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Role for Vitamin B_{12} in Light Induction of Gene Expression in the Bacterium *Myxococcus xanthus*

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A light-inducible promoter (P_B) drives the *carB* operon (carotenoid genes) of the bacterium *Myxococcus xanthus*. A gene encoding a regulator of carotenoid biosynthesis was identified by studying mutant strains carrying a transcriptional fusion to P_B and deletions in three candidate genes. Our results prove that the identified gene, named *carA*, codes for a repressor of the P_B promoter in the dark. They also show that the *carA* gene product does not participate in the light activation of two other promoters connected with carotenoid synthesis or its regulation in *M. xanthus*. CarA is a novel protein consisting of a DNA-binding domain of the family of MerR helix-turn-helix transcriptional regulators, directly joined to a cobalamin-binding domain. In support of this, we report here that the presence of vitamin B_{12} or some other cobalamin derivatives is absolutely required for activation of the P_B promoter by light.

Cells of the gram-negative bacterium *Myxococcus xanthus* respond to blue light by accumulating significant amounts of carotenoids. This response relies on the transcriptional activation of the structural genes for carotenoid synthesis (Fig. 1A) (see reference 17 for a review). All but one of the carotenoid biosynthesis genes are clustered together at the *carB* operon. The exception is gene *crtI*, unlinked to *carB*. Regulatory genes participating in the activation of the *crtI* promoter (P_I) by blue light have been identified. Two of them, *carQ* and *carR*, form part of the *carQRS* operon, the promoter of which (P_{QRS}) is also regulated by blue light. Protein CarQ is a member of the extracytoplasmic function (ECF) subfamily of σ factors. CarR, a membrane-spanning protein, acts as an anti-σ factor, sequestering protein CarQ to the membrane in the dark. Illumination of the cells somehow results in the loss of CarR, leaving CarQ free to activate the P_{QRS} and P_I promoters. These two promoters share two DNA segments, centered at the −31 and −10 positions, which correspond to the binding sites of CarQ. Another protein, CarD, which contains a DNA-binding domain similar to that of eukaryotic high-mobility group A proteins, is required independently for light activation of the P_I and P_{QRS} promoters. The latter also requires the normal action of integration host factor (10, 12, 23, 27).

Less is known on the regulation of the *carB* promoter (P_B), but previous lines of evidence indicated that the mechanism of the action of light is different from the one involved in the activation of P_{QRS} and P_I. On the one hand, the P_B promoter lacks the binding sites for the ECF-σ factor CarQ, showing instead a −35 sequence that perfectly matches the −35 consensus for the major bacterial σ factor (6). On the other hand, the P_B promoter, but not P_{QRS} nor P_I, is affected by mutations at two unlinked genes. One is *carS*, the third gene of the *carQRS* operon mentioned above. The first known *carS* mutation (*carS1*) was identified because it caused constitutive expression from the normally light-inducible P_B promoter (4). Mutation *carS1* is, however, a gain-of-function mutation, as lack of expression of *carS* blocks the activation of P_B by light, whereas expressing *carS* from a heterologous, light-independent promoter produces high expression of P_B in the dark (26). Thus, the *carS* gene product is a positive regulator of P_B.

Mutations affecting the expression of the *carQRS* operon in different ways, such as those in *carQ*, *carR*, or *carD*, affect the activity of P_B in the same way, but that can be explained by the effect of those mutations on the expression of protein CarS. This has been directly proved in the case of *carD* mutations (28). The second locus specifically involved in the regulation of P_B is named *carA*, and it is located directly downstream of the *carB* operon. The *carA* locus was defined by a mutation *carA1*, which results in high, light-independent expression from the P_B promoter (5). The effect of the *carA1* mutation is brought about even when the entire *carQRS* operon is deleted. This confirms that P_B is not driven by the σ factor CarQ and leads to a model in which CarA normally acts as a negative regulator of P_B in the dark, whose action is somehow counteracted by protein CarS in the light (25, 26).

The *carA1* mutation has been sequenced and shown to be a double mutation, which may affect three contiguous genes at the *carA* locus (6) (see Results). Here, we report the generation of *M. xanthus* strains carrying nonpolar deletions within each one of these three genes. This allowed us to identify which of the candidate genes encodes the negative regulator CarA. At the time of publication, no similarity was detected between CarA and other known proteins apart from the presence of a DNA-binding domain at the N terminus of CarA (6). We now report that the amino acid sequence and the predicted structural conformation of the carboxyl half of CarA and of another gene product from the *carA* locus, named Orf11, are strikingly similar to those of the methylcobalamin-binding domain of methionine synthase (9). In accordance with this, we also report here that cyanocobalamin (vitamin B_{12}), or some other cobalamin derivative, is absolutely required for the normal activation of the *carB* promoter by blue light. These results lead to a model in which cobalamin acts as a CarA prosthetic

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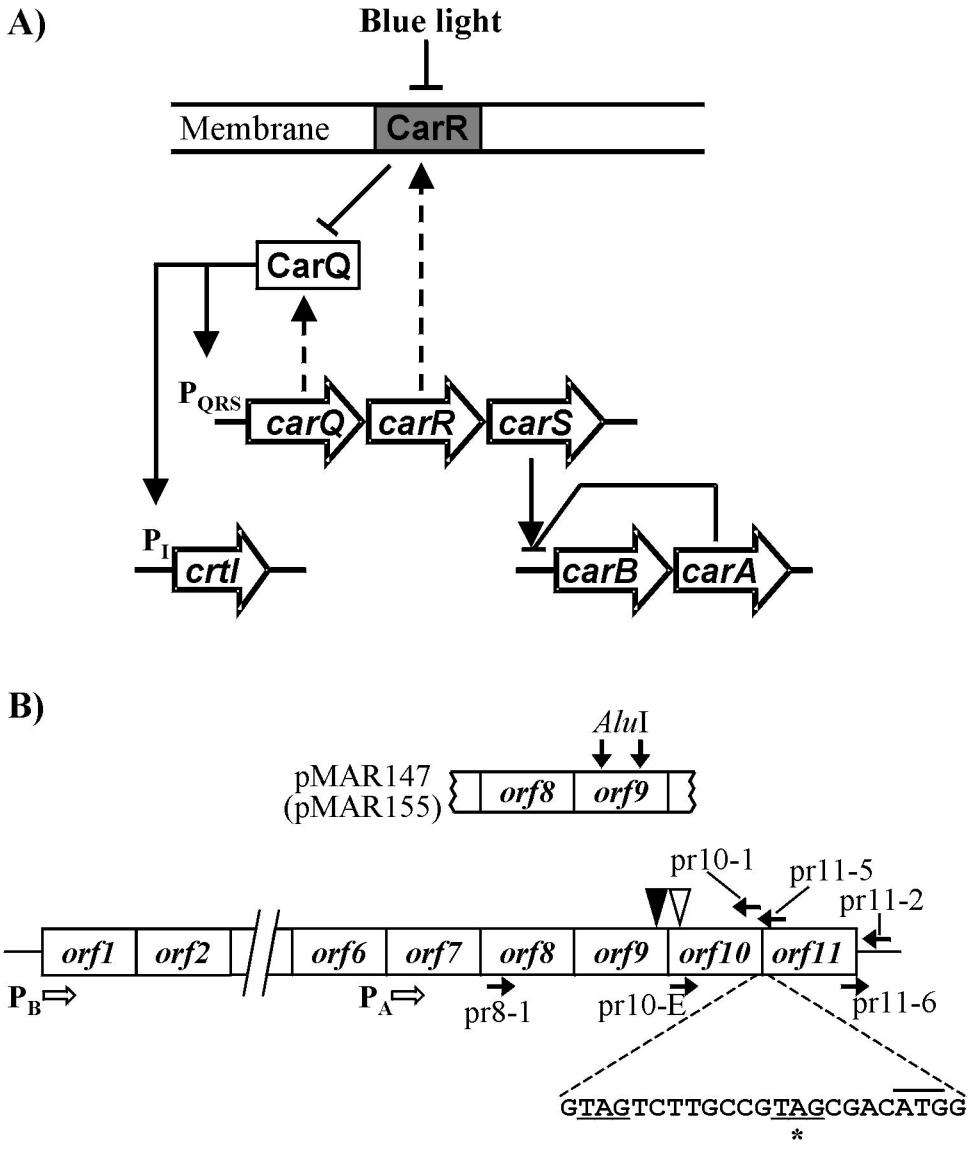

FIG. 1. (A) Light induction of gene expression in *M. xanthus*. Three unlinked loci are represented (*carQRS*, *crtI*, and *carB-carA*). Genes are indicated by big arrows, which also indicate the direction of transcription. Discontinuous arrows connect some genes with their gene products. Continuous arrows, positive regulation; blunt-ended lines, negative regulation (see the introduction). The promoters of the *carQRS* operon (P_{QRS}) and gene *crtI* (P_I) are indicated. The *carB* and *carA* gene clusters (represented by single arrows) are expanded in panel B. (B) *carB*, *carA* gene clusters. Genes are represented by boxes. The *carB* operon contains six open reading frames. The steps for carotenoid synthesis controlled by four of them, named *crtE* (*orf1*), *crtB* (*orf3*), *crtD* (*orf4*), and *crtC* (*orf5*), are known, but not those controlled by *orf2* and *orf6* (6). The *carB* operon is driven by the light-inducible promoter P_B. The *carA* operon contains five genes (*orf7* to *orf11*) and is driven by the light-independent promoter P_A, located within *orf6*. Genes *orf9* and *orf10* are translationally coupled. The positions of the two nucleotide changes found in the *carA1* mutant, a G deletion (solid triangle) and an A→T transversion (open triangle), are shown. Below, the stop codon of *orf10* (underlined) and the start codon of *orf11* (upper line) are shown. Also shown is the −1-shifted stop codon (asterisk) mentioned in Materials and Methods. On top, the 1.77-kb fragment cloned in plasmid pMAR147 is shown, as well as the two *Alu*I sites that were used to generate the *orf9* in-frame deletion (plasmid pMAR155). Horizontal solid arrows indicate the approximate positions of primers used to generate (or check) the *orf10* and *orf11* deletions (Materials and Methods).

group that, in conjunction with protein CarS, mediates the inactivation by light of CarA.

MATERIALS AND METHODS

Bacterial strains, transducing phages, and growth conditions. The *M. xanthus* strains used in this study are listed in Table 1, together with their phenotype, genotype, and origin. The standard strain DK1050 shows normal light-induced synthesis of carotenoids (Car^+ phenotype). Car^- stands for lack of carotenoid synthesis, and Car^c stands for light-independent production of carotenoids. Some strains carried in vitro-constructed *lacZ* fusions that were integrated into the *M. xanthus* chromosome by homologous recombination (see below). The reporter gene retained the normal translation start signal preceded by stop codons in all three reading frames; therefore, it produced transcriptional but not translational fusions. For cloning purposes, *Escherichia coli* strains DH5α (13) and MC1061 (8) were used.

TABLE 1. *M. xanthus* strains used in this study

Strain	Phenotype[a]	Genotype[b]	Reference or source[c]
DK1050	Car^+		31
MR397	Car^+ $LacZ^i$ Km^r	*carQ*::*lacZ* (pDAH217)	28
MR401	Car^- $LacZ^i$ Km^r	*carB*::Tn*5-lac*	4
MR418	Car^- $LacZ^i$ Tc^r	*carB*::Tn*5-132lac*	24
MR553	Car^+ $LacZ^i$ Km^r	*crtI*::*lacZ* (pMAR206)	10
MR819	Car^c Tc^r	*crtI*::Tn*5-132*	M. Fontes
MR844	Car^c	Δ*orf10*	This study
MR845	Car^c $LacZ^i$ Km^r	Δ*orf10 crtI*::*lacZ*	pMAR206 × MR844
MR846	Car^c $LacZ^c$ Km^r	Δ*orf10 carB*::Tn*5-lac*	MR401 × MR844
MR847	Car^c $LacZ^i$ Km^r	Δ*orf10 carQ*::*lacZ*	pDAH217 × MR844
MR848	Car^+	Δ*orf11*	This study
MR856	Car^- $LacZ^i$ Km^r Tc^r	Δ*orf10 carQ*::*lacZ crtI*::Tn*5-132*	MR819 × MR847

[a] $LacZ^i$, light-inducible synthesis of β-galactosidase; $LacZ^c$, high level of β-galactosidase in the dark and in the light.
[b] The integrated plasmid that carries the *lacZ* transcriptional fusion is indicated in parentheses.
[c] Strains MR845 and MR847 were generated by P1-mediated transduction into MR844 of the integrative plasmids pMAR206 (Km^r *crtI*::*lacZ*) and pDAH217 (Km^r *crtQ*::*lacZ*), respectively. Strain MR846 was constructed by generalized transduction into MR844 of the Tn*5-lac* (Km^r) insertion present in strain MR401. Strain MR856 was constructed by generalized transduction into MR847 of the Tn*5-132* (Tc^r) insertion present in strain MR819.

The rich medium CTT (7) and the exact culture conditions for growth of *M. xanthus* in the dark and in the light have been described previously (10). Coliphage P1*clr100*Cm (hereafter called P1) was used to transfer plasmids from *E. coli* strain MC1061 to *M. xanthus* (11). For generalized transduction between *M. xanthus* strains, phage Mx4-LA27 was used. The phages and the conditions used for the transduction of resistance to kanamycin (Km^r) or tetracycline (Tc^r) have been described previously (3). Electroporation was also carried out as described earlier (21).

For the vitamin assays, we used CAA medium, containing 5 mg of Vitamin Assay Casamino Acids (Difco) per ml in 10 mM Tris–1 mM KH_2PO_4-K_2HPO_4–8 mM $MgSO_4$ buffer (pH 7.6). After autoclaving and cooling to 50°C, filter-sterilized solutions were used to add L-asparagine (0.1 mg/ml), sodium pyruvate (1 mg/ml), and sodium citrate (2 mg/ml). The CAA cultures were inoculated by diluting (10,000-fold) a suspension of cells growing exponentially in CTT (2×10^8 to 5×10^8 cells/ml) that had been washed and resuspended in an equal volume of CAA. The cultures were incubated at 33°C in an orbital shaker (300 rpm) until they reached approximately the same cell density as the original inoculum. This point (time zero in Fig. 5) was attained after a time period that varied between 2.5 and 3.5 days. The same variation was observed for all strains tested.

Plasmid and DNA manipulation. Cloning vector pDAH160 (16) carries a Km^r gene and the incompatibility region of P1 for transfer of the plasmid from *E. coli* to *M. xanthus* by P1-specialized transduction. Plasmid pDAH217 contains a *lacZ* transcriptional probe fused to the light-inducible promoter of the *carQRS* operon. Like all other plasmids used here, pDAH217 cannot replicate in *M. xanthus* cells but can integrate into the *M. xanthus* chromosome by homologous recombination. Integration of pDAH217 produces a tandem duplication of the cloned DNA; therefore, a normal copy of the *carQRS* operon is generated (16). Similarly, plasmid pMAR206 contains a *lacZ* transcriptional fusion to the *crtI* promoter (10). Plasmid pMAR976 is a pBJ113 derivative (20) lacking the *Eco*RI restriction site. It contains a positive-negative cassette, with a Km^r gene for positive screening and a galactose sensitivity (Gal^s) gene for negative screening (36). For standard cloning procedures, plasmids pUC19 (29) and pUC9 (37) were used. Nucleic acid and enzymatic manipulations were done according to standard procedures (33).

Strain construction. Plasmids carrying a long deletion within one of three different genes from the *carA* locus (*orf9*, *orf10*, and *orf11*; see Fig. 1B) were generated in the following way.

(i) *orf9* deletion. Plasmid pMAR147 is a pUC19 derivative carrying a 1.77-kb fragment from the *carA* locus that extends from a site within *orf7* to a site within *orf10* (Fig. 1B). The only two *Alu*I sites present in that fragment are located within the coding region of *orf9*, being separated by 243 bp. The 1.77-kb fragment was isolated from pMAR147 after digestion with *Bam*HI and *Hin*dIII (pUC19 polylinker sites) and treated with *Alu*I. The *Bam*HI-*Alu*I and *Hin*dIII-*Alu*I subfragments were isolated, ligated by their *Alu*I ends, and recloned into *Bam*HI- and *Hin*dIII-digested plasmid pDAH160 to generate plasmid pMAR155. The presence in pMAR155 of the expected deletion was first checked by Southern analysis. Sequencing of the appropriate DNA stretch of pMAR155 confirmed that the correct deletion within *orf9* had been generated. The deletion covers the expected 81-codon-long stretch, leaving 59 amino acids from the N terminus and 83 amino acids from the C terminus of the normal *orf9* gene product.

(ii) *orf10* deletion. Plasmid pMAR172 is a pUC19 derivative which carries a *Kpn*I restriction fragment (4.3 kb) from the *carA* locus. This fragment extends from a site within *orf7* to a site located about 1.4 kb downstream of *orf11* (see Fig. 2). A two-step procedure was used in this case to remove an *Rca*I-*Nru*I restriction fragment from pMAR172 to generate the *orf10*-deleted plasmid pMAR173. For clarity, the procedure is depicted in Fig. 2A. The two steps were required because the *Nru*I site was unique, but several *Rca*I restriction sites were present in pMAR172. The deletion started right after the initiation codon of *orf10* and covered 758 of its 864 bp. So, translation of the mutated gene should initiate normally, but will then immediately suffer a −1 nucleotide shift. This would not, however, compromise the normal translation of the downstream gene *orf11*. The shifted translation of the deleted *orf10* will stop at a new (−1 shifted) UAG triplet located between the normal stop codon of *orf10* and the *orf11* initiation codon (see Fig. 1B). The *M. xanthus* DNA present in pMAR173 was recloned into the plasmid vector pDAH160 (*Eco*RI and *Bam*HI double digestion of both plasmids) to generate plasmid pMAR174. The presence in pMAR174 of the expected deletion was checked by sequencing.

(iii) *orf11* deletion. The same 4.3-kb fragment present in pMAR172 (Fig. 2) was cloned into pMAR976 to generate plasmid pMAR188. Using pMAR188 as a template and the appropriate oligonucleotides (pr11-5 and pr11-6; Fig. 1B), inverse PCR was carried out (see below). The oligonucleotides included *Eco*RI overhangs for further digestion and self-ligation of the amplified fragment to generate plasmid pMAR189. This carries a deletion of *orf11*, which extends from the ATG start codon to a position located 22 nucleotides upstream of the stop codon.

To introduce the three indicated deletions into *M. xanthus*, each deleted plasmid was separately transferred (Km^r selection) to the standard strain DK1050. Plasmids pMAR155 and pMAR173 were transferred by P1-mediated transduction, and plasmid pMAR189 was transferred by electroporation. Km^r cells will arise by integration of the incoming plasmid by homologous recombination, so they should carry both the wild-type and the deleted copies of the cloned DNA. Cells carrying only the deleted copy can arise by intramolecular recombination events that remove the plasmid vector and the wild-type version of the corresponding gene (see Fig. 2B for the case of the *orf10* deletion). Those cells were selected in two different ways. For the *orf9* and *orf10* deletions, independent merodiploid transductants were grown for at least 60 generations in the absence of kanamycin, plated for single colonies, and screened for a Car mutant phenotype (see Results). For the *orf11* deletion, merodiploid transductants were grown in the absence of kanamycin and plated in CTT supplemented with 10 mg of galactose per ml. Several Gal^r Km^s colonies were then checked (PCR) for the presence of the expected *orf11* deletion.

PCR. PCR was used to check for the presence of the *orf10* and *orf11* deletions. DNA from the corresponding *M. xanthus* strain (Promega genomic DNA purification kit) was used as a template. Synthetic primers were as follows (see Fig. 1B): pr8-1 (5′-CGCCGCGAAGGCGCGTCGACGGCTCGG-3′) and pr10-1 (5′-AAACTCGAGCTACGGGCGATTCTGAGCGT-3′) were used to detect the *orf10* deletion; and pr10-E (5′-AAAGAATTCATGACGTTGCGCATCCG

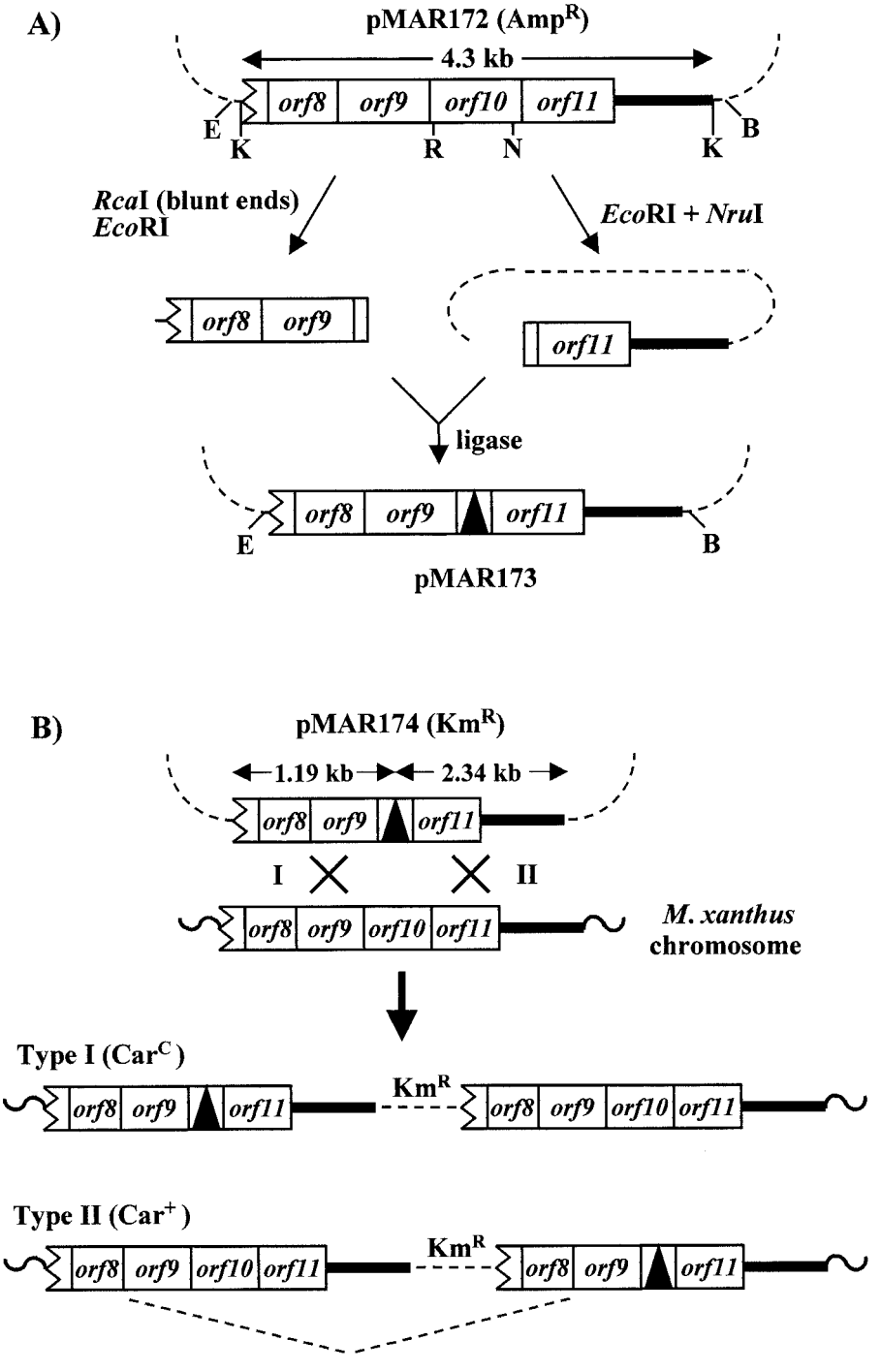

FIG. 2. Deleting the *orf10* gene of *M. xanthus*. (A) Plasmid pMAR172 is a pUC19 derivative which carries the indicated *Kpn*I fragment from the *carA* locus. Only part of the vector DNA is shown. Boxes represent genes from the *carA* operon (see Fig. 1B). A thick line represents DNA downstream of the *carA* operon. The indicated two-step procedure was used to obtain plasmid pMAR173, which carries the Δ*orf10* deletion (see the text). B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; N, *Nru*I; R, *Rca*I. (B) The *Eco*RI-*Bam*HI fragment of pMAR173 was transferred to the plasmid vector pDAH160 (kanamycin resistance) to obtain plasmid pMAR174. The figure shows the two types of merodiploids expected from the integration of pMAR174 into the *M. xanthus* chromosome by a single homologous recombination event to the left (type I) or to the right (type II) of the Δ*orf10* deletion. Indicated in parentheses are the expected phenotypes (Car^C or Car^+) of the merodiploids, assuming that the truncated copy of the *carA* operon lacks a promoter (see Fig. 1B). The discontinuous line shown below represents an intramolecular recombination event that will generate a plasmid-free strain carrying the Δ*orf10* deletion.

CAC-3′) and pr11-2 (5′-GTCTTCAATCACTCCGATATCAACACGCCCTGA C-3′) were used to detected the *orf11* deletion. Polymerase mix and reaction buffer were from Roche Molecular Biochemicals. The samples were subjected to 30 cycles of denaturation (45 s, 95°C), primer hybridization (60 s, 65°C), and polymerization (2 min, 72°C).

Inverse PCR (18) was used to obtain the *orf11* deletion. Plasmid pMAR188 (see above) was used as a template. The following synthetic oligonucleotides were used as primers (see Fig. 1B): pr11-5 (5′-AAAGGAATTCGTCGCTAGC GCAAGACTACG-3′) and pr11-6 (5′-AAAGGAATTCTGGGACCGCCTCGC GGGTAC-3′). The sample was subjected to 18 cycles of denaturation (30 s, 95°C), primer hybridization (60 s, 62°C), and polymerization (9 min, 68°C). The Expand long template PCR system from Roche Molecular Biochemicals was used.

Protein sequence analysis. For comparison with databases and multiple protein alignments, the Blast and PIMA1.4 programs provided by the BCM search launcher were used (http://dot.imgen.bcm.tmc.edu/1) (34). For protein secondary-structure predictions, the nearest-neighbor prediction method (32) provided by the same launcher was used.

Expression of β-galactosidase. Rapid determination of β-galactosidase production was carried out by examining colony color on plates containing 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Quantitative analysis of β-galactosidase of dark-or light-grown liquid cultures was performed as previously described (5). At least three independent determinations were done in all cases.

RESULTS

A deletion within *orf10* but not within *orf9* or *orf11* causes light-independent expression from the *carB* promoter. The *carB-carA* gene cluster from wild-type *M. xanthus* as well as the *carA* locus from the *carA1* mutant strain have been cloned and sequenced. The cluster contains 11 different open reading frames (ORFs), named here, for simplicity, *orf1* to *orf11* (Fig. 1B). The first six ORFs are under the control of the light-inducible promoter P_B. The rest are driven by a light-independent promoter located within *orf6* (6; our unpublished results). When compared with the wild type, two nucleotide changes, very close to each other, were detected in the *carA1* mutant (6) (Fig. 1B). One is a single-nucleotide deletion, located 18 nucleotides upstream of the *orf9* stop codon. This mutation should result in the modification of the last six amino acids of Orf9 and in a 76-residue-long extension of its carboxyl end, due to the (frameshifted) reading through of gene *orf10*.

Genes *orf9* and *orf10* are translationally coupled, and gene *orf11* lacks the consensus sequence for a ribosome-binding site (6), so the *orf9* frameshift mutation may be producing a polar effect by affecting the correct translation of both *orf10* and *orf11*. It should be said that the predicted amino acid sequences of proteins Orf10 and Orf11 show an overall identity of 35.2%, with several regions which are particularly well conserved (6) (see below). The second mutation found in the *carA1* mutant is a transversion at the fifth codon of *orf10*, which causes an Ile-Phe substitution. The N terminus of Orf10 contains a helix-turn-helix (HTH) DNA-binding domain similar to that found in the MerR family of transcriptional regulators (6). The aforementioned Ile is one of the conserved residues in the HTH domain. This complex situation made it difficult to assign to a particular gene the effect of the *carA1* mutation on the P_B promoter (6). To clarify this point, we have now generated *M. xanthus* strains carrying long, nonpolar deletions within each of the candidate genes (see Materials and Methods).

To generate the *orf9*-deleted strain, Km^r plasmid pMAR155, which carries an in-frame deletion within *orf9* (Δ*orf9*, Fig. 1B), was transduced into wild-type strain DK1050. Transductants should arise by integration of the plasmid into the *M. xanthus* chromosome by homologous recombination. All the Km^r merodiploid transductants developed an intense orange color in the dark, indicative of light-independent accumulation of carotenoids (Car^C phenotype). In the light, the orange colonies turned more reddish. Intramolecular recombination between the directed repeat generated by the integration of pMAR155 would result in loss of plasmid DNA and the normal or deleted copy of *orf9*, according to whether the recombination event takes place at one or the other side of Δ*orf9*.

Several of the Km^r Car^C merodiploids were grown separately in the absence of kanamycin, plated for single colonies, and checked for a change in phenotype. Colonies were found from all cultures which showed either a Car^+ Km^s or a Car^- Km^s phenotype. Southern analysis (not shown) demonstrated that the Car^+ Km^s cells contained a single, wild-type copy of the *carA* operon, whereas the Car^- Km^s cells contained a single copy of the *carA* operon carrying the Δ*orf9* deletion. This proves that the *orf9* gene product is not the negative regulator of P_B. Further analyses have shown that the Car^- phenotype caused by the Δ*orf9* deletion is due to a negative effect of this mutation on the activation of the *carQRS* operon by blue light (to be described elsewhere). As shown in Fig. 1B, the *M. xanthus* DNA present in pMAR155 is truncated at the *orf10* gene. Therefore, all the Km^r merodiploids from the pMAR155 × DK1050 transduction were also truncated at the *orf10* gene. The Car^C phenotype of all those transductants suggested that *orf10* is the negative regulator of the P_B promoter.

A procedure similar to that described above was used to introduce the *orf10* deletion (Δ*orf10*) in *M. xanthus* (Fig. 2B). First, plasmid pMAR174 (Km^r) was transferred to wild-type strain DK1050. Two classes of Km^r merodiploids were expected, depending on whether integration of the plasmid had occurred to the left or to the right of the Δ*orf10* site. In the first case (type I in Fig. 2B), the Δ*orf10* deletion will replace the endogenous *orf10* allele, this being displaced to the truncated copy of the *carA* operon. This copy lacks a promoter, located within *orf6* (Fig. 1B), so the type I merodiploids might be deficient in protein Orf10. The reverse situation is expected in the second class of merodiploids, which should carry a normal version of the entire *carA* locus (type II in Fig. 2B). Given the length of the DNA stretches available for homologous recombination at the sides of Δ*orf10*, the type II merodiploids were expected to be the most frequent.

More than 100 independent transductant colonies were checked. About 80% of them showed a normal (Car^+) phenotype. The remaining 20% showed the Car^C phenotype described above for the *orf9* merodiploids. Therefore, one might conclude that the class I transductants are those showing a Car^C phenotype and that the *orf10* gene product is the one responsible for repressing the P_B promoter in the dark. As mentioned above, intramolecular recombination events would result in the removal of both the plasmid vector and either the wild-type or the deleted version of the *orf10* gene. Four of the Car^+ merodiploids were grown in the absence of kanamycin and plated for a Car^C phenotype. Colonies showing that phenotype were obtained from all cultures, and all of them proved to be sensitive to kanamycin (Km^s).

The presence of a single copy of the *carA* operon carrying the expected Δ*orf10* deletion was confirmed by PCR analysis.

TABLE 2. Effects of deletions Δ*orf10* and Δ*orf11* on the light-inducible promoters

Host strain (genotype)	Transcriptional fusion	Mean β-galactosidase sp act[a] ± SD		
		Dark		Light, 8 h
		0 h	8 h	
DK1050 (wild type)	P_B::*lacZ*	30 ± 4	34 ± 3	186 ± 16
MR844 (Δ*orf10*)	P_B::*lacZ*	158 ± 14	174 ± 15	188 ± 16
MR848 (Δ*orf11*)	P_B::*lacZ*	11 ± 1	12 ± 2	144 ± 14
DK1050 (wild type)	P_I::*lacZ*	14 ± 1	15 ± 1	439 ± 48
MR844 (Δ*orf10*)	P_I::*lacZ*	18 ± 2	17 ± 2	371 ± 39
DK1050 (wild type)	P_{ORS}::*lacZ*	8 ± 1	6 ± 1	218 ± 19
MR844 (Δ*orf10*)	P_{ORS}::*lacZ*	9 ± 1	9 ± 4	23 ± 6
MR856 (Δ*orf10 crtI*)	P_{ORS}::*lacZ*	7 ± 1	8 ± 1	163 ± 17

[a] At time zero, cell samples growing exponentially in the dark (CTT medium) were divided in two, one to be kept in the dark and the other to be exposed to light. Strains carrying a P_I::*lacZ* fusion were at the late exponential phase, as entry into the stationary phase is required for activation of the P_I promoter by blue light (10). The same was true for the strains carrying a P_{ORS}::*lacZ* fusion (see the text). Samples for β-galactosidase assay were taken at time zero and after 8 h of additional growth in the indicated conditions. Means and standard deviations are given; units are as defined for Fig. 5.

For this, we used DNA from several independent Car^C Km^s colonies and the oligonucleotides pr8-1 and pr10-1 (Fig. 1B) (results not shown). The Car^C Km^s strain was named MR844. The *orf10* deletion moves the *orf10* stop codon closer to the *orf11* start codon (see Materials and Methods). The possibility that the Car^C phenotype of MR844 stems from an unusual polar effect of Δ*orf10* on *orf11* was ruled out, as deleting *orf11* did not affect the normal response to blue light (see below).

M. xanthus strain MR401 carries a Tn*5*::*lac* (Km^r) insertion at the *carB* operon that generates a *lacZ* transcriptional fusion to the P_B promoter (5) (Table 1). We tested the effect of the Δ*orf10* mutation on the activity of P_B by transducing the indicated insertion (selection for Km^r) into strain MR844. Quantitative analysis of *lacZ* expression confirmed that Δ*orf10* causes high expression in the dark from the normally light-inducible promoter P_B (Table 2).

Plasmid-based *lacZ* fusions to the P_{ORS} and P_I promoters (Fig. 1A) are available. Integration of the plasmids into the *M. xanthus* chromosome by homologous recombination generates *lacZ* transcriptional fusions, leaving an intact copy of the corresponding gene or operon. Plasmid pMAR206 (Km^r P_I::*lacZ*; Table 1) was transferred to strain MR844 by P1-mediated transduction and selection for Km^r. On X-Gal plates, all the transductants behaved like a wild-type derivative carrying the same *lacZ* fusion (a pale blue color in the dark that turned intense blue in the light). The results of β-galactosidase assays confirmed that mutation Δ*orf10* does not affect the normal regulation of P_I (Table 2).

An experiment similar to the one just described was done using plasmid pDAH217 (Km^r P_{ORS}::*lacZ*; Table 1). In the quantitative analysis of *lacZ* expression, we noticed that the activation of the P_{ORS} promoter in the MR844-derived transductants was similar to that of the control sample only when the cells were illuminated at the early exponential phase (not shown). When illumination was at the late exponential phase, the activation of P_{ORS} was much lower in the MR844-derived transductants (Table 2). It is known that the activation of P_{ORS} by light decreases with the progressive accumulation of colored carotenoids (16). Since Δ*orf10* caused light-independent carotenoid synthesis, the reduced activation of P_{ORS} might be an indirect consequence of the progressive accumulation of carotenoids during growth in the dark.

To avoid this interference, a transposon insertion in the *crtI* gene was used. The *crtI* gene codes for an enzyme acting at an early step of the carotenoid pathway (10). The inserted transposon, a tetracycline resistance (Tc^r) derivative of Tn*5*, did not affect the light activation of P_{ORS} (not shown). The Tc^r transposon was transduced into the strain carrying the Δ*orf10* deletion and the P_{ORS}::*lacZ* fusion to obtain the Car^- strain MR856. The behavior of all transductants on X-Gal plates and the results of β-galactosidase assays confirmed that mutation Δ*orf10* by itself does not have a significant effect on the normal regulation of P_{ORS} (Table 2).

An experiment similar to the ones described above was attempted to generate an *M. xanthus* strain carrying an *orf11* deletion. In this case, all the merodiploid transductants were Car^+. This indicated that *orf11* does not play a critical role in light induction of gene expression in *M. xanthus*, but it also made it difficult to screen for cells having a single copy of the *carA* locus mutated at the *orf11* gene. We then took advantage of plasmid pMAR976, which carries a positive-negative cassette (see Materials an Methods). As expected, the DK1050-derived strain, which carried a single copy of the *carA* locus with the *orf11* deletion, named MR848, showed a Car^+ phenotype. This clearly indicated that *orf11* does not play a critical role in the normal regulation of the P_B promoter or the other promoters known to participate in the carotenogenic response of *M. xanthus*. This was confirmed for P_B by generating the same Δ*orf11* deletion in *M. xanthus* strain MR418, which carries a *lacZ* fusion to the indicated promoter (Table 1). The β-galactosidase expression data are shown in Table 2.

Cobalamin-binding domain in the *orf10 and orf11* gene products. At the time the predicted amino acid sequences of Orf10 and Orf11 were published, we noticed the overall similarity of both sequences and the presence of a helix-turn-helix DNA-binding domain at their N termini (Fig. 3A). No other similarities to the then-known protein domains were noticed (6). In later, computer-aided searches, we found that both Orf10 and Orf11 contained a cobalamin-binding domain. The crystal structure of a methylcobalamin-containing fragment of

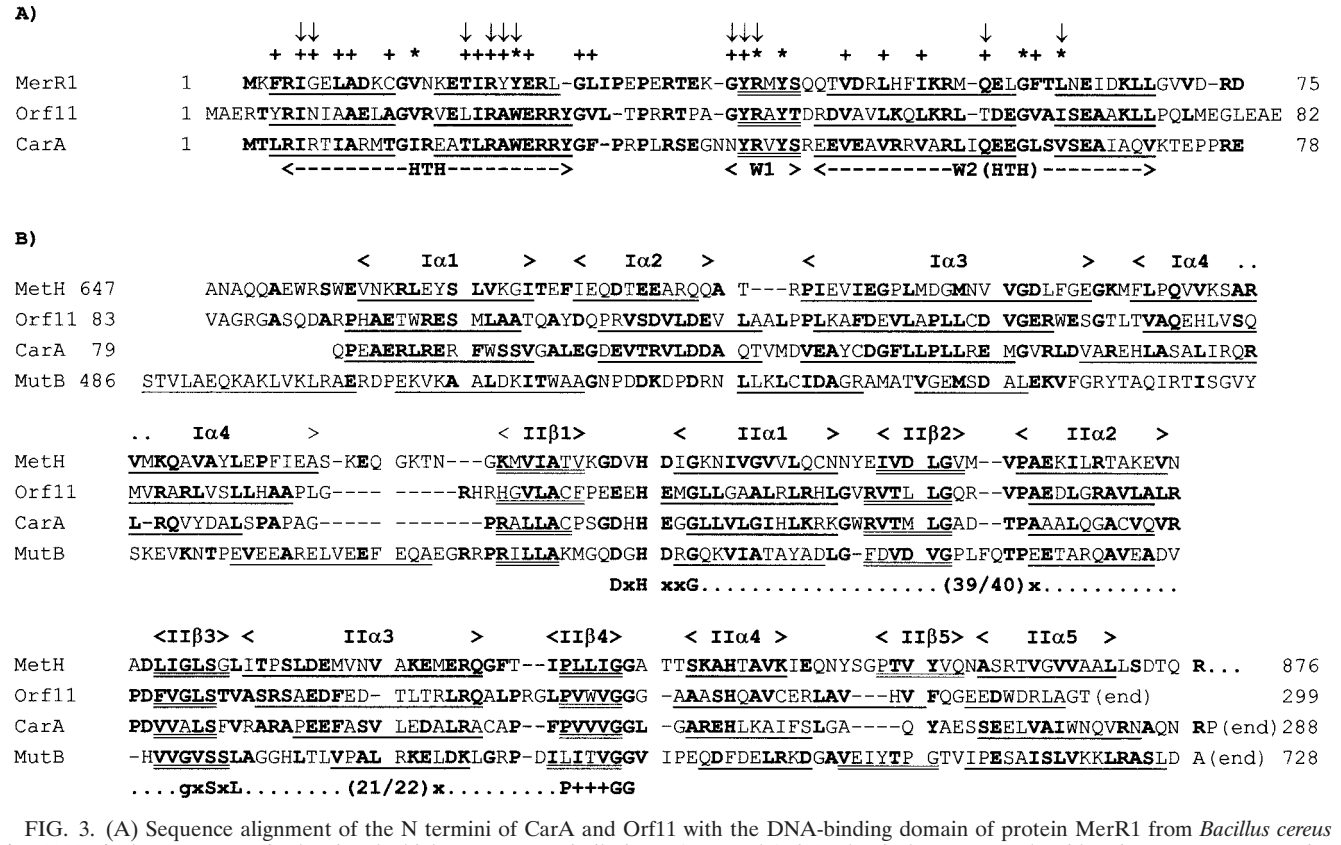

FIG. 3. (A) Sequence alignment of the N termini of CarA and Orf11 with the DNA-binding domain of protein MerR1 from *Bacillus cereus* (AF138877), the MerR protein showing the highest sequence similarity to CarA and Orf11. Identical or conserved residues in two or more proteins are in boldface. An HTH motif (α-helices are underlined) and two "wings" (W1 and W2) form the DNA-binding domain. W1 is a β-strand (doubly underlined), and W2 is another HTH motif. The asterisks and the + symbols indicate invariant and conserved residues, respectively, in 11 other members of the MerR family; the arrows point to positions that contact DNA, according to crystallographic data for one member of the family (reference 38 and Fig. 2 of the Supplementary Information in this reference). (B) Sequence alignment of the C termini of CarA and Orf11, the methylcobalamin-binding domain of methionine synthase from *E. coli* (MetH), and the adenosylcobalamin-binding domain of methylmalonyl-coenzyme A mutase from *Propionibacterium shermanii* (MutB). Identical or conserved residues in two or more proteins are in boldface. Notice that in a few positions, two proteins share a particular amino acid, and the other two proteins share a different one. The structural elements indicated on top of the alignment correspond to those in Fig. 4. The α-helices (underlined) and β-sheets (doubly underlined) of MetH and MutB are from crystallographic studies (9, 22). Those of CarA and Orf11 are computer-aided predictions (32). The cobalamin-binding sequence fingerprint proposed by Drennan et al. (9) is shown below the alignment (x indicates any amino acid). Added to the fingerprint line are a proline followed by three hydrophobic residues (+ symbols), which are much conserved in methylcobalamin-binding domains (see entry pfam02310 of the Pfam data bank at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). (Note: amino acid residues A_{271}, A_{272}, and A_{273} in Orf11 rectify the previously incorrect G_{271}, R_{272}, and R_{273}; M. Elías, personal communication).

the *E. coli* methionine synthase (MetH) has been solved (9) (Fig. 4). The structure revealed an amino-terminal domain, formed by two pairs of antiparallel helices (domain I), and an α/β carboxyl-terminal domain (domain II).

Sequence comparison with other methylcobalamin- and adenosylcobalamin-dependent enzymes (9) allowed Drennan et al. to propose a sequence fingerprint from domain II: Dx-HxxG-(39/40)x-gxSxL-(25/26)x-GG, where x is any amino acid. The conserved residues included in this fingerprint are critical for the binding to or the reactivity of the prosthetic group. As shown in Fig. 3B, the amino acid sequences of both Orf10 and Orf11 are predicted to generate the helical bundle and the α/β region that form, respectively, domains I and II of a cobalamin-binding peptide. The indicated sequence fingerprint is also present, at the right position, in both proteins. Moreover, 51% of the Orf10 residues located between the predicted structural elements IIβ1 and IIβ4 are identical or similar to those found at the same positions in MetH from various bacterial species (45% for Orf11) (entry pfam02310 in the Pfam Data Bank; see the legend to Fig. 3).

Vitamin B_{12} is required for normal activation of the *carB* promoter by blue light. The role of protein Orf10 in the regulation of the P_B promoter and the presence in that protein of a cobalamin-binding domain prompted us to test the effect of cobalamin on the activation of P_B by light. Three different cobalamin derivatives, cyanocobalamin (vitamin B_{12}), methylcobalamin, and adenosylcobalamin (coenzyme B_{12}), were tested. The data reported are those obtained with vitamin B_{12}, but similar results were observed when either of the other two compounds was used.

In all previous works on light induction of gene expression in *M. xanthus*, the standard rich medium CTT, which supports fast growth of the cells, had been used. In preliminary experiments using strain MR401 (P_B::*lacZ* in a wild-type back-

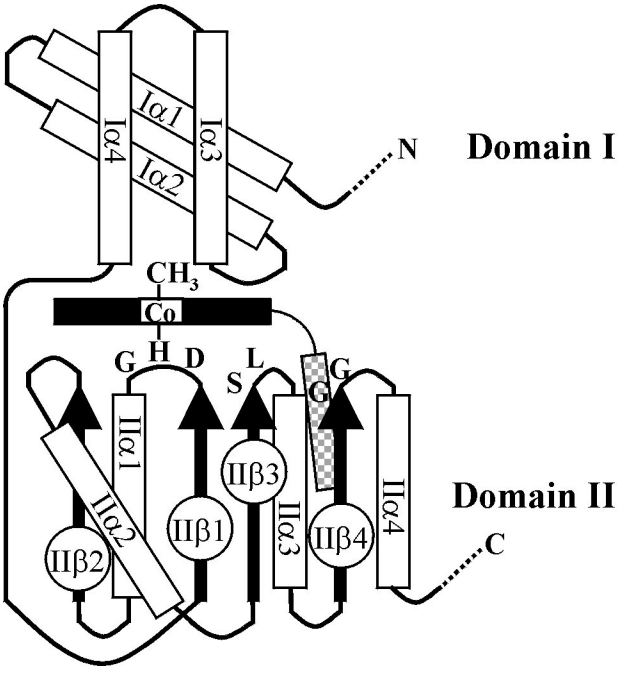

FIG. 4. Topology diagram of the methylcobalamin-binding domain of methionine synthase from *E. coli*. Domain I, formed by two pairs of antiparallel helices (Iα1 to Iα4), contacts the upper phase of the corrin macrocycle (represented by a black rectangle with a central cobalt atom). The α/β domain II (IIβ1 to IIα4) contacts the lower phase of the corrin macrocycle and accommodates the dimethylbenzimidazole nucleotide tail (shaded rectangle). The invariant residues that form a structure-based sequence fingerprint for cobalamin binding are indicated. Domain II is folded in such a way that the serine and the three glycines line and pack the nucleotide tail. The histidine residue is the cobalt lower ligand, which is adjoined by invariant leucine and aspartate residues. The aspartate is part of a hydrogen-bonded proton pathway from solvent to the buried histidine (adapted from Fig. 3 of reference 9).

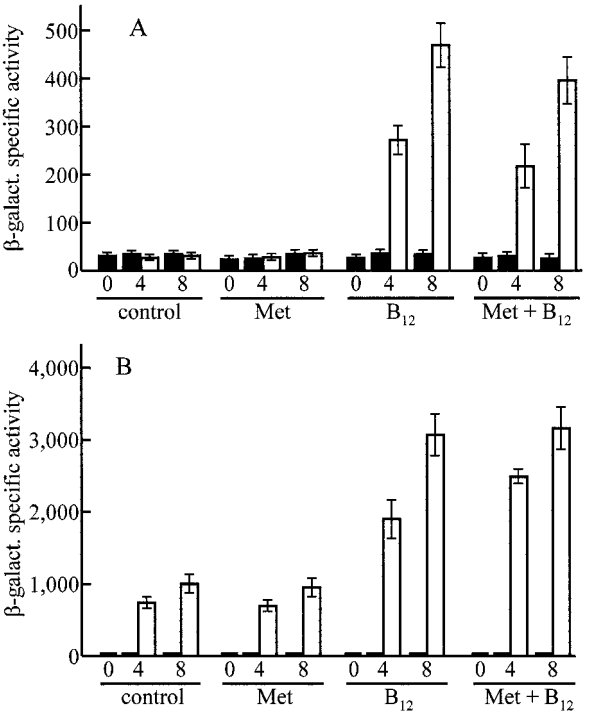

FIG. 5. Effect of B_{12} on activation of P_B and P_{ORS} promoters by light. Strains MR401 (P_B::*lacZ*, panel A) and MR397 (P_{ORS}::*lacZ*, panel B) were grown separately in the dark on vitamin-free CAA medium (control) or CAA supplemented with 100 μg of methionine (Met) or 0.5 μg of cyanocobalamin (B_{12}) or both (Met + B_{12}) per ml. At time zero (exponential phase), each culture was divided in two, one for the dark and the other for the light. Samples for β-galactosidase assays were taken at time zero and after 4 and 8 h of additional growth in the dark (solid bars) or the light (open bars). Enzyme specific activities are given in nanomoles of *o*-nitrophenol produced per minute per milligram of protein. Bars indicate standard deviations.

ground), we checked the effect of adding vitamin B_{12} to CTT on the activation of P_B by light. The vitamin did not affect the low activity of P_B in the dark, but it did stimulate the activation of that promoter by light. A reproducible effect was observed at a vitamin concentration as low as 50 ng/ml. The effect reached a plateau, a twofold increase in β-galactosidase specific activity with respect to the control, at about 500 ng of the vitamin per ml (not shown).

CTT medium contains an undetermined amount of vitamins. For better quantification of the effect of vitamin B_{12}, a culture medium based on a vitamin-free mixture of amino acids (CAA) was used. It should be noted that *M. xanthus* requires vitamin B_{12} for growth only in the absence of methionine (7). This amino acid is present in the CAA medium, but to avoid a possible nutritional effect of the vitamin, control samples specifically supplemented with methionine were included in the experiments. As shown in Fig. 5A, activation of the P_B promoter by light was negligible in the absence of vitamin B_{12}. A very intense activation was observed, however, in the presence of the vitamin. The addition of methionine produced no significant effect.

The product of *carS*, one of the genes of the light-inducible *carQRS* operon, acts as a positive element for the activation of P_B by light (see the introduction). To test whether the lack of response of P_B to illumination in the absence of vitamin B_{12} was the consequence of a primary effect of the vitamin on the P_{ORS} promoter, an experiment identical to the one described above was done with the wild-type strain carrying the P_{ORS}::*lacZ* fusion. Vitamin B_{12} was not required for the activation of P_{ORS}, although it stimulated the response of that promoter to illumination (Fig. 5B).

DISCUSSION

In wild-type *M. xanthus*, the accumulation of carotenoids is absolutely dependent on the activation of the single gene *crtI* and the *carB* operon by blue light. The *carA* gene of this bacterium was originally defined as the one impaired in a mutant strain that accumulated slightly colored, partially dehydrogenated carotenoids in the dark. In the light, the mutant accumulated the same, fully dehydrogenated carotenoids as the wild type grown in the same conditions. The mutation, named *carA1*, was found to cause constitutive expression from the normally light-inducible promoter of the *carB* operon (P_B) (5, 24, 25). This explains the phenotype of the *carA1* mutant, as the *carB* operon contains six genes responsible for the synthesis of phytoene, the first carotene precursor, as well as for the partial dehydrogenation of phytoene and other steps down-

stream in the pathway. The accumulation of fully dehydrogenated carotenoids requires the *carA*-independent activation of the *crtI* gene (6, 10) (Fig. 1A).

Sequence analysis had revealed that *carA1* is in fact a double mutation that could affect more than one gene. Here we have shown that a deletion affecting one of the candidate genes (*orf10* in Fig. 1B) reproduces exactly the known effects of mutation *carA1*, both on the Car phenotype and on the activity of the P_B promoter. Therefore, we retained the original *carA* designation for *orf10*. The previous proposal that protein CarA is a negative regulator of P_B in the dark (see the introduction) was based on the simple but unproven assumption that *carA1* was a lack-of-function mutation. Now, our results with the Δ*orf10* deletion confirm that CarA normally acts as a negative regulator of the P_B promoter in the dark. They also confirm that CarA does not participate directly in the regulation of the other light-inducible promoters known in *M. xanthus*, P_{QRS} and P_I.

Downstream of *carA*, a gene was found (*orf11*) which was predicted to generate a protein strikingly similar to CarA (Fig. 3). Thus, *carA* and *orf11* may have originated from the duplication of a common ancestral gene. Deleting the *orf11* gene produced no effect on the regulation of the P_B promoter, nor did it affect the normal accumulation of carotenoids by *M. xanthus* cells in the light. The function of the *orf11* gene is presently unknown.

The sequence and the structural elements predicted for the N termini of CarA and Orf11 closely resemble the DNA-binding domain found at the N termini of the MerR-like proteins (Fig. 3A). This is a family of bacterial transcriptional activators that regulate the response to stress arising from exposure to toxic compounds or oxygen free radicals. The nonhomologous carboxy-terminal domain of the MerR proteins contains the binding site for the coactivator-toxic compound or a redox [2Fe-2S] center, as in the case of the SoxR protein from *E. coli* (2, 14, 35). Binding to the coactivator or oxidation of the redox center converts the MerR proteins to active forms.

Many lines of evidence, including data on the crystal structure of a member of the family bound to DNA, indicate that MerR proteins activate transcription initiation by remodeling the unusually long (19 bp) spacer found between the −35 and −10 promoter elements (38 and references therein). CarA is not an activator, but probably acts as a truly classical bacterial repressor. This unusual behavior of CarA as a MerR-like protein might be related to the particular position of its binding site relative to the promoter or to certain structural peculiarities of the protein. For example, the MerR proteins contain an 11-turn-long α-helix that separates the DNA-binding region from the coactivator-binding domain (38). This long linker region is absent in CarA, whose DNA-binding domain is immediately linked to the cofactor-binding domain.

As shown in Fig. 3, CarA is predicted to contain a cobalamin-binding domain that is separated from its DNA-binding region by only a few amino acids. Cobalamin is a complex prosthetic group formed by a heme-like corrin macrocycle linked to the nucleotide dimethylbenzimidazole (15). The crystal structure of the methylcobalamin-binding domain of protein MetH from *E. coli* reveals the corrin macrocycle lying between an amino-terminal domain (domain I), formed by two pairs of antiparallel helices, and an α/β carboxyl-terminal domain (domain II). A bond to a conserved histidine residue replaces the lower axial bond of dimethylbenzimidazole to the cobalt which is found in free methylcobalamin. As a result, the nucleotide is displaced and extended as a tail that penetrates into a deep pocket of domain II (9) (Fig. 4).

The crystal structure of the adenosylcobalamin-binding domain of methylmalonyl-coenzyme A mutase (MutB) has also been solved (22). Not many residues are conserved, but extensive structural similarities exist between the cobalamin-binding domains of MetH and MutB (Fig. 3B). In particular, MutB contains an α/β domain whose tertiary structure superimposes very well on domain II of MetH (22). In this domain, the best-conserved residues are the aforementioned histidine and several other residues that play critical roles in the cobalamin-protein functional interaction (Fig. 4). CarA contains all but one of the conserved residues, at the right spacing and within the expected structural elements (Fig. 3 and 4). The one exception is a conservative leucine-to-valine substitution. A second conservative substitution, aspartic to glutamic acid, is found in Orf11 (Fig. 3B). The four-helix domain I of MetH interacts with the upper face of the methylcobalamin molecule (9). The motif used by MutB to interact with the upper face of adenosylcobalamin shows no sequence or structural similarity to the MetH domain I (22) (Fig. 3B). CarA is predicted to form a four-helix bundle with a spacing similar to that of the MetH domain I.

Cobalamin is absolutely required for the light activation of P_B, the *M. xanthus* promoter regulated by CarA. That requirement is not observed for the CarA-independent, light-inducible promoter P_{QRS}, although cobalamin increases the effect of light on this promoter (Fig. 5). This increase is likely to be an indirect consequence of the effect of cobalamin on the P_B promoter. We have data showing that transcription from P_B goes through the *carA* operon and that the gene *orf9* from this operon (Fig. 1B) is positively required for the activation of P_{QRS} by light (unpublished data).

The concurrence of structural and physiological data leads to a model in which cobalamin acts as a prosthetic group of CarA that mediates a light-activated on-off shift of this protein as a repressor of the P_B promoter. This process will depend on protein CarS in an as yet unknown way. As already mentioned (see the introduction), the overexpression of CarS counteracts the negative action of CarA on P_B in the dark, and the same effect is produced by the gain-of-function mutation *carS1*, which does not affect the low expression of *carS* in the dark. The model reinforces the versatility of the MerR-like DNA-binding domain, regarding the cofactor-binding domains with which they have become successfully associated during evolution (38). More interestingly, it points to a rather surprisingly novel capacity of cobalamin, that of acting as a cofactor of regulatory, DNA-binding proteins. To our knowledge, this is the first cobalamin-binding protein known to regulate gene expression. A search of the data banks for a CarA-like product, one having a cobalamin- and a DNA-binding domain in the same molecule, was not fruitful.

The precise mechanism of action of cobalamin in the light-mediated inactivation of CarA is an open question. Three cobalamin derivatives have been effective in our tests. This might suggest that the breaking of a specific cobalt-carbon bond is not involved, contrary to what happens in the well-known cobalamin-dependent enzymatic reactions (9, 22). Very little is known about the metabolism of the cobalamins in *M.*

xanthus, although it is known that it can convert vitamin B_{12} into methylcobalamin (7). Other interconversions of the different cobalamins within the *M. xanthus* cells cannot be discarded. A distinctive property of methylcobalamin is particularly propitious for the light-driven inactivation of an associated protein. The cob(I)alamin form of MetH can suffer a photolytic conversion to an oxidized form, cob(II)alamin, that renders the enzyme inactive. Interestingly, the enzyme-bound cob(I)alamin but not other forms of protein-bound cobalamins exhibits strong blue light absorbance, with a maximum near 400 nm (19 and references therein).

Current work in our laboratory is aimed at delimiting the *carB* promoter and purifying the CarA and CarS proteins. This will allow in vitro studies that should bring forth new insights into the novel action of the cobalamins that we have uncovered here.

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ADDENDUM IN PROOF

While this paper was under review, we confirmed DNA binding by CarA to an operator site in P_B and that CarS abrogates this by interacting with the CarA DNA-binding domain. We also showed that the cobalamin-binding domain is involved in CarA-CarA interaction (J. J. López-Rubio, M. Elías-Arnanz, S. Padmanabhan, and F. J. Murillo, J. Biol. Chem. **277:**7262–7270, 2002). Another recent study has also confirmed CarA-CarS interaction and its effect on CarA-DNA binding, but varies from ours in, among others, the details of the protein-DNA and protein-protein interactions (D. E. Whitworth and D. A. Hodgson, Mol. Microbiol. **42:**809–819, 2001).

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