Indeed a similar amount of photosynthetic apparatus is present in intact cells of the WT or of the canthaxanthin *minus* mutant (278Δ*crtY*) (see Fig. 2*B*) demonstrating that the lack of canthaxanthin does not affect its formation. In contrast, the amount of photosynthetic apparatus is reduced by at least a factor of 3–5 in the spirilloxanthin *minus* mutant (278Δ*crtCD*) compared with the WT as shown by the comparison of their absorption spectra (see Fig. 2*B*) or measurement of light-induced photooxidation of the cytochrome on intact cells (data not shown). In fact, the chemical analysis of the carotenoid content of this mutant reveals the presence of a significant amount of lycopene (0.05 mg/g of cells) (data not shown). This carotenoid is associated with the small fraction of photosystem present in the 278Δ*crtCD* mutant as demonstrated by the absorption spectrum of the chromatophore fraction and of purified isolated RC-LH1 complexes (Fig. 3, *A* and *D*) and the fluorescence excitation spectrum (Fig. 3*C*), which all present the characteristic bands of lycopene around 520, 487, and 460 nm.

Canthaxanthin and RC-LH1 complexes can be easily extracted from the cytoplasmic membranes. The fraction containing the canthaxanthin does not enter the sucrose gradient, while the RC-LH1 complexes sediment at the 0.6–1.2 M inter-

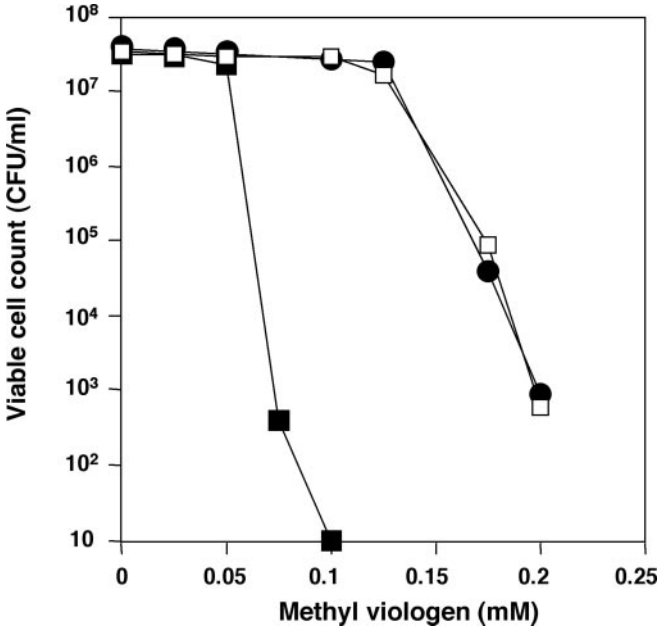

FIG. 4. **Effect of methyl viologen on the viability of *Bradyrhizobium* ORS278 wild-type strain (*open squares*) and the mutants 278Δ*crtCD* (*filled circles*) and 278Δ*crtY* (*filled squares*).** Exponentially growing cells (WT, 278Δ*crtCD*, and 278Δ*crtY*) were diluted and plated onto yeast extract mannitol agar medium containing various concentrations of methyl viologen. Plates were incubated at 37 °C, and after 1 week the colony-forming units (*CFU*) were counted.

face (not shown). Further purification of the canthaxanthin fraction on a Mono-Q column followed by gel electrophoresis shows that the canthaxanthin is not associated with protein. This suggests that, unless the detergent has destroyed a weak protein-canthaxanthin association, canthaxanthin is present in the lipid phase of the membrane and not associated with specific polypeptides.

One putative function of canthaxanthin is to act as strong antioxidant protecting against (photo)oxidative damage. To test this hypothesis, we measured the survival of bacteria exposed to an oxidative stress caused by addition of methyl viologen for both the WT and the 278Δ*crtY* and 278Δ*crtCD* mutants. As seen on Fig. 4, the canthaxanthin *minus* mutant (278Δ*crtY*) is more sensitive to the addition of increasing concentrations of methyl viologen than either the WT strain or the spirilloxanthin *minus* mutant (278Δ*crtCD*). In addition, the canthaxanthin *minus* mutant is less resistant than the WT strain in patch assays with H_2O_2 as the oxidative stress inducer (data not shown). These results are a clear indication that canthaxanthin acts as a protective agent against oxidative stress in *Bradyrhizobium* ORS278 cells.

Regulation of Canthaxanthin and Spirilloxanthin Synthesis by Light—We have previously shown that the photosynthetic activity in *Bradyrhizobium* is stimulated by far-red light through the action of the bacteriophytochrome BrbphP (25). The *BrbphP* gene is localized close to the photosynthesis gene cluster and contiguous to an open reading frame homologous to the transcription factor PpsR. The PpsR protein is known to repress *crt* genes in *Rhodobacter* species at high oxygen tension or high light intensity (29, 30). The up-regulation of photosynthesis genes by far-red light illumination observed in *Bradyrhizobium* ORS278 strain results from the antirepressor effect of the Pr form of BrbphP on PpsR (25). This prompted us to clarify the role of both BrbphP and PpsR in the biosynthesis of spirilloxanthin and canthaxanthin in this species. In a first set of experiments we investigated the effect of light quality in the red/far-red region on the production of both carotenoids. Fig. 5*A* clearly shows that the production of both carotenoids synthesized is enhanced in the 740–780 nm region, *i.e.* by the formation of the Pr form of BrbphP. Under far-red light, production of canthaxanthin is double, whereas the production of spirilloxanthin is triple, compared with the level measured in the dark (Fig. 5, *B* and *C*). To prove that the increase of carotenoids synthesis induced by far-red light is under the control of BrbphP and PpsR, we analyzed the carotenoid content (Fig. 5, *B* and *C*) of the *BrbphP* and *ppsR* null mutants obtained previously (25). The *BrbphP* minus mutant displays a very low level of production of spirilloxanthin independent of the growth conditions, whereas deletion of the *ppsR* gene leads to the overproduction of this carotenoid again whatever the growth conditions (Fig. 5*B*). Both mutants also have opposite effects on canthaxanthin production with a decrease for the 278Δ*BrbphP* mutant and an increase for the 278Δ*ppsR* mutant, once more in both cases independent of the growth conditions (Fig. 5*C*). Together these results show clearly that far-red light controls the synthesis of canthaxanthin and spirilloxanthin via the dual actions of the bacteriophytochrome BrbphP and the transcription factor PpsR.

To further elucidate the molecular mechanisms that lead to the stimulation of spirilloxanthin and canthaxanthin by far-red light, we determined the effect of far-red light on the expression of the *crt* genes of both clusters with the help of mutants carrying *lacZ* fusions. The expression of both of the spirilloxanthin *crt* genes assayed (*crtI.s* and *crtD*) is strongly stimulated by far-red light with an action spectrum corresponding to the Pfr form of BrbphP (Fig. 5*D*). In contrast, the expression of the canthaxanthin *crt* genes tested (*crtE.c* and *crtY*) remains at the same level whatever the light conditions (Fig. 5*D*). These data demonstrate that only the spirilloxanthin *crt* genes are up-regulated by far-red light via the action of the Pr form of BrbphP. These results are in agreement with the profiles of the promoter regions of the *crt* genes of both clusters. The repressive action of PpsR in *Rhodobacter* species results from its ability to stop transcription by binding DNA with the palindromic sequence $TGTN_{12}ACA$ present in the promoter regions of some photosynthesis genes (30). One PpsR DNA binding motif can be identified in the spirilloxanthin *crt* gene cluster, whereas such a motif is not found in the canthaxanthin *crt* gene cluster. The interaction of PpsR with the promoters of the spirilloxanthin pathway was confirmed by DNA footprint analyses with purified PpsR protein (data not shown). PpsR binds to the intergenic region between *crtE.s* and *crtC* that overlaps the promoter regions of the two putative operons *crtEF* and *crtCD* in the spirilloxanthin cluster. In contrast, a DNA probe that overlaps the promoter regions between the two putative operons *crtE* and *crtYIBW* of the canthaxanthin cluster is not protected from DNase I digestion by addition of PpsR. We therefore conclude that only the expression of the spirilloxanthin *crt* genes are under the control of PpsR and BrbphP. There is a clear discrepancy between this conclusion and the observed enhancement of the production of canthaxanthin by far-red light (Fig. 5*A*) and the carotenoid content of the *BrbphP* and *ppsR* null mutants (Fig. 5*C*). To resolve this apparent contradiction, we hypothesize that the up-regulation of spirilloxanthin *crt* genes by the phytochrome leads to the overproduction of some common precursors to the biosynthesis pathway of canthaxanthin and spirilloxanthin. For example lycopene produced by the CrtE.s, CrtB.s, and CrtI.s enzymes could be partially rerouted by the CrtY and CrtW enzymes to form canthaxanthin. To test this hypothesis we measured the carotenoid content in mutants deleted in one of the genes of the enzymes of lycopene synthesis in each carotenoid pathway (278Δ*crtE.c* and 278Δ*crtI.s*). These two mutants produce both canthaxan-

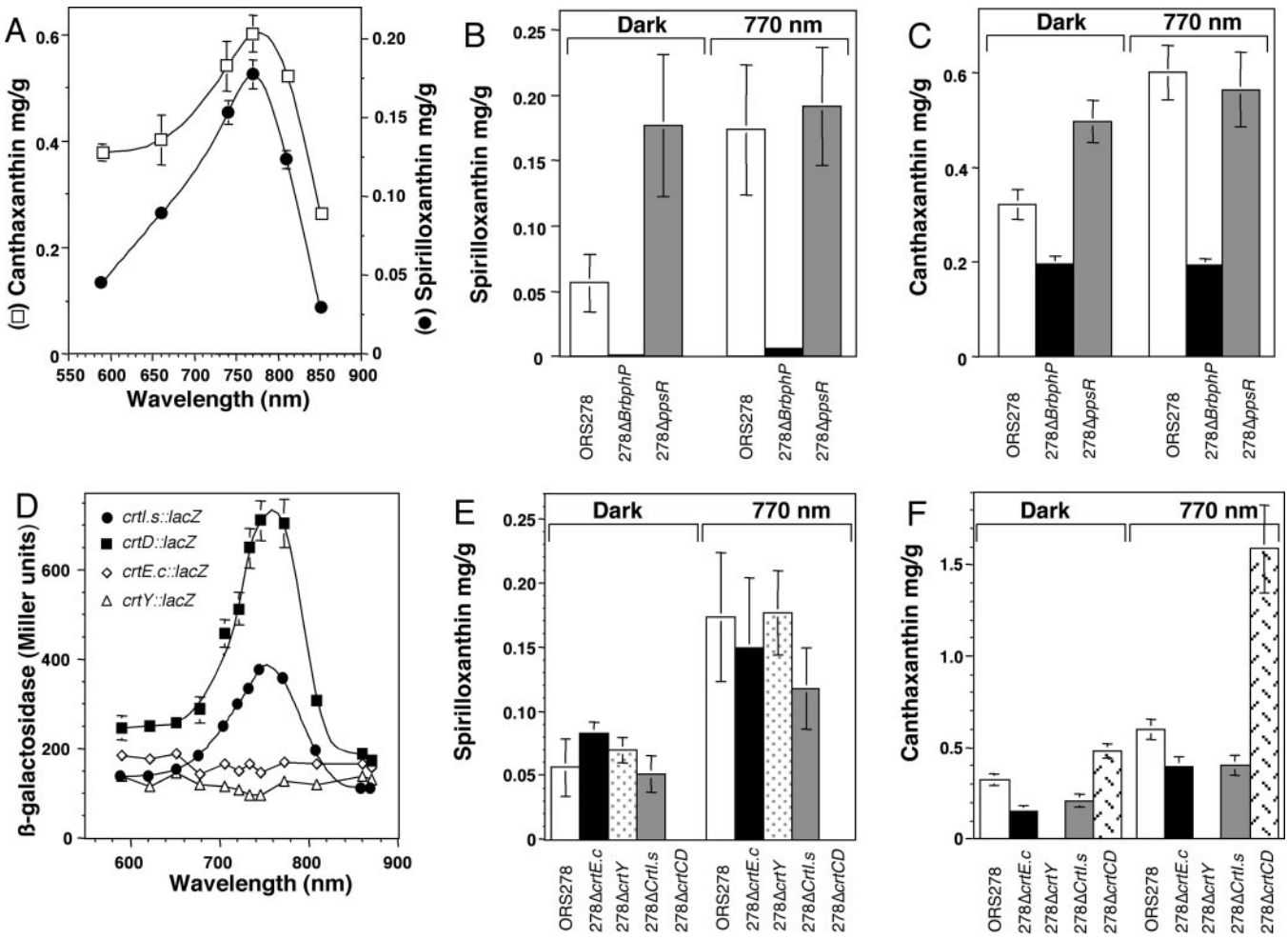

FIG. 5. **Effect of illumination on canthaxanthin and spirilloxanthin production.** The data in all the different panels represent the mean of three experiments (*errors bars* indicate ±S.D.). The amount of carotenoids is indicated in milligrams per gram of dry cell weight. *A*, light action spectra for canthaxanthin (*open squares*) and spirilloxanthin (*filled circles*) production by *Bradyrhizobium* ORS278 wild-type strain. *B*, production of spirilloxanthin in WT strain (ORS278) and the 278Δ*BrbphP* and 278Δ*ppsR* mutants after growth under complete darkness or far-red light (770 nm). *C*, production of canthaxanthin in WT strain (ORS278) and the 278Δ*BrbphP* and 278Δ*ppsR* mutants after growth under complete darkness or far-red light (770 nm). *D*, light action spectra of various *crt* gene expression. β-Galactosidase activity of the strains harboring the various *lacZ-crt* fusions is indicated in miller units. *E*, production of spirilloxanthin in WT strain (ORS278) and the 278Δ*crtE.c*, 278Δ*crtY*, 278Δ*crtI.s*, and 278Δ*crtCD* mutants after growth under complete darkness or far-red light (770 nm). *F*, production of canthaxanthin in WT strain (ORS278) and the 278Δ*crtE.c*, 278Δ*crtY*, 278Δ*crtI.s*, and 278Δ*crtCD* mutants after growth under complete darkness or far-red light (770 nm).

thin and spirilloxanthin demonstrating a cross-talk between the two carotenoid synthesis pathways (Fig. 5, *E* and *F*). Other proofs of this link are the lower level of canthaxanthin produced by the 278Δ*crtI.s* mutant (Fig. 5*F*) and the strong enhancement by far-red light of canthaxanthin production especially in the 278Δ*crtCD* mutant. These different observations imply that *crtE.s*, *crtI.s*, or *crtB.s* genes can complement, respectively, for the deletion of the *crtE.c*, *crtI.c*, or *crtB.c* genes and vice versa. We therefore conclude that the biosynthesis of spirilloxanthin and canthaxanthin are strongly connected at the level of lycopene or one of its precursors.

DISCUSSION

Aerobic phototrophic bacteria present a surprising complex carotenoid composition. In the present study, our goal was to clarify this complexity using the bacterial model *Bradyrhizobium* ORS278, which synthesizes two major carotenoids, the linear spirilloxanthin and the bicyclic canthaxanthin. Combining biophysical, biochemical, and genetic approaches, we determined the function of each of these carotenoids, characterized their biosynthesis genes, and described their regulation. We demonstrate the presence of two distinct carotenoids gene clusters involved in the biosynthesis of spirilloxanthin and of canthaxanthin, respectively. One striking result is the presence in each of these clusters of the three genes, *crtE*, *crtB*, and *crtI*, implicated in the synthesis of the precursor lycopene, common to the two biosynthetic pathways. Altogether these results, discussed in more detail below, give the first indications to an understanding of the presence of different carotenoids in a photosynthetic bacterium.

Functions of Spirilloxanthin and Canthaxanthin—Biochemical analysis and phenotypes of mutants clearly demonstrate that the spirilloxanthin molecules are the only carotenoid associated with the photosynthetic apparatus in *Bradyrhizobium* ORS278. Comparison between the excitation spectrum and the absorption spectrum leads to the conclusion that only about 30% of the light energy absorbed by the spirilloxanthin molecules are transferred to the bacteriochlorophyll molecules. This efficiency is typical to spirilloxanthin molecules associated with LH1 as already observed in the case of *Rhodospirillum rubrum* for example (31). When the synthesis of spirilloxanthin is blocked at the CrtC and CrtD enzyme level, spirilloxanthin is replaced by lycopene in the LH1 complexes and probably in the RC. Lycopene has also been shown recently to be an integral part of the LH2 complexes in a mutant of *Rhodobacter spha-*

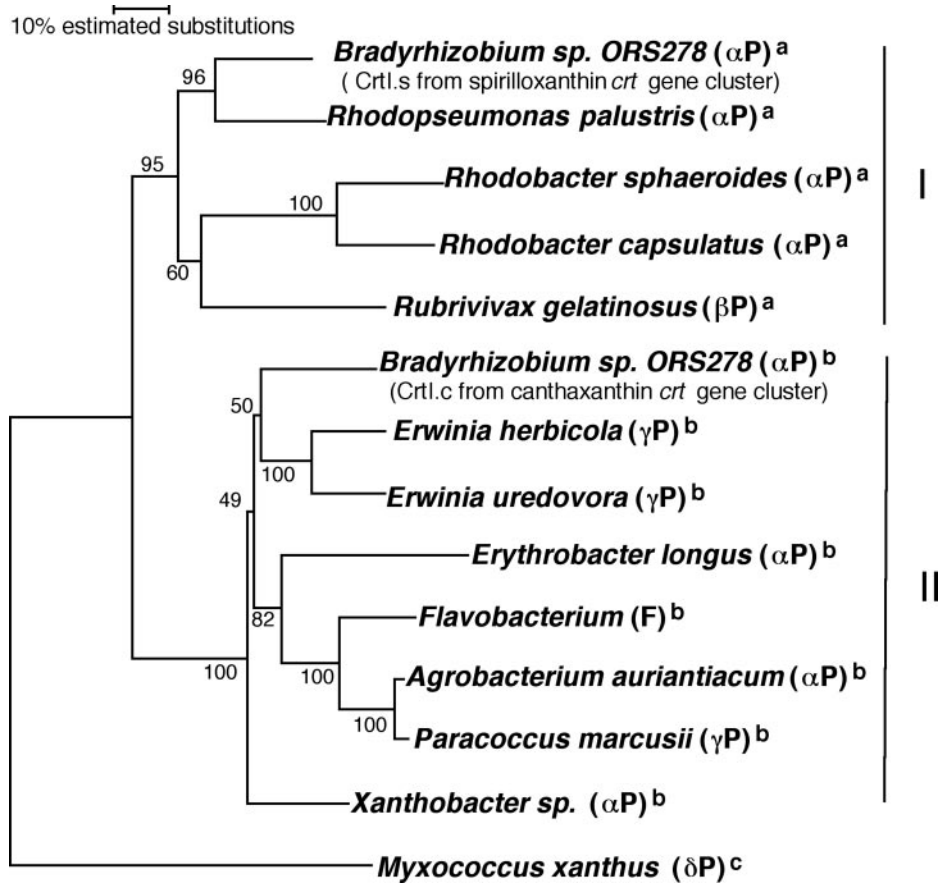

FIG. 6. **Phylogenetic tree based on the CrtI sequences.** This tree has been constructed by using the neighbor-joining method (40). Bootstrap values (41), expressed as percentages of 1000 replications, are given at the *branching points*. *P*, Proteobacteria; *F*, Flavobacteria; *a*, bicyclic carotenoid; *b*, acyclic carotenoid; *c*, monocyclic carotenoid. The GenBank™ accession numbers are: AF218415, CrtI.c *Bradyrhizobium* sp. ORS278; AF182374, CrtI.s *Bradyrhizobium* sp. ORS278; D58420, *Agrobacterium aurantiacum*; Y15112, *Paracoccus marcusii*; U62808, *Flavobacterium* sp.; M87280, *E. herbicola* EHO10; D90087, *Erwinia uredovora*; ZP_00011091, *R. palustris*; AF195122, *R. sphaeroides*; X52291, *R. capsulatus*; AB034704, *R. gelatinosus*; AF408840 *Xanthobacter* sp.; Z21955, *Myxococcus xanthus.* Note that the *crtI* gene from the aerobic photosynthetic bacterium *E. longus* Och101, a species that produces about 20 different kinds of carotenoids, has been found contiguous to a *crtY* gene that encodes the lycopene cyclase enzyme (42). It is therefore likely that this *crtI* gene is implicated in the synthesis of cyclic carotenoids. *I*, group I; II, group II.

eroides in which the native three-step phytoene desaturase (CrtI) was replaced with the four-step enzyme from *Erwinia herbicola* (32). However, to our knowledge, this is the first example of the presence of lycopene in LH1 complexes. Although the energy transfer between lycopene and bacteriochlorophylls is less efficient than the one measured for spirilloxanthin, lycopene and spirilloxanthin present a similar arrangement and conformational state as shown by linear dichroism measurements on intact cells oriented in polyacrylamide gels (data not shown). Furthermore the amount of photosynthetic apparatus per cell is reduced by a factor of 3–5 in the 278Δ*crtCD* mutant. Therefore, our results not only demonstrate that the spirilloxanthin is the major carotenoid associated with the photosystem in *Bradyrhizobium* but also that this molecule plays an important role in the structural stabilization of the bacteriochlorophyll LH1 complexes. In contrast, canthaxanthin appears to be localized predominantly in the cytoplasmic part of the membrane and to protect the cells against oxidative stress in agreement with different *in vitro* studies (33, 34).

Regulation of Carotenoid Biosynthesis—In this study, we clearly show that the canthaxanthin and spirilloxanthin *crt* gene clusters are differently regulated. Indeed only the spirilloxanthin *crt* genes are under the control of the light via the dual action of the phytochrome BrbphP and the transcriptional factor PpsR. The protein PpsR is known, in purple bacteria, to repress photosynthesis by binding to the promoter regions of some photosynthesis genes including *crt* genes (30). The light action spectrum of the spirilloxanthin *crt* gene expression and the phenotypes of *ppsR* and *BrbphP* minus mutants provide evidence that the Pr form of BrbphP activates the expression of spirilloxanthin *crt* genes alleviating the repression by PpsR. We found only one binding site of PpsR in the spirilloxanthin *crt* gene cluster of ORS278. Since this site is present in the intergenic region between the two putative operons *crtEF* and *crtCD*, we can speculate that the fixation of PpsR on this site blocks the transcription of these four genes. On the other hand no PpsR binding site is found upstream of the putative operon *crtIB.s*, although the expression of the *crtI.s* gene is strongly enhanced by far-red light. One possible explanation is that these genes are co-transcribed with other *bch* genes present upstream and under the control of PpsR.

The molecular mechanism by which BrbphP activates the expression of spirilloxanthin *crt* genes and antirepresses the action of PpsR remains unknown. This mechanism may be close to the dual mechanism of action between AppA and PpsR recently described in *R. sphaeroides* (35). Like BrbphP, Appa is a light photoreceptor that activates the expression of photosynthesis genes including *crt* genes. It has been shown that Appa antagonizes the repressive effect of PpsR by forming a blue light-sensitive and redox-dependent AppA-PpsR complex. By analogy, one can speculate that BrbphP and PpsR form a light-dependent complex.

Although the canthaxanthin *crt* gene cluster is not regulated via BrbphP and PpsR, we observe an increase in production of canthaxanthin caused by far-red light in the WT strain. We propose that a part of the common intermediates synthesized by the CrtE.s, CrtI.s, and CrtB.s enzymes could be diverted toward canthaxanthin production. The production of both canthaxanthin and spirilloxanthin by the *crtE.c* and *crtI.s minus* mutants is in agreement with such a hypothesis. Despite this interconnection between the two biosynthesis pathways, the amount of spirilloxanthin does not exceed 0.2 mg/g of dry cells even in the mutant (278Δ*crtY*), which does not synthesize canthaxanthin. This observation has two important implications: i) canthaxanthin is not made at the expense of spirilloxanthin synthesis and ii) the level of spirilloxanthin is limited to the quantity bound to the photosynthetic apparatus present in the cell. Such control could result

from the inhibition of some enzymes of the biosynthesis pathway by the end product spirilloxanthin. This proposal is in line with the observation that some phytoene desaturases are inhibited by carotenoids such as neurosporene, lycopene, and β-carotene (36, 37). In contrast no such limitation is observed for the biosynthesis of canthaxanthin as the total amount of canthaxanthin can reach 1.5 mg/g of cells in the case of the 278Δ*crtCD* mutant under far-red light illumination. This strong enhancement may be the result of an overexpression of the CrtE.s, CrtI.s, and CrtB.s enzymes, not repressed in this case because of the absence of spirilloxanthin production.

Origin of Carotenoid Gene Clusters—A recent analysis of the diversity of photosynthetic *Bradyrhizobium* sp. shows a monophyletic origin of the strains producing canthaxanthin (38). It is therefore very tempting to suggest that an ancestral photosynthetic *Bradyrhizobium* acquired, by lateral gene transfer, the canthaxanthin *crt* gene cluster. Phylogenetic analysis based on CrtI (Fig. 6) or CrtB sequences (data not shown) provide strong support for this hypothesis. Indeed the trees obtained clearly show the presence of two distinct groups that are not related to the taxonomical position of the various species but to the function or the nature of their carotenoid (cyclic or non-cyclic). Group I contains only sequences from photosynthetic bacteria, and group II contains sequences from pigmented non-photosynthetic bacteria except for the two aerobic photosynthetic bacteria *Bradyrhizobium* ORS278 and *E. longus*. The fact that CrtI.s and CrtI.c (or CrtB.s and CrtB.c) from *Bradyrhizobium* ORS278 strain are classified separately into these two groups confirms that these genes have evolved independently. Moreover the fact that each group contains bacteria phylogenetically distant suggests that lateral gene transfers have occurred within each group. The *crtI.s* or *crtB.s* genes of *Bradyrhizobium* ORS278 congregate with *crt* genes implicated in the synthesis of photosynthetic pigment and are branched with those of the closely related anaerobic photosynthetic bacteria *Rhodopseudomonas palustris* (supported by a bootstrap value of 96%). This is in agreement with previous phylogenetic analysis based on 16 S rDNA or *puf* genes (39, 24). The congruence between these different phylogenetic analyses using different genetic markers strengthens the hypothesis that photosynthetic *Bradyrhizobium* sp. inherited photosynthesis genes including spirilloxanthin *crt* genes directly from a photosynthetic ancestor common to *R. palustris*. In contrast the *crtI.c* or *crtB.c* genes are grouped with *crt* genes implicated in the synthesis of cyclic carotenoids found in non-photosynthetic bacteria and are phylogenetically closer to the distant species of *Erwinia.* This is a strong argument for acquisition of the canthaxanthin *crt* gene cluster by lateral gene transfer.

The presence of two distinct *crt* gene clusters found in *Bradyrhizobium* ORS278 is surely not unique to some photosynthetic strains of this species. This is certainly also the case for other aerobic photosynthetic bacteria, such as *E. longus*, which have been reported to produce different classes of carotenoids (4, 10). One can wonder what is the physiological and evolutionary basis for such a complex carotenoid composition found specifically in some aerobic photosynthetic bacteria. In contrast to the purple bacteria, the photosystem of these bacteria is active only under aerobic condition. In the presence of oxygen and light, the photosynthetic apparatus generates triplet states of bacteriochlorophyll molecules, which can react with singlet oxygen to form harmful reactive oxygen species toxic to cells. The acquisition by lateral gene transfer of an additional *crt* gene cluster that permits the synthesis of supplementary carotenoids may constitute a major selective advantage for these bacteria protecting them against the generation of reactive oxygen species during photosynthesis. This may be especially the case when the bacteria are developing *ex planta* where they have to cope with high light intensity at elevated oxygen tension. The observation that the amount of canthaxanthin increases in parallel with the synthesis of the photosynthetic apparatus and the weaker resistance of the canthaxanthin *minus* mutant (278Δ*crtY*) to oxidative stress are in agreement with such a hypothesis.

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