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Photooxidative stress stimulates illegitimate recombination and mutability in carotenoid-less mutants of *Rubrivivax gelatinosus*

Soufian Ouchane, Martine Picaud, Claudie Vernotte and Chantal Astier[1]

Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique (UPR 9061) associé à l'Université Pierre et Marie Curie, Bâtiment 24, Avenue de la Terrasse, 91198 Gif sur Yvette Cedex, France

[1]Corresponding author
e-mail: astier@cgm.cnrs-gif.fr

Carotenoids are essential to protection against photooxidative damage in photosynthetic and non-photosynthetic organisms. In a previous study, we reported the disruption of *crtD* and *crtC* carotenoid genes in the purple bacterium *Rubrivivax gelatinosus*, resulting in mutants that synthesized carotenoid intermediates. Here, carotenoid-less mutants have been constructed by disruption of the *crtB* gene. To study the biological role of carotenoids in photoprotection, the wild-type and the three carotenoid mutants were grown under different conditions. When exposed to photooxidative stress, only the carotenoid-less strains (*crtB*$^-$) gave rise with a high frequency to four classes of mutants. In the first class, carotenoid biosynthesis was partially restored. The second class corresponded to photosynthetic-deficient mutants. The third class corresponded to mutants in which the LHI antenna level was decreased. In the fourth class, synthesis of the photosynthetic apparatus was inhibited only in aerobiosis. Molecular analyses indicated that the oxidative stress induced mutations and illegitimate recombination. Illegitimate recombination events produced either functional or non-functional chimeric genes. The *R.gelatinosus crtB*$^-$ strain could be very useful for studies of the SOS response and of illegitimate recombination induced by oxidants in bacteria.

Keywords: carotenoid genes/illegitimate recombination/photooxidative stress/photosynthesis/purple bacteria

Introduction

The photosynthetic apparatus in many purple bacteria contains three types of pigment–protein complexes. The two light-harvesting LHII and LHI antenna complexes capture light and transfer energy to the third complex, the reaction centre (RC), in which charge separation occurs. The three complexes contain pigments, bacteriochlorophyll and carotenoids, which are essential in the photosynthetic process. The synthesis and the assembly of these different complexes are regulated primarily by two environmental signals, light and oxygen (for reviews, see Bauer, 1995; Bauer and Bird, 1996).

The three functions of carotenoids in bacterial photosynthesis are the absorption of energy for use in photosynthesis (Cogdell and Frank, 1987; Frank and Cogdell, 1993), participation in the assembly of the light-harvesting antenna (Jirsakova and Reiss-Husson, 1994; Lang and Hunter, 1994) and protection from photodamage (Frank and Cogdell, 1993). The harmful effects of excess light energy arise from the production of bacteriochlorophyll triplet states. In aerobic conditions, the triplet-excited states of bacteriochlorophylls sensitize oxygen to produce singlet molecular oxygen ($^1O_2$), a powerful oxidant which damages DNA, proteins, carbohydrates and lipids, causing death of the cells (Shigenaga *et al.*, 1994). In the RC and the antenna complexes, carotenoids bound in proximity to bacteriochlorophylls prevent formation of this reactive oxygen (Figure 1). It has been known for many years that carotenoids are capable of quenching bacteriochlorophyll triplet states. This photochemical reaction, resulting in triplet-excited carotenoids (^{3}Car), provides the mechanism by which carotenoids protect photosynthetic systems from singlet oxygen (Frank and Cogdell, 1993). The photoprotective role of carotenoids against oxidative damage is essential and is largely documented in various organisms from photosynthetic prokaryotes, anoxygenic bacteria and cyanobacteria, through photosynthetic eukaryotes, algae and higher plants, as well as numerous non-photosynthetic organisms from bacteria to mammalian cells (Armstrong and Hearst, 1996). Recent work provides increasing evidence that some carotenoids are effective antioxidants which can afford protection against some diseases and especially against some forms of cancer (Sies *et al.*, 1992).

Rubrivivax gelatinosus is a facultative phototrophic non-sulfur bacterium belonging to the β subclass of purple bacteria. This strain, like *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* and unlike *Rhodopseudomonas viridis*, can grow very easily under aerobic conditions in the dark as well as under photosynthetic conditions (anaerobiosis and light). These trophic characteristics are very useful for studies of the photosynthetic processes using mutants. Gene transfer systems in *R.gelatinosus* have been established (Ouchane *et al.*, 1996), and used to study the organization of the genes coding for the subunits of LHI and RC. The first studies of the *puf* operon of two strains of *R.gelatinosus*, strain IL144 (Nagashima *et al.*, 1994) and strain 1 (Ouchane *et al.*, 1995, 1996), have shown that two unknown open reading frames (ORFs) are present in these strains in addition to the five photosynthetic genes previously reported in species belonging to the α subclass, which include *pufB*, *A*, *L*, *M* and *C* coding for the β and α subunits of the LHI antenna and for the L, M and cytochrome subunits of the RC complex, respectively. In addition, we recently have demonstrated that the *puf* operon of *R.gelatinosus* strain 1 contained two genes of the carotenoid biosynthesis pathways. The *crtD* and *crtC* genes are localized downstream of the *pufC* gene and are co-transcribed with the *puf* operon (Ouchane *et al.*,

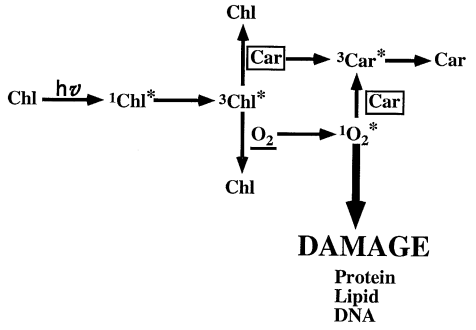

Fig. 1. Schematic representation of singlet oxygen formation upon excitation of chlorophyll (Chl) and the role of carotenoids (Car) in protection against photooxidative damage. The symbol * indicates excited states.

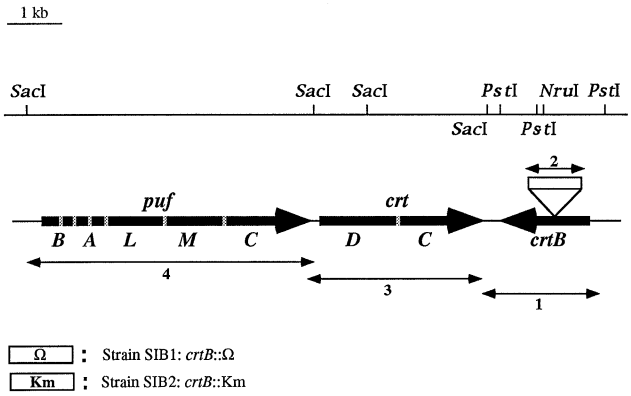

Fig. 2. Genomic organization of the *puf* operon and the downstream carotenoid genes in *R.gelatinosus* S1. The strains SIB1 and SIB2 were constructed by insertion of the drug cartridges in the *Nru*I site of the *crtB* gene. The different probes used in this work are indicated by the numbered lines with two arrows.

1997). Genetic evidence for an organization of the *puf* operon and of the upstream *bch* genes in a 'superoperon' has also been reported (Ouchane *et al.*, 1996).

In this study, we report the localization of a third gene of the carotenoid biosynthesis pathway of *R.gelatinosus*, the *crtB* gene coding for the phytoene synthase involved in the first step of carotenoid biosynthesis (Armstrong, 1995). Two *crtB*$^-$ mutants were constructed by insertion of antibiotic resistance cartridges in the *crtB* gene. These mutants were blue and completely devoid of coloured carotenoids and of LHII antennae, confirming the role of carotenoids in LHII assembly. The *crtD*$^-$, *crtC*$^-$ and *crtB*$^-$ mutants were used to study the biological role of carotenoids in photoprotection. Only the carotenoid-less mutants (*crtB*$^-$) were highly sensitive to photooxidative stress which induced mutagenesis with a high frequency, due in several cases to chromosomal rearrangements.

Results

Localization, nucleotide and deduced amino acid sequences of the crtB gene

Cloning of the *crtD* and *crtC* genes localized downstream of the *puf* operon from *R.gelatinosus* was achieved using insertional strains in which recombinant plasmids were inserted downstream of the *puf* operon (Ouchane *et al.*, 1997). The pSO22 plasmid containing a genomic DNA fragment of 5 kb was obtained and sequenced on both strands. In addition to the *crtD–C* operon (Figure 2), this fragment contains an ORF corresponding to the *crtB* carotenoid gene. The assignment of the ORF was based on the alignment with *R.capsulatus* and *R.sphaeroides* photosynthetic gene clusters. The nucleotide and deduced amino acid sequences were deposited in the DDBJ/EMBL/GenBank Database under the accession No. U87626.

The *crtB* gene was localized immediately downstream of the *crtC* gene; it has the opposite transcriptional orientation to that of the *crtD–C* operon, and a putative transcriptional terminator was found between them. An ATG initiating codon is proposed. No alternative initiation codon could be found. The putative translation start of this gene was based on the position of a Shine–Dalgarno ribosome-binding site (RBS) evaluated by comparison with other sequenced genes from *R.gelatinosus* known to encode proteins and by comparison with genes from *R.capsulatus* (Armstrong *et al.*, 1989). In *R.gelatinosus*, the codon usage deduced from the *pufBALMC*, *crtD* and *crtC* genes is characterized by the presence of a majority (89%) of C and G at the third position. The *crtB* sequence has a value of 85% comparable with the mean value. No putative promoter sequence with significant homology to the known *puf* promoter or to the *crt* promoters from *R.capsulatus* and *R.sphaeroides* was found for the *crtB* gene, suggesting that the *crtB* gene could be part of an operon.

The *crtB* gene is 1032 bp long, and encodes a protein with a predicted mol. wt of ~37.5 kDa. The CrtB protein from *R.gelatinosus* is highly similar (61%) to the corresponding protein from *R.capsulatus* (Figure 3). The consensus GX3QX6D domain found in the phytoene synthase from *R.capsulatus* and other organisms and required for the binding of isoprenoid pyrophosphate substrates (Armstrong *et al.*, 1993) is also conserved in *R.gelatinosus* (underlined in Figure 3). The hydropathy plot of the protein, determined according to Kyte and Doolittle (1982), did not reveal any putative transmembrane domain.

Involvement of crtB in the carotenoid biosynthesis pathway

To inactivate the *crtB* gene, the Ω or the Km cartridges were inserted in the unique *Nru*I site of a DNA fragment from *crtB* in the pSO26 plasmid. The resulting pSO40 and pSO41 plasmids were used for transformation of *R.gelatinosus* to create the *crtB*$^-$ strains, SIB1 and SIB2, respectively (Figure 2). Sp^r or Km^r and Ap^s transformants resulting from double crossover events were selected. All of them were photosynthetic. Southern blot analyses were performed to confirm the inactivation of the *crtB* gene by a double crossover event in the transformants. Figure 4 shows an example of Southern blot analysis. Genomic DNAs from wild-type and SIB1 strains were digested either by *Sac*I or by *Pst*I and probed with the *Pst*I fragment containing a large part of the *crtB* and with the Ω cartridge. The 8 kb *Sac*I band seen in the wild-type was replaced in the case of the SIB1, as expected, by a 10 kb band resulting from the 2 kb Ω cartridge insertion in the gene. The 1.5 kb *Pst*I band seen in the wild-type was replaced in SIB1 strain, as expected, by two small 1.3 and 0.2 kb (not visible in the figure) bands because of the two *Pst*I sites of the polylinkers flanking the Ω cartridge. The 2 kb band corresponded to the Ω cartridge. The other two

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CrtB RG    MPADLRVSSELQACRELMRGGSKSFFAASLLLPQRVRAPATALYAFCRVADDAVDLSGDP
CrtB RC    ----MIAEADMEVCRELIRTGSYSFHAASRVLPARVRDPALALYAFCRVADDEVDEVGAP
               .   .... ****.* ** ** *** .** *** ** ********** **  * *

CrtB RG    ---HAAMAELRTRLDGVYAGTPAPIAADRALACTVHRYGVPRVLLDALLEGFLWDADGRR
CrtB RC    RDKAAAVLKLGDRLEDIYAGRPRNAPSDRAFAAVVEEFEMPRELPEALLEGFAWDAEGRW
               **.  *  **. .*** *    .*** *. *  . .** * .****** ***.**

CrtB RG    YDTIADVEAYGARVAGTVGAAMALIMGVRSPQALARACELGVAMQFTNIARDVGEDARNG
CrtB RC    YHTLSDVQAYSARVAAAVGAMMCVLMRVRNPDALARACDLGLAMQMSNIARDVGEDARAG
           * *..**.** **** .*** *...* ** * ******.**.*** .********** *

CrtB RG    RLYLPRGWLVEAGLDVDAWLQNPVHCPPVAQTVRRLLRAADELYERSEHGIAALPRDCRP
CrtB RC    RLFLPTDWMVEEGIDPQAFLADPQPTKGIRRVTERLLNRADRLYWRAATGVRLLPFDCRP
           **.**  *.** *.*  * *  *  .  . .   ***  ** ** *.  *.  ** ****

CrtB RG    AIRAARWSTAEIGKRLERDGLDSVNRRVVVPARRKAALMARAVRRLQHARARLHLDAALP
CrtB RC    GIMAAGKIYAAIGAEVAKAKYDNITRRAHTTKGRKLWLVANSA----MSATATSMLPLSP
            * **    * **  . .   * ..**      **  *.* .      . .   .    *

CrtB RG    AIQYLVDASVAAPGPTVPHR--PPQRSFDERIGWVIELMERQAARRGI---
CrtB RC    RVHAKPEPEVAHLVDAAAHRNLHPER--SE--VLISALMALKARDRGLAMD
            ..   .  **    .  **   *.*   *    .  **  .*  **.
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Fig. 3. Comparison of the deduced *crtB* gene products of *R.capsulatus* (RC) and *R.gelatinosus* (RG). The symbols * and • indicate identical and similar amino acids respectively (using the ClustalV program). The underlined stretch of residues corresponds to a highly conserved region in the CrtB proteins from *R.gelatinosus*, *R.capsulatus* and other organisms.

bands (2.2 kb in the wild-type and 4.2 kb in SIB1) corresponded to partial *Pst*I digestions (see Figure 2).

As the *crtB* gene encodes a phytoene synthase, the first enzyme involved in carotenoid biosynthesis, we expected no carotenoid to be produced in the mutants. Pigment modifications in the mutants SIB1 and SIB2, which are blue instead of purple, were first checked by recording absorption spectra of their membranes and comparing them with that of the wild-type membranes (Figure 5). In the wild-type membranes, the three main carotenoid absorption peaks are located at 455, 481 and 512 nm. In both mutants, no peaks were identified in the absorption region of carotenoids. This indicated that the mutants corresponded effectively to carotenoid-less strains. A pleiotropic effect of the *crtB* inactivation was the loss of the absorption peaks at 800 and 850 nm, corresponding to the absorption peaks of the LHII antenna. This indicated that carotenoids are essential to the formation of LHII antenna complexes but not to the assembly of LHI antenna and RC complexes. The pigment composition of the mutant cells was analysed by thin layer chromatography (TLC) of the acetone–methanol extract of membranes. Only bacteriochlorophyll and bacteriopheophytin were found.

Involvement of carotenoids in photooxidative stress resistance

Several carotenoids have been described to be very efficient antioxidants, providing protection against reactive molecular oxygen. In purple bacteria, singlet oxygen production occurs during photosynthesis activity. The role of carotenoids in the photoprotection of these organisms poses two questions: (i) could any carotenoid pigment quench the bacteriochlorophyll triplet state *in vivo*, preventing molecular singlet oxygen formation in photooxidative stress conditions?; (ii) in the absence of carotenoids,

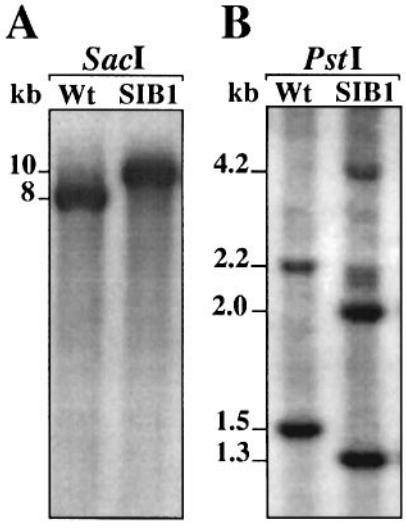

Fig. 4. Southern blot analysis of genomic DNAs of the wild-type (Wt) and the SIB1 strains digested either by *Sac*I (**A**) or *Pst*I (**B**). The probes used (1 and 2, see Figure 2) were labelled with [α-^{32}P]CTP by nick translation.

do photosynthetic bacterial cells have other pathways of resistance to photooxidative stress? To answer these questions, we studied the behaviour of the wild-type, of the carotenoid mutants *crtB*$^{-}$ and of the *crtD*$^{-}$ and *crtC*$^{-}$ strains previously constructed (Ouchane *et al.*, 1997) when exposed to photooxidative stress. The carotenoid pigments accumulated in each strain are summarized in Table I. Serial cell dilutions from the five strains were spread on plates and put in photosynthetic conditions (light and anaerobiosis), non-photosynthetic conditions (dark and aerobiosis) and photooxidative stress conditions (light and aerobiosis) to assess cell survival. In photosynthetic and non-photosynthetic conditions, all the strains grew and

remained stable. This was also the case under photooxidative stress conditions for the wild-type, the *crtD*$^-$ and the *crtC*$^-$ strains. For the carotenoid-less mutants, a survival rate of ~10^{-3} was observed and the surviving cells displayed new pigmentation phenotypes. These results confirm the essential role of carotenoids in photoprotection and indicate that the carotenoid intermediates present in the *crtD*$^-$ and the *crtC*$^-$ strains and mentioned in Table I are able to prevent photooxidative damage. An interesting point was the appearance, in the case of the carotenoid-less cells, of mutants resistant to photooxidative stress. Analysis of the mutants provides very exciting data concerning the behaviour of carotenoid-less cells when exposed to photooxidative stress.

One could ask whether highly selective, but non-photooxidative stress conditions could also produce increased mutability or not. One way to answer this question is to expose non-photosynthetic interposon mutants to photosynthetic conditions (light and anaerobiosis). Strains SIL1 and SIL2, in which Ω and Km cartridges respectively were inserted in the *pufL* gene

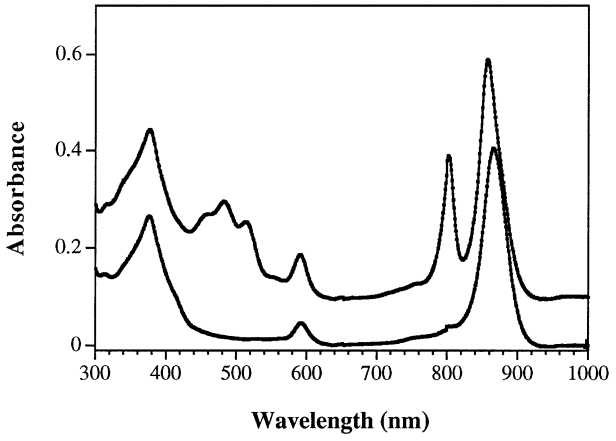

Fig. 5. Absorption spectra of chromatophores from *R.gelatinosus* wild-type (top) and SIB1 mutant (bottom), grown photosynthetically. SIB2 chromatophores produced the same spectrum as SIB1 (not shown). The zero level of the upper spectrum was shifted for better viewing.

(Table II), were placed in photosynthetic conditions. We did not obtain any surviving mutant. Therefore, we concluded that the mutagenic events observed in *crtB*$^-$ strains were due to the photooxidative stress selection and that singlet oxygen is responsible for mutagenesis in the carotenoid-less strains.

Selection of the photooxidative stress resistance mutants

To select mutants resistant to photooxidative stress, *crtB*$^-$ cells (SIB1 and SIB2), grown in photosynthetic conditions, were exposed to light and oxygen. Three days later, differently coloured colonies appeared, with a very high frequency of ~10^{-3}. They were streaked in the same conditions to ascertain that they corresponded to stable mutants, before any other characterization. The blue *crtB*$^-$ cells gave rise to seven different coloured colonies: green, green–brown, brown, beige, green–yellow, brilliant green and grey. The different mutants (called OSR for oxidative stress resistant) were resuspended and streaked to grow in photosynthetic and non-photosynthetic conditions. Some mutants were unable to grow photosynthetically. Among the mutants that were photosynthetic, some displayed the same phenotype when grown under photosynthetic or non-photosynthetic conditions whereas others displayed different phenotypes under the two conditions. The non-photosynthetic mutants appeared with the highest frequency (50%), followed by the green and green–brown mutants (20%). The frequency of the other mutants was lower. The mechanisms by which the mutants resist photooxidative stress and by which the cells mutated to acquire this resistance have been characterized further by studying a selection of mutants exhibiting various phenotypes.

Phenotypic characterization of the mutants

One preliminary and easy way to analyse the mutants is to record the absorption spectra of cells of these strains and to compare them with spectra of a carotenoid-less strain. Strains were grown under photosynthetic or non-photosynthetic conditions and the absorption spectra were

Table I. Major carotenoids, bacteriochlorophyll and precursors identified in the differents strains

	Wt	SΔP	SID1	SID2	SIB	OSR								
						1	2	3	4	5	6	7	8	9
Carotenoids														
Hydroxyspheroidene	+													
Spirilloxanthin	+													
Spheroidene	+							+						
Neurosporene		+	+	+		+	+	+						
Lycopene		+	+	+		+	+							
Methoxyneurosporene				+			+							
Hydroxyneurosporene				+			+							
Rhodopin				+			+							
Anhydrorhodovibrin								+						
Bacteriochlorophyll and precursors														
Bacteriochlorophyll *a*	+	+	+	+	+	+	+	+					+	+
DVHEC												+		
Chlorophyllide *a*											+			
Protoporphyrin IX									+	+				

DVHEC, desvinyl hydroxyethyl chlorophyllide *a*; OSR, oxidative-stress-resistant.

Table II. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
Escherichia coli		
C600	F^-, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44	Stratagene
XL1-Blue	*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*-1, relA1, *lac*$^-$F′ [*pro*AB$^+$ *lac*I^q, *lac*ZΔM15 Tn*10* (Tc^r)]	Stratagene
Rubrivivax gelatinosus		
S1	wild-type	Uffen (1976)
SID1	interposon strain (*crtD*::Ω)	Ouchane *et al.* (1997)
SID2	interposon strain (*crtD*::Km)	Ouchane *et al.* (1997)
SID3	insertion strain: plasmid pSO21 integrated into *crtD* on the chromosome	Ouchane *et al.* (1997)
SIL1	interposon strain (*pufL*::Ω)	Ouchane *et al.* (1997)
SIL2	interposon strain (*pufL*::Km)	Ouchane *et al.* (1997)
SIB1	interposon strain (*crtB*::Ω)	this work
SIB2	Interposon strain (*crtB*::Km)	this work
OSR1	*crtD*::Ω (green)	this work
OSR2	*crtD*::Km (green–brown)	this work
OSR3	nd (brown)	this work
OSR4	non-photosynthetic mutant, *puf*$^-$ (beige)	this work
OSR5	non-photosynthetic mutant, *bch* mutant, (beige)	this work
OSR6	bch mutant (green–yellow)	this work
OSR7	nd (brilliant green)	this work
OSR8	nd (grey)	this work
OSR9	nd (grey)	this work
Plasmids		
Bluescript KS$^+$	cloning vector (Ap^r)	Pharmacia
pBBR1MCS-2	expression vector, (bom$^+$, Km^r)	Kovach *et al.* (1994)
pDW9	plasmid with Ω cartridge (Sp, Sm)^r	Golden (1988)
pSO5	pBBR1MCS-2 + 5 kb *Sac*I fragment containing the *puf* operon	Ouchane *et al.* (1996)
pSO22	Bluescript KS$^+$+ 5 kb *Sac*I fragment downstream of the *puf* operon	this work
pSO26	Bluescript KS$^+$+ 2.5 kb *Pst*I fragment downstream of the *crtC* gene	this work
pSO40	(KS$^+$+ *crtB*::Ω). Ω cartridge cloned in the *Nru*I site within *crtB*	this work
pSO41	(KS$^+$+ *crtB*::Km). Km cartridge cloned in the *Nru*I site within *crtB*	this work
pSO50	pBBR1MCS-2 + 3 kb *Sac*I fragment containing the *crtD–C* operon under *lac* promoter control	this work
pSO51	pBBR1MCS-2 + 3 kb *Sac*I fragment containing the *crtD–C* operon cloned in the opposite orientation	this work
pUC4K	plasmid with Km cartridge	Pharmacia

Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant, Sm^r, streptomycin-resistant; Sp^r, spectinomycin-resistant; Tc^r, tetracycline-resistant; nd, not determined.

recorded. According to their spectra, the mutants were grouped into four classes. The first class included mutants (OSR1, OSR2 and OSR3) which have recovered the synthesis of carotenoid intermediates and consequently the assembled LHII antenna. The second class included photosynthetic-deficient mutants (OSR4 and OSR5). The third class was composed of photosynthetic mutants that excreted bacteriochlorophyll intermediates in the growth medium (OSR6, OSR8 and OSR9). The fourth class included a mutant (OSR7) which displayed different phenotypes (colour and photosynthetic apparatus synthesis) when grown under photosynthetic or non-photosynthetic conditions.

Mutants that recovered carotenoid biosynthesis (OSR1, OSR2 and OSR3)

OSR1 is a mutant from SIB1 (*crtB*::Ω) and is green–brown. OSR2 and OSR3 are mutants from SIB2 (*crtB*::Km) and are green and brown respectively. The absorption spectra of the whole cells (Figure 6A) show that these mutants had recovered the biosynthesis of carotenoid intermediates and the LHII antenna assembly. Pigment extracts from the three mutants were prepared and analysed by absorption spectroscopy (Figure 6B).

OSR1 and OSR2. Extracts from these two mutants presented the same absorption peaks at 415, 440 and 470 nm, with a minor peak at 500 nm, as observed in the carotenoid extracts from the SID1 (*crtD*::Ω) and SID2 (*crtD*::Km) strains (Ouchane *et al.*, 1997). Pigment extracts from the mutants were also analysed by TLC (Table I). Comparison with SID1 and SID2 strains indicated that OSR1 and OSR2 had the same phenotype as the SID1 (*crtC*$^-$) and SID2 mutant (*crtD*$^-$) respectively. SID1 and SID2 are disrupted at the same *crtD* gene but the difference in their phenotypes was a result of the cartridge (Ω or Km) used to inactivate the gene (Ouchane *et al.*, 1997). We conclude that the two mutants OSR1 and OSR2 have recovered a functional *crtB* gene and at the same time have mutated the *crtD* or the *crtC* gene. One possible interpretation of these results is to suppose that the drug cartridge had been removed from *crtB* and inserted in the *crtD* or in the *crtC* gene to disrupt it by a recombination process. These hypotheses will be confirmed by further genetic and molecular experiments (see below). Absorption spectra and chromatographic analyses of the pigment extracts (data not shown) also indicated that in these mutants, the amount of carotenoid, on the basis of the amount of bacteriochlorophyll, was lower than in the SID1

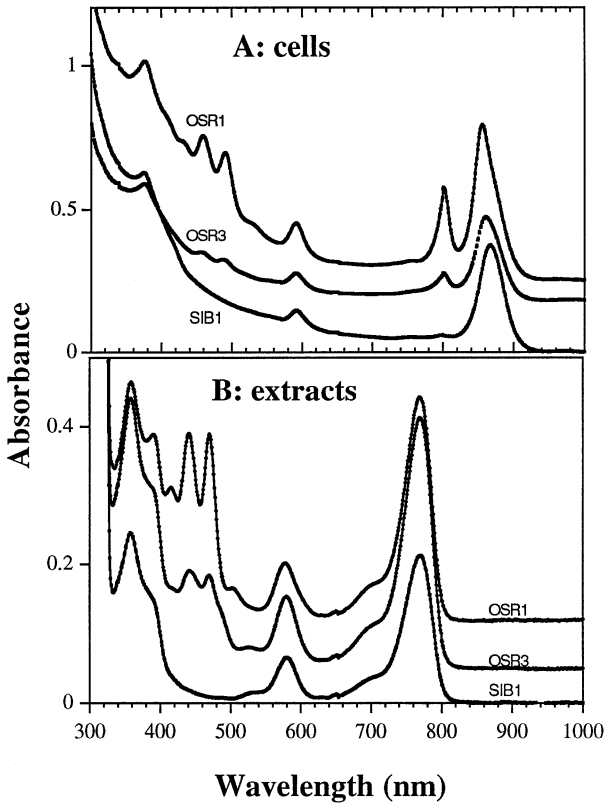

Fig. 6. Absorption spectra of whole cells (**A**) and of pigment extracts (**B**) from SIB1, OSR3 and OSR1. OSR2 cells and pigment extract gave spectra similar to those of OSR1 (not shown). The zero levels of the spectra were shifted for better viewing.

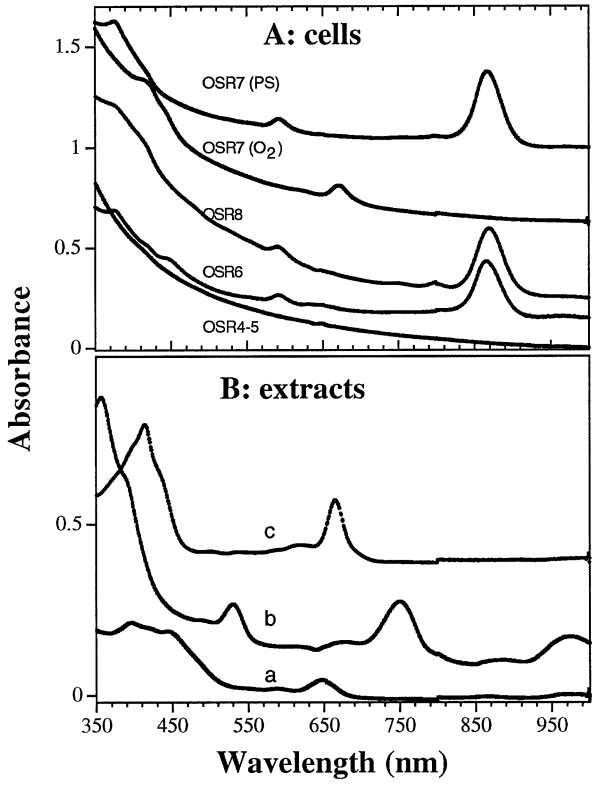

Fig. 7. (**A**) Absorption spectra of whole cells from OSR4 (OSR5 cells gave the same spectrum as OSR4), OSR6, OSR7 grown either in anaerobiosis (PS, photosynthetic conditions) or in aerobiosis (O_2, darkness or light), and OSR8. (**B**) Absorption spectra of the methanol pigment extract of the growth medium of OSR6 (a), OSR8 (b) and OSR7 grown in aerobiosis (c). The zero levels of the spectra were shifted for better viewing.

and SID2 mutants, and therefore that the chimeric CrtB protein generated in the mutants would be less efficient than the wild-type CrtB protein.

OSR3. Pigment extract from this mutant, as compared with OSR1 and OSR2, presented two peaks at 440 and 470 nm, with a minor peak at 525 nm, as shown in Figure 6B. These results indicated that the carotenoid content of OSR3 was different from that of the other mutants. TLC of the OSR3 pigment extract (Table I) indicated that OSR3 has recovered a functional *crtB* gene and at the same time has mutated another carotenoid gene, probably by the same mechanism as in OSR1 and OSR2.

To confirm our hypotheses on the genotypes of these three carotenoid mutants, the complementation of the mutants with the *crtD–C* genes was performed. As no promoter sequences have been found for the *crtD–C* operon (Ouchane *et al.*, 1997), the operon was cloned in the replicative pBBR1MCS-2 plasmid in both orientations (Table II). In plasmid pSO50, the direction of transcription of the *crtD–C* operon was the same as that of the *lac* promoter of pBBR1MCS, whereas in pSO51 it was in the opposite direction. As expected, the transformants obtained from complementation with pSO50 were purple like the wild-type, whereas transformants obtained from complementation with pSO51 did not change their respective phenotypes. These genetic results confirm first the sequence data concerning the absence of the promoter of *crtD–C*, and second, that in the carotenoid mutants, the observed phenotypes were due to lesions within the *crtD–C* operon.

As we have demonstrated above that the carotenoid intermediates from *crtD*$^-$ or *crtC*$^-$ are able to protect cells against photooxidative damage, the recovery of carotenoid intermediate biosynthesis in these mutants explained the acquired resistance against photooxidative stress.

Photosynthetic-deficient mutants (OSR4 and OSR5)

OSR4 and OSR5 are mutants from SIB1 and SIB2, respectively. Both are beige and are non-photosynthetic. Figure 7A presents their absorption spectra. The mutants did not produce any carotenoid and therefore no LHII complexes. In addition, they were devoid of LHI and RC complexes. These results indicated that in these mutants the *crtB* gene function was not reconstituted and that biosynthesis of the photosynthetic apparatus was somehow blocked. Spectral analysis of pigment extracts of OSR4 and OSR5 showed a complete absence of absorption at wavelengths >500 nm. Upon excitation at 400 nm, fluorescence emission spectra of pigments accumulated by both mutants indicated the presence of a pigment with an emission peak at 630 nm, characteristic of protoporphyrin IX. The presence of this bacteriochlorophyll intermediate could result either from the inactivation of the *puf* operon or from the inactivation of a bacteriochlorophyll gene involved in the early step of bacteriochlorophyll synthesis.

Two hypotheses can explain these phenotypes. The first hypothesis is that drug cartridges remained in the *crtB*

gene and a mutation has occurred in the *puf* operon or in a bacteriochlorophyll gene. The second hypothesis is that recombination processes allowing cartridge exchange between the disrupted *crtB* gene and the *puf* operon or a bacteriochlorophyll gene have occurred, as described for OSR1 and OSR2 mutants.

The non-photosynthetic phenotype observed in OSR4 and OSR5 could be due either to inactivation of the photosynthetic structural *puf* genes or to an inactivation of the early genes of bacteriochlorophyll biosynthesis. To check the genetic modifications occurring in these mutants, both strains were complemented with the *puf* operon. Selection of transformants was achieved in photosynthetic conditions. Only mutant OSR4 complementation with the *puf* operon restored photosynthetic growth, giving blue photosynthetic colonies exhibiting the *crtB*$^-$ phenotype. This indicated that in OSR4 the non-photosynthetic phenotype was due to a genetic lesion within the *puf* operon. For the OSR5 mutant, *puf* operon complementation did not restore the photosynthetic phenotype, indicating that in this strain, the non-photosynthetic phenotype was probably a result of a bacteriochlorophyll gene inactivation. These results will be confirmed by genomic DNA analyses.

Since photooxidation and production of singlet oxygen occur within the photosynthetic complexes, resistance to photooxidative stress in those mutants is simply due to the fact that they are not photosynthetic.

Bacteriochlorophyll mutants (OSR6, OSR8 and OSR9)

Both OSR6 and OSR9 are mutants from SIB2, and OSR8 is a mutant from SIB1. All are photosynthetic; OSR6 is green–yellow and OSR8 and OSR9 are grey. The absorption spectrum from OSR6 cells (Figure 7A) shows that in addition to the bacteriochlorophyll peak at 580 nm, an additional peak at ~640 nm corresponding to a bacteriochlorophyll intermediate was observed in OSR6. This bacteriochlorophyll intermediate was also found in the growth medium (spectrum a of Figure 7B). The absorption spectrum of a pigment extract from OSR6 was compared with those of *R.sphaeroides* strains whose bacteriochlorophyll lesions have been genetically mapped and bacteriochlorophyll precursors identified (Lang *et al.*, 1994). In OSR6, the precursor absorbing at 650 nm could correspond to chlorophyllide *a*.

The absorption spectra of OSR8 and OSR9 were comparable with that of a *crtB*$^-$ strain, except that they displayed maxima at 800 and 780 nm, which we attribute to RC monomeric bacteriochlorophylls and bacteriopheophytins, respectively. This observation suggests that these mutants produced a lower LHI/RC molar ratio in comparison with the *crtB*$^-$ strain. To examine this hypothesis, membranes from the *crtB*$^-$ strain and the mutant were isolated and the LHI antenna/RC ratio was determined as described in Materials and methods. We found ~22 and 47 bch/RC for OSR8 and SIB1 respectively. Another difference with the *crtB*$^-$ strain was that the mutants excreted in the growth medium a pigment which had a maximum absorption at 750 nm when extracted with methanol (spectrum b of Figure 7B). This pink pigment could correspond to bacteriopheophytin *a*. If the reduced size of the LHI antenna in OSR8 compared with SIB1 resulted from its instability, the presence of bacteriopheophytin *a* could result from the degradation of LHI.

Concerning the OSR6 mutants, the spectroscopic data did not provide any explanation as to how they resist photooxidative stress, as their LHI antenna/RC ratio (~85 bch/RC) appeared greater than in SIB2. One possibility could be that this strain reduces its amount of RC and that its LHI antennae are not all connected with the remaining RCs.

Mutant repressing the synthesis of the photosynthetic complexes in aerobiosis (OSR7)

OSR7 was a SIB2 mutant, isolated as a brilliant green clone. When grown in the presence of oxygen (darkness or light) it remained brilliant green, while when grown in the absence of oxygen it became blue. When the blue colonies were re-streaked again in the presence of oxygen, brilliant green colour was restored. Absorption spectra from this mutant (Figure 7A) showed that when grown in aerobiosis the mutant did not produce any photosynthetic complex, and only an absorption peak at 665 nm was observed. The mutation in this mutant resulted in a phenotype similar to that reported for a *bchXYZ* mutant from *R.rubrum* (Hessner *et al.*, 1991). Pigment extracts of the OSR7 strain exhibited an absorption peak at 660 nm which is characteristic of DVHEC (desvinyl hydroxyethyl chlorophyllide *a*) (spectrum c of Figure 7B). Fluorescence spectra of this intermediate exhibited a major peak at 680 nm. The OSR7 mutant thus appeared to be a *bchXYZ* mutant. However, when grown photosynthetically, the mutant presented the same absorption spectra as the *crtB*$^-$ strain, producing bacteriochlorophyll *a*. These results indicated that in this mutant, oxygen induced an inhibition of bacteriochlorophyll biosynthesis at the level of DVHEC and, consequently, the assembly of the photosynthetic complexes was impaired, allowing resistance to photooxidative stress. When grown in anaerobiosis the cells produced normal bacteriochlorophylls and their photosynthetic apparatus allowed photosynthetic growth. The factor and the gene that lead to inhibition of bacteriochlorophyll biosynthesis in OSR7 in aerobiosis remain to be determined. A *R.capsulatus* mutant called APP11 constructed by Gomelsky and Kaplan (1995) exhibited the same phenotype as OSR7. The *appA* gene which was inactivated in the APP11 strain corresponds to a bacteriochlorophyll biosynthesis regulator.

Molecular analysis of the mutants

To verify the hypotheses postulated above to explain the mutant phenotypes, the genomic DNAs from the wild-type, the *crtB*$^-$ strains and the mutants were digested with appropriate restriction enzymes and blots were hybridized with the different probes shown in Figure 2. Cartridge probes (probes 2) were used to check general genomic DNA rearrangements. Probes 1, 3 and 4 were used to check genomic DNA rearrangements within the *crtB* gene, within the *crtD* and *crtC* genes and within the *puf* operon, respectively.

The three mutants, OSR1, OSR2 and OSR3, have recovered carotenoid intermediate synthesis, and we postulated that recombination has occurred between the disrupted *crtB* gene and the upstream *crtD* or *crtC* genes. Genomic DNAs from the wild-type, the *crtB*$^-$ strains and

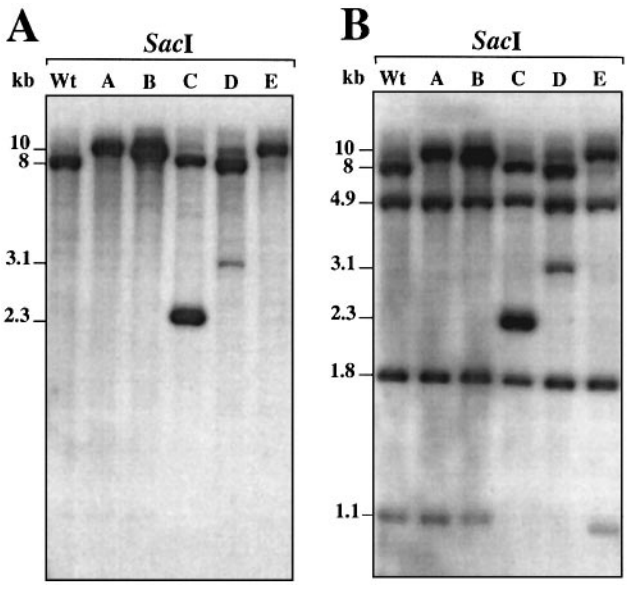

Fig. 8. Southern blot analysis of genomic DNAs of wild-type (Wt), SIB1 (A), SIB2 (B), OSR2 (C), OSR1 (D) and OSR3 (E) strains, digested by *Sac*I. The different bands were revealed by probes 1 and 2 in (**A**) and by probes 3 and 4 in (**B**). For probe numbers, see Figure 2.

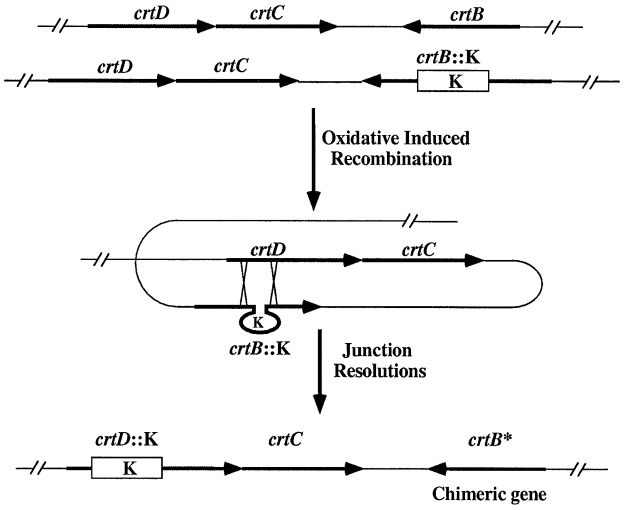

Fig. 9. Schematic representation of the illegitimate recombination induced by the photooxidative stress, between the disrupted *crtB* gene and the *crtD* gene. This recombination results in the formation of a chimeric *crtB** gene. The drug cartridge (Ω or Km) used to inactivate the *crtB* gene is indicated by the letter K.

the mutants were digested with *Sac*I and transferred DNAs were hybridized with probes 1, 2 and 3 (Figure 8). Figure 8A shows hybridization with probes 1 and 2. When probed with probe 1, the 10 or 9.2 kb bands seen in SIB1 and SIB2 respectively were replaced in OSR1 and OSR2 by a band at 8 kb, as in the wild-type, indicating that the *crtB* gene has been reconstituted in these mutants. The chimeric *crtB* gene corresponded to a simple exchange event between the two *crt* genes. In OSR3, *crtB* remained inactivated, as shown by the 9.2 kb band. Hybridization with probe 2 confirmed this result; indeed, in OSR3 no additional band was revealed, while in OSR1 and OSR2 bands at 3.1 and 2.3 kb respectively were revealed, confirming that the drug cartridges were removed from *crtB* and placed elsewhere on the chromosome.

As these mutants displayed *crtD*$^-$ or *crtC*$^-$ phenotypes, the cartridges could be re-inserted in *crtD*, *crtC* or *pufC* genes since Ω insertion within *crtD* or *pufC* leads to the *crtC*$^-$ phenotype (Ouchane *et al.*, 1997). The blot was probed with probes 3 and 4 to examine these hypotheses. As shown in Figure 8B, probe 4 gave a band at 4.9 kb for all the tested strains, indicating that the *puf* operon was not modified. Probe 3 gave a band at 1.8 kb for all the strains, indicating that the 3′ end of *crtD* and the whole *crtC* gene were not affected. This probe also hybridized with the *Sac*I fragment of the *crtD* gene (5′ end), giving, in the wild-type and the *crtB*$^-$ strains, a band at 1.1 kb. The same band was present in OSR3 but, in OSR1 and OSR2, this band was shifted to 3.1 and 2.3 kb, respectively. These bands also hybridized with probe 2. Thus, the phenotypes seen in OSR1 and OSR2 result from the disruption of the *crtD* gene by the drug cartridges according to a recombination event between *crtB*::cartridge and the *crtD* gene (Figure 9). We therefore looked for sequence homologies between *crtB* and *crtD* genes. The two genes only shared 47% homology and the maximal homologous stretches were about seven nucleotides. These findings demonstrate that photooxidative stress induces a illegitimate recombination system in *R.gelatinosus*.

For OSR3, since no modification in the fragment hybridization pattern compared with the *crtB*$^-$ strain was obtained, the only way to explain the presence of carotenoid intermediates is to suppose that a mutation has occurred within a carotenoid gene allowing the conversion of geranyl geranyl-pyrophosphate (GGPP) to phytoene. Because OSR3 seems to have a hydratase (CrtC) less efficient than the wild-type enzyme, one possibility is that the *crtC* gene has been mutated in such a way that the CrtC protein has lost its efficiency for converting its own substrates but has acquired the new phytoene synthase function. Further experiments are needed to verify this assumption.

OSR4 and OSR5 mutants are non-photosynthetic. The genetic data indicated that the *puf* operon and a *bch* gene were mutated in OSR4 and OSR5 respectively. One possibility is that similar recombination processes to those in OSR1 have occurred within the *crtB*$^-$ strain, allowing a cartridge exchange between the disrupted *crtB* gene and the *puf* operon or the *bch* gene. Genomic DNAs from the wild-type, a *crtB*$^-$ strain and the two mutants, were digested with *Sac*I and transferred DNAs were hybridized with probes 1, 2 and 4 (Figure 10). Figure 10A shows the hybridization with probes 1 and 2. When probed with probe 1, the band at 10 kb seen in the SIB1 strain was shifted to 9 kb in OSR4, indicating that the Ω cartridge has been removed from the *crtB* gene. In OSR5, the revealed band was still at 9.2 kb, as in SIB2, indicating that the Km cartridge has remained in the *crtB* gene. Hybridization with probe 2 confirmed these results and revealed that in OSR4, the Ω cartridge was removed from *crtB* giving a band at ~6 kb, and that in OSR5, the Km cartridge was still in the *crtB* gene. Additional hybridization with probe 4 (*puf* probe) revealed a shift for the 4.9 kb band seen in the wild-type, SIB1 and OSR5 to a band at 6 kb in OSR4 (Figure 10B). This band is the same as that revealed in Figure 10A with probe 2. These results demonstrated that in the OSR4 mutant, the drug

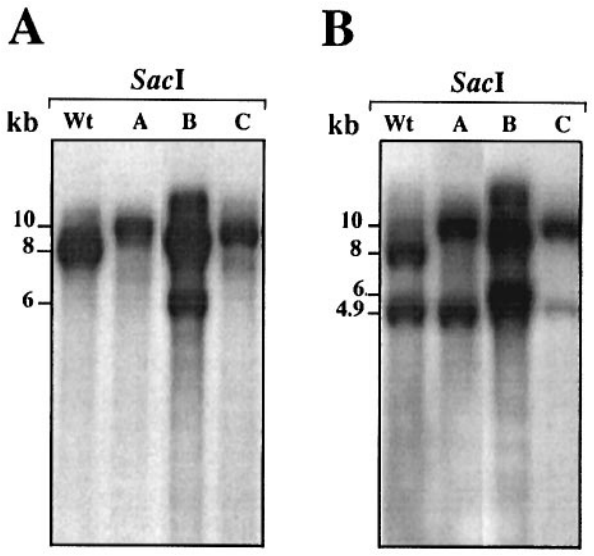

Fig. 10. Southern blot analyses of genomic DNAs of wild-type (Wt), SIB1 (A), OSR4 (B) and OSR5 (C) strains, digested by *Sac*I. The different bands were revealed by probes 1 and 2 in (**A**) and by probe 4 in (**B**). For probe numbers, see Figure 2.

cartridge was removed from the *crtB* gene and inserted into the *puf* operon, probably by the same mechanism as in OSR1 and OSR2. Contrary to OSR1 and OSR2 strains, this recombination gave rise in OSR4 to a non-functional *crtB* gene. As above, we looked for sequence identities between *crtB* and the *puf* genes. The two sequences only shared 46% identity and the maximal identical stretches were seven nucleotides. For the other mutants, no modification in the hybridization pattern with the four probes was revealed, indicating that in these mutants, the phenotypes observed probably result from accumulation of new mutations.

Discussion

Carotenoid gene organization in R.gelatinosus

We recently have reported that the localization and the expression of the two carotenoid genes *crtD* and *crtC* from *R.gelatinosus* were different from those known of the other purple bacteria. These two genes, localized downstream of the *puf* operon, were co-transcribed with this operon (Ouchane *et al.*, 1997). We wondered if they were the only carotenoid genes localized in this region of the chromosome or if carotenoid genes are organized in a cluster. Previous data in *R.capsulatus* and *R.sphaeroides* indicated that all the carotenoid genes are clustered upstream of the *puf* operon within the large 45 kb photosynthetic cluster (Armstrong, 1995). Partial genetic maps in *R.centenum* and *R.rubrum* support these conclusions (Bauer *et al.*, 1993). In this work, we report the localization of a third carotenoid gene, *crtB*. This gene is linked to *crtD* and *crtC* genes but not co-transcribed with them, suggesting that the carotenoid genes of *R.gelatinosus* could be organized in a cluster as in the previously mentioned purple bacteria. However, in contrast to these bacteria, the *crt* genes are localized in *R.gelatinosus* downstream of the *puf* operon. The product of the *crtB* gene corresponds to a phytoene synthase, the first enzyme implicated in carotenoid biosynthesis. This enzyme catalyses condensation of two GGPPs to produce phytoene.

Role of carotenoids in the stability of the photosynthetic complexes

To study the biological role of the carotenoids in purple bacteria, two carotenoid mutants, *crtC*$^-$ and *crtD*$^-$ strains, had been previously constructed (Ouchane *et al.*, 1997). Here, *R.gelatinosus* carotenoid-less strains have been constructed by inactivating the *crtB* gene. The presence of coloured carotenoid intermediates in *crtC*$^-$ and *crtD*$^-$ strains allowed formation of all the photosynthetic complexes, LHII, LHI and RC, in amounts comparable with those of the wild-type. However, purification of the various complexes has shown that they are less stable than those of the wild-type (I.Agalidis and F.Reiss-Husson, personal communication). The *crtB*$^-$ strains exhibited the same phenotypes as the previously characterized carotenoid-less strains described from *R.capsulatus* and *R.sphaeroides* (Hunter, 1995). In these strains, a pleiotropic effect of the *crtB* inactivation was the absence of the LHII antenna complexes. Indeed, it was reported that in the *crtB*$^-$ strain from *R.sphaeroides*, the LHII subunits were synthesized but rapidly turned over and were not inserted into the membrane (Lang and Hunter, 1994). Our results confirm that the formation of the LHII antenna complexes required the synthesis of coloured carotenoids. In contrast, the formation of the LHI and RC complexes did not require any coloured carotenoids. However, *in vitro*, the LHI antenna of *R.gelatinosus* required specific carotenoids to be assembled (Jirsakova and Reiss-Husson, 1994).

Role of carotenoids against oxidative stress

Reactive molecular oxygen is a product of aerobic metabolism with potential toxicity towards macromolecules, including lipids, proteins and DNA (Shigenaga *et al.*, 1994). Oxidative DNA damage is the most frequent type of damage encountered by aerobic cells and may play an important role in mutagenesis processes, carcinogenesis and ageing (Ames *et al.*, 1993). In photosynthetic bacteria, as in plants, in the presence of oxygen, an excess of light (photooxidative stress conditions) results in the production of such reactive molecules. To gain insight into the photoprotective role of carotenoids against molecular oxygen in purple bacteria and into the mutagenic effects of reactive oxygen, we have studied the behaviours of the *crtB*$^-$, *crtC*$^-$ and *crtD*$^-$ strains when exposed to photooxidative stress conditions. Our results show that different carotenoid intermediates (at least those accumulated in *crtD*$^-$ and *crtC*$^-$ cells) are able to quench triplet states and molecular oxygen, preventing photooxidative damage. Both strains (*crtC*$^-$ and *crtD*$^-$) accumulated lycopene. This carotenoid was found to be the most efficient biological singlet oxygen quencher among several carotenoids (Di Mascio *et al.*, 1989). The *crtB*$^-$ strains, completely devoid of carotenoids, were unable to survive in photooxidative conditions and, in these conditions, resistant mutants appear with a high frequency.

Photooxidative stress resistant mutants of crtB$^-$ *strains in R.gelatinosus*

In addition to mutants which have recovered carotenoid biosynthesis, such as OSR1, OSR2 and OSR3, other types

of mutants have appeared which provide interesting data about the strategies that cells can develop to survive when depleted of carotenoids. As photooxidation and production of singlet oxygen occur within the photosynthetic complexes, an inhibition of the synthesis or assembly of these complexes could allow aerobic growth of the cells, even in photooxidative stress conditions. These inhibitions could be carried out by inactivating the structural genes of the RC (*puf* operon) as in OSR4, by inactivating an early step of the bacteriochlorophyll biosynthesis pathway as in OSR5 or by repressing the synthesis of the photosynthetic complexes, but only in aerobiosis, as in OSR7. An interesting alternative that allows resistance to photooxidative stress is to reduce the light energy arriving at the RC by reducing the amount of the LHI antenna as in OSR8 and OSR9 strains. Probably other strategies have been developed in OSR6, but further experiments are needed to elucidate the mechanisms involved.

Mutagenesis mechanisms induced by photoxidative stress

Several groups have reported that oxidative stress induces the SOS response and other repair systems (Goerlich *et al.*, 1989; Bol and Yasbin, 1990; Farr and Kogoma, 1991; Wang and Humayun, 1996). The SOS response inducing mutagenesis contributes to adaptation processes. In *R.gelatinosus* cells, two types of mutagenesis events have been induced in the absence of carotenoids as photoprotectors. The first type corresponds to the accumulation of new mutations independently of the initial construction, probably as a consequence of the induction of the SOS response (OSR3, OSR6, OSR7, OSR8 and OSR9). The second type corresponds to chromosomic rearrangements involving illegitimate recombination processes, i.e. recombination requiring little or no DNA homology. In the *crtB*$^{-}$ strains, we have observed and characterized two cases of illegitimate recombination. The first one (in OSR1 and OSR2 strains) corresponds to recombination between *crtB* and *crtD* genes. This illegitimate recombination has made a functional chimeric *crtB* gene. The second case corresponds to recombination between the *crtB* gene and the *puf* operon, as the cartridge initially in the *crtB* gene was found after recombination into the *puf* operon (OSR4 strain). However, in this latter case, the chimeric *crtB* gene produced was not functional. Cloning and sequencing of the chimeric *crtB* genes in the three mutants are in progress. This will allow identification of the regions involved in the recombination processes and will give information on the molecular basis of the interactions between non-homologous DNA sequences. Induction of such processes by environmental conditions could play an important biological role in gene and chromosome evolution.

Materials and methods

Bacterial strains, plasmids and growth media

Escherichia coli strains were grown at 37°C on LB medium (Sambrook *et al.*, 1989). *Rubrivivax gelatinosus* strain S1 (Uffen, 1976) and the constructed strains were grown anaerobically (photoheterotrophic conditions) or aerobically (light or darkness) at 32°C in malate (ML) medium (Agalidis *et al.*, 1990). Antibiotics were used at the following concentrations for *E.coli* and *R.gelatinosus*: spectinomycin 50 μg/ml, streptomycin 50 μg/ml, ampicillin 100 μg/ml, kanamycin 50 μg/ml and tetracycline 10 μg/ml. Bacterial strains and plasmids used in this work are listed in Table II.

Gene transfer

Plasmid DNA was introduced into *R.gelatinosus* cells using the electroporation system described in Ouchane *et al.* (1996). Following the electric pulse, cells were diluted in 10 ml of ML medium. After incubation at 32°C for 6 h in darkness, serial cell dilutions were plated on non-selective ML plates to assess cell survival and on selective ML plates to select transformants. Two different antibiotic resistance markers were used to distinguish a double crossover event from a single crossover event, the first one being located on the vector and the second one as the cartridge inserted in the gene to be inactivated.

Membrane isolation and carotenoid extraction: spectrophotometric measurements

Membranes from *R.gelatinosus* were prepared by differential centrifugation after disruption of cells with a French press and were resuspended in 10 mM Tris–HCl pH 8 buffer.

Carotenoids were extracted from membranes with acetone/methanol (7/2 v/v) and separated by TLC in an acetone/petroleum ether (1/9) solvent, as described in Jirsakova and Reiss-Husson (1994). Each carotenoid spot was recovered, eluted from silica by a small volume of acetone and analysed by spectroscopy. Spectral analysis was carried out on a Cary-2300 spectrophotometer interfaced with a computer.

Whole-cell spectra were obtained from cells that were resuspended in 60% sucrose. Fluorescence emission spectra of the extracts were recorded with a home-made fluorescence spectrophotometer (Ajlani *et al.*, 1995).

For estimation of the number of bacteriochlorophylls per RC in the mutant membranes, the bch concentration was determined at 870 nm using an extinction coefficient of 128/mM/cm (Clayton and Clayton, 1981). To estimate the concentration of RC, difference absorption spectra between membranes with and without ferricyanide were obtained. For this estimation, the shift at 800 nm induced by charge separation in the RC with an extinction coefficient of 80/mM/cm was used. This coefficient was deduced from the room temperature photobleaching difference spectrum (P^{+}–P) of *R.sphaeroides* wild-type chromatophores (Wachtveitl *et al.*, 1993). The amplitude of the shift at 800 nm was saturated with 1 mM ferricyanide.

Molecular biology techniques

Standard methods were used, unless otherwise indicated, according to Sambrook *et al.* (1989). Plasmid DNAs were purified using Qiagen columns (Diagen). DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes (Klenow fragment, alkaline phosphatase, T4 DNA ligase) according to the manufacturer's specifications. DNA fragments were analysed on agarose gel, and different restriction fragments were purified using Geneclean kit (Bio. 101). Genomic DNA was purified as described in Ouchane *et al.* (1996). Southern hybridization analysis of genomic DNA was performed as indicated by Amersham, and probes were labelled with [α-^{32}P]CTP by nick translation.

Cloning of the crtB gene and construction of plasmids

To clone the *crtB* carotenoid gene from *R.gelatinosus*, genomic DNA from the SID3 strain (Ouchane *et al.*, 1997) was digested with different restriction endonucleases and ligated. The ligation product was used to transform *E.coli*. Transformants were analysed and several plasmids were obtained. Plasmid pSO22, containing a 5 kb DNA fragment, was digested and resulting fragments were subcloned in Bluescript. Plasmid pSO26, bearing a 2.5 kb DNA fragment containing the *crtB* gene, was sequenced on both strands using the dideoxy chain termination method of Sanger with the Sequenase version 2.0 kit from Amersham.

To inactivate the *crtB* gene, plasmid pSO26 was linearized with *Nru*I and ligated with the *Sma*I Ω cartridge from pDW9 encoding spectinomycin and streptomycin resistance, or the Km cartridge from pUC4K encoding kanamycin resistance, to create pSO40 and PSO41 respectively. Construction of pSO5 has been described previously in Ouchane *et al.* (1996). To construct plasmids pSO50 and pSO51, the (*Sac*I) *crtD–C* operon was cloned in the linearized *Sac*I pBBR1MCS-2. The orientation of the *crtD–C* operon in the plasmids was determined by digestions with appropriate restriction enzymes.

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