Two distinct *crt* gene clusters for two different functional classes of carotenoid in *Bradyrhizobium*

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**Running title:** carotenoid biosynthesis in *Bradyrhizobium*
SUMMARY

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, able to photosynthesize only under condition where harmful reactive oxygen species are generated.

INTRODUCTION

Carotenoids comprise a large class of pigments which 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 photo-protectors 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 *E. longus* or *E. ramosum*, which have been reported to produce about twenty different carotenoids (4, 5). Another example comes from various strains of photosynthetic *Bradyrhizobia*, 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 catalyzed the condensation of farnesyl pyrophosphahate 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 3 desaturation steps or to lycopene in 4 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 catalyses cyclisation of lycopene leading to β-carotene and CrtW which oxygenates β-carotene to form canthaxanthin (Fig. 1A), (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 (Fig. 1A), (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 (PGC)- a 45 kb DNA region which contains the essential genes involved in the synthesis of the photosynthetic apparatus (14, 15, 16, 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, \( \text{crtE} \), \( \text{crtY} \), \( \text{crtI} \), \( \text{crtB} \) and \( \text{crtW} \), necessary for the canthaxanthin biosynthesis. Neither photosynthesis genes nor specific genes of spirilloxanthin biosynthesis from lycopene (crtC, \( \text{crtD} \) or \( \text{crtF} \)) 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 YM-agar medium with addition of appropriated 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 provided by light emitting diodes (LEDs) of different wavelength between 590 and 870 nm with an irradiance of 6.6 µmoles photons/m²/s. *Escherichia coli* was grown in Luria-Bertani (LB) medium supplemented with the appropriated antibiotics.

Pigments analysis.

Cells grown at the surface of the Petri dishes in the dark or under different light conditions were re-suspended in 6 ml of water + NaCl 9 g/l and spun down for 10 min at 4,000 g. The pellets were extracted 3 times in the dark with 1 ml of cold acetone/methanol (7/2, vol/vol). The carotenoids in the pooled extracts were analysis by HPLC using an ALLIANCE Waters 2690 Channel. The conditions were: 5 µm Hypersil C₁₈ column (250 by 4.6 mm, Alltech France), acetonitrile/methanol/isopropanol (40/50/10, vol/vol/vol) as eluent, flow rate 0.8 ml/min. The eluted fractions were monitored using a Waters 996 photodiode array detector, scanning from 270 to 600 nm every 2s. 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 (France). 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 on crt genes expression**

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

**Construction of crt mutant strains.**

Constructions of mutants deleted in the bacteriophytochrome (278BrbphP) and in the transcriptional factor PpsR (278ppsR) have been previously described (25). For the construction of a crtCD mutant (278crtCD), a region of about 3.5 kb containing crtC and crtD genes of ORS278 was amplified by PCR using the primers 5TAGTCGACGCAATGGCGCGCCACGATCTATC3 and 5ACAGTCGACCCTTGGAGCGGTGATAATG3 and subsequently cloned in the pGEM-T vector (Promega, Madison, WI, USA). 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-KmR 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’–GGTAGATCTGCTCTGATGCGATGACACGG–3’ and 5’–GGAAGATCTCGAAGGCAGGTTCAGAGTATG–3’, digested by BglII and cloned into the BamHI sites of pJQ200mp18. The 4.7-kb BamHI LacZ-KmR 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-KmR cassette of pKOK5. To mutate the crtI.s gene of the spirilloxanthin gene cluster, a region of 1.4 kb was amplified by PCR using the primers 5’–CGGGATCCCTGGCTGGCGAAAGCGTCAATTTC–3’ and 5’–CGGGATCCAGGACGACAGGCGCTGCTCGAAATC–3’, digested by BamHI and cloned into the BamHI sites of pJQ200mp18. The 4.7-kb SalI LacZ-KmR cassette of pKOK5 was then inserted into the unique XhoI site of crtI.s. For each construction, we have verified by PCR and sequencing that the lacZ reporter gene was in the correct orientation. The pJQ200 derivatives obtained, which encoded a counter selective sacB marker, were transformed into E. coli S17-1 for mobilization into ORS278 as previously described (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* (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 MPa. 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; Tris-HCl buffer 50 mM, pH 8) and centrifuged at 255,000 g for 90 min. The membranes localized at the interface of the two sucrose layers constitute the chromatophores fraction while the pellet contains the cytoplasmic membranes. Each fraction is diluted in 25 ml Tris-HCl buffer (50
mM, pH 8), spun down at 255,000 g for 90 min to removed the sucrose and then re-
suspended 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 discontinuous sucrose gradient (0.1, 0.2, 0.3, 0.6 M sucrose; Tris-HCl, 50 mM; 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 spun
down 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 were performed using a mono-Q column coupled to a FPLC (Pharmacia) and
submitted to a NaCl gradient.

**Absorption and fluorescence spectroscopy**

Absorption spectra and light-induced absorption changes in intact cells were measured as
previously described (24). Fluorescence excitation and emission spectra were recorded on a
Spex Fluorolog 3 spectrofluorimeter (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 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 carotenoid biosynthesis genes have been found linked to the photosynthesis gene cluster (PGC). We have previously isolated, from a genomic DNA library of the ORS278 strain, a cosmid (pSTM1) with an insert of approximately 35-kb which contains some photosynthesis genes (24). Its partial sequencing reveals a gene arrangement close to that of purple photosynthetic bacteria with the superoperon-like structure $\text{bchCXYZpufBALM}$ conserved. This prompted us to check if the upstream region of $\text{bchC}$ gene contains some of the carotenoid synthesis genes as observed in other photosynthetic bacteria. The sequencing of this region reveals the presence of 6 open reading frames (ORFs) encoding proteins with similarities to known Crt enzymes assigned to $\text{crtE}$, $\text{crtF}$, $\text{crtC}$, $\text{crtD}$, $\text{crtI}$ and $\text{crtB}$ genes (Fig. 1B). The orientation of these ORFs suggests the existence of a minimum of 3 operons ($\text{crtEF}$, $\text{crtCD}$ and $\text{crtIB}$) as usually observed in purple photosynthetic bacteria (2). The overlap observed between $\text{crtD}$ and $\text{crtC}$ (348 bp) was also reported in $\text{Rubrivivax gelatinosus}$ (28). The canthaxanthin $\text{crt}$ gene cluster (Fig. 1C) 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 $\text{crt}$ 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, 43% and 51.6% for the CrtB and CrtI pairs respectively). To distinguish these genes, we call the genes from the spirilloxanthin *crt* cluster *crtE.s*, *crtI.s* and *crtB.s* and those from the canthaxanthin *crt* cluster, *crtE.c*, *crtI.c*, *crtB.c*.

To demonstrate that the canthaxanthin and the spirilloxanthin *crt* gene clusters we have identified are each responsible to the biosynthesis of the appropriate one of these two carotenoids, we constructed a mutant deleted in *crtY* gene (278*crtY* strain, canthaxanthin minus) and a mutant deleted in the *crtCD* genes (278*crtCD* strain, spirilloxanthin minus). The pigmentation of both mutants is clearly different from the wild type (WT) strain (Fig. 2A). The 278*crtCD* mutant presents an orange color characteristic of canthaxanthin while the 278*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. 2B). HPLC analysis of the carotenoids extracted from each mutant (Fig. 2C) confirmed the absence of spirilloxanthin in the 278*crtCD* mutant as well as the absence of canthaxanthin in the 278*crtY* mutant.

**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. 3A). In contrast the cytoplasmic membrane contains a large amount of canthaxanthin in addition to the photosynthetic pigments (Fig. 3B). 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. 3C). Comparison between the absorption spectrum of intact cells (Fig. 2B) and the excitation spectrum associated with the LH1 complexes fluorescence emission (Fig. 3C) 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 278crtY mutant (canthaxanthin minus) (Fig. 3C). These results clearly demonstrate that spirilloxanthin, but not canthaxanthin, transfer 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. 3D) and analysis of their carotenoid content (data not shown). The phenotypes of mutants deleted in one of the gene of the canthaxanthin (278crtY) or the spirilloxanthin (278crtCD) 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 (278crtY) (see Fig. 2B) 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 to 5 in the spirilloxanthin minus mutant (278crtCD) compared to the WT as shown by the comparison of their absorption spectra (see fig. 2B) or measurement of light-induced photooxidation of the cytochrome on intact cells (data not shown). In fact, the chemical analysis of the carotenoids content of this mutant reveals the presence of significant amount of lycopene (0.05 mg/g cells) (data not shown). This carotenoid is associated with this small fraction of photosystem present in the 278crtCD mutant, as demonstrated by the absorption spectrum of the chromatophore fraction and of purified isolated RC-LH1 complexes (Fig. 3A...
and D), and the fluorescence excitation spectrum (Fig. 3C), 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 interface (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 anti-oxidant protecting against (photo)-oxidative damage. To test this hypothesis, we have measured the survival of bacteria exposed to an oxidative stress caused by addition of Methyl Viologen (MV), for both the WT and the 278crtY and 278crtCD mutants. As seen on figure 4, the canthaxanthin minus mutant (278crtY) is more sensitive, to the addition of increasing concentrations of MV, than either the WT strain or the spirilloxanthin minus mutant (278crtCD). In addition, the canthaxanthin minus mutant is less resistant than the WT strain in patch assays with H$_2$O$_2$ 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 ORF homologous to the transcription factor PpsR. The PpsR protein is known to repress \textit{crt} genes in \textit{Rhodobacter} species at high oxygen tension or high light intensity \cite{29, 30}. The up regulation of photosynthesis genes by far-red light illumination observed in \textit{Bradyrhizobium} ORS278 strain results from the anti-repressor effect of the Pr form of BrbphP on PpsR \cite{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. 5A 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 to the level of measured in the dark (Fig 5B 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 carotenoids content (Fig 5B and C) of the BrbphP and PpsR null mutants previously obtained \cite{25}. The BrbphP minus mutant displays a very low level of production of spirilloxanthin independent of the growth conditions, whereas deletion of the \textit{ppsR} gene leads to the overproduction of this carotenoid, again, whatever the growth conditions (Fig. 5B). Both mutants have also opposite effects on canthaxanthin production with a decrease for the 278\textit{BrbphP} mutant and an increase for the 278\textit{ppsR} mutant, once more in both cases independent of the growth conditions (Fig. 5C). 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, which 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. 5D). In contrast, the expression of the canthaxanthin *crt* genes tested (*crtE.c* and *crtY*) remains at the same level whatever the light conditions (Fig. 5D). 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 TGT-N$_{12}$-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. 5A) and the carotenoid content of the *BrbphP* and *ppsR* null mutants (Fig. 5C). To resolve this apparent contradiction, we hypothesize that the up regulation of spirilloxanthin *crt* genes by the phytochrome leads to the over production 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 re-routed by the CrtY and CrtW enzymes to form canthaxanthin. To test this hypothesis we have measured the carotenoid content in mutants deleted in one of the genes of the enzymes of lycopene synthesis in each carotenoid pathway (278crtE.c and 278crtI.s). These two mutants produce both canthaxanthin and spirilloxanthin demonstrating a crosstalk between the two carotenoid synthesis pathways (Fig. 5E and F). Other proofs of this link are the lower level of canthaxanthin produced by the 278crtI.s mutant (Fig. 5F) and the strong enhancement by far-red light of canthaxanthin production, especially in the 278crtCD 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, 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 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 enzymes 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 *Rhodobactersphaeroides* in which the native 3-step phytoene desaturase (CrtI) was replaced with the 4-step enzyme from *Erwiniaherbicola* (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 to 5 in the 278*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 carotenoids 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* genes 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 genes 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 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 anti-represses 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, which 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 BrbphP and PpsR form a light-dependent complex.

Although the canthaxanthin crt genes 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 towards 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 (278crtY) which does not synthesize canthaxanthin. This observation has two important implications: i) canthaxanthin is not made at the expense of spirilloxanthin synthesis, 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 neuroposorene, lycopene and ß-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 278crtCD mutant under far-red light illumination. This strong enhancement may be the result of an over expression of the CrtEs, CrtIs and CrtBs 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 *Bradyrhizobia* 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 which 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 *Erythrobacter 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 close related anaerobic photosynthetic bacteria *Rhodopseudomonas palustris* (supported by a bootstrap value of 96%). This is in agreement with previous phylogenetic analysis based on 16s rDNA or *puf* genes (39, 24). The congruence between these different phylogenetic analyses using different genetic markers strengthens the hypothesis that photosynthetic *Bradyrhizobia* inherited their photosynthesis genes including spirilloxanthin *crt* genes directly from a photosynthetic ancestor common to *R. palustris*. In contrast the *crtI.c* or *crtB.c* genes group 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 (ROS) toxic to cells. The acquisition by lateral gene transfer of an additional \textit{crt} gene cluster which permits the synthesis of supplementary carotenoids may constitute a major selective advantage for these bacteria protecting them against the generation of ROS during photosynthesis. This may be especially the case when the bacteria are developing \textit{ex planta} where they have to cope with high light intensity at elevated oxygen tension. The observations that the amount of canthaxanthin increases in parallel with the synthesis of the photosynthetic apparatus and the weaker resistance of the canthaxanthin minus mutant (278\textit{crtY}) to oxidative stress are in agreement with such a hypothesis.

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**FOOTNOTE**

GenBank Accession Number AF182374.
FIGURE LEGENDS

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.

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

Fig. 3 Absorption spectra of the intracytoplasmic (A) and cytoplasmic membranes (B) isolated from the Bradyrhizobium ORS278 wild type strain (continuous line) and the mutants
278crtCD (dashed line) and 278crtY (dotted line).

C: Fluorescence excitation spectra for intact cells of the WT (continuous line) and the mutants 278crtCD (dashed line) and 278crtY (dotted line). The fluorescence emission is measured around 870 nm, between 425 and 630 nm the fluorescence spectra have been multiplied by a factor 3.

For part A, B, and D: The absorbency level at 950 nm has been arbitrary adjusted to avoid overlapping spectra.

D: Absorption spectra of the purified RC-LH1 particles isolated from the WT strain (continuous line) and the mutant 278crtCD (dashed line).

**Fig. 4** Effect of Methyl Viologen on the viability of *Bradyrhizobium* ORS278 wild-type strain (open squares) and the mutants 278crtCD (filled circles) and 278crtY (filled squares). Exponentially growing cells (WT, 278crtCD, 278crtY) were diluted and plated onto YM agar medium containing various concentration of Methyl Viologen. Plates were incubated at 37°C and, after one week, the CFU were counted.

**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 278BrbphP and 278ppsR mutants after growth under complete darkness or far-red light (770 nm).

C. Production of canthaxanthin in WT strain (ORS278) and the 278BrbphP and 278ppsR mutants after growth under complete darkness or far-red light (770 nm).

D. Light action spectra on various crt genes expression. β galactosidase activity of the strains harboring the various lacZ-crt fusion is indicated in miller unit.

E Production of spirilloxanthin in WT strain (ORS278) and the 278crtE.c, 278crtY, 278crtI.s, and 278crtCD mutants after growth under complete darkness or far-red light (770 nm).

F. Production of canthaxanthin in WT strain (ORS278) and the 278crtE.c, 278crtY, 278crtI.s, and 278crtCD mutants after growth under complete darkness or far-red light (770 nm).

**Fig. 6** Phylogenetic tree based on the CrtI sequences.

This tree has been constructed by using Neighbour-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, CrtIc *Bradyrhizobium* sp. ORS278; AF182374, CrtIs *Bradyrhizobium* sp. ORS278; D58420, *Agrobacterium aurantiacum*; Y15112, *Paracoccus marcusii*; U62808, *Flavobacterium* sp.; M87280, *Erwinia herbicola* EHO10; D90087, *E. uredovora*; ZP_00011091, *Rhodopseudomonas palustris*; AF195122, *Rhodobacter sphaeroides*; X52291, *R. capsulatus*; AB034704, *Rubrivivax gelatinosus*; AF408840 *Xanthobacter* sp.; Z21955, *Myxococcus xanthus*. Note that the crtI
gene from the aerobic photosynthetic bacterium *Erythrobacter longus* Och101, a species which produces about 20 different kinds of carotenoids, has been found contiguous to a *crtY* gene which encodes the lycopene cyclase enzyme (42). It is therefore likely that this *crtI* gene is implicated in the synthesis of cyclic carotenoids.
Two distinct crt gene clusters for two different functional classes of carotenoid in bradyrhizobium
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