A Repressor-Antirepressor Pair Links Two Loci Controlling Light-induced Carotenogenesis in *Myxococcus xanthus*

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The light-inducible *carB* operon encodes all but one of the structural genes for carotenogenesis in *Myxococcus xanthus*. It is transcriptionally controlled by two proteins expressed from two unlinked genetic loci: CarS from the light-inducible *carQRS* operon, and CarA from the light-independent *carA* operon. CarA represses transcription from the *carB* promoter (P\(_{\text{B}}\)) in the dark, and CarS counteracts this on illumination. The CarA sequence revealed a helix-turn-helix DNA-binding motif of the type found in bacterial MerR transcriptional factors, whereas CarS contains no known DNA-binding motif. Here, we examine the molecular interplay between CarA and CarS. We demonstrate the following: (i) Whereas CarS exhibits no DNA binding *in vitro*, CarA binds specifically to a region encompassing P\(_{\text{B}}\) to form at least two distinct complexes. (ii) A palindrome located between positions −46 and −63 relative to the transcription start point is essential but not sufficient for the formation of the two CarA-DNA complexes observed. (iii) CarS abrogates the specific DNA binding of CarA. CarA is therefore a repressor and CarS an antirepressor. (iv) CarS physically interacts with CarA; thus, the functional interaction between them is mediated by protein-protein interactions.

One of various cellular responses to blue light is the induction of the synthesis of carotenoids. These protect cells against photo-oxidative damage by quenching singlet oxygen and other free radicals produced on illumination (1, 2). The Gram-negative bacterium *Myxococcus xanthus* is a model prokaryotic system for investigating how blue light switches on the network of cellular activities leading to carotenoid synthesis (3). Genetic analyses have revealed a number of regulatory and structural genes involved in this response (Ref. 4; see Fig. 1). One enzyme involved in carotenoid synthesis is encoded by gene *crtI*, and all the rest by the unlinked *carB* operon (5, 6). Photoinduction of these structural genes is mediated by at least six regulatory genes as follows: the *carQ*, *carR*, and *carS* gene cluster and the unlinked *carD*, *ihfA*, and *carA* genes.

Transcriptional activation of *crtI* is mediated by the extra-cytoplasmic function-σ factor CarQ (7–9) and by CarD, a multifunctional transcriptional factor of considerable resemblance to eukaryotic high mobility group A proteins (10–12). Light up-regulates *crtI* expression by triggering the liberation of CarQ from CarR, a membrane-associated protein that sequences CarQ in the dark (8). The released CarQ is then free to activate transcription from the *crtI* promoter (P\(_{\text{B}}\)). CarQ, in conjunction with CarD and the histone-like protein IhfA, also promotes transcription from its own promoter (P\(_{\text{QRS}}\)), leading to increased production in the light of the three proteins encoded in the operon (10, 13, 14).

Photoinduction of the structural genes in the *carB* operon depends on a different set of regulatory proteins: CarS, encoded by the third gene of the *carQRS* operon (13), and CarA, produced independently of light from an unlinked operon (Fig. 1). A non-polar deletion within the *carA* gene leads to light-independent expression of the *carB* operon, indicating that CarA acts as a negative regulator of the *carB* promoter (P\(_{\text{B}}\)) in the dark. Cells bearing a lack-of-function mutation in *crtI*, on the other hand, do not display light activation of the *carB* operon; CarS thus functions as a positive regulator of P\(_{\text{B}}\) in the light (13). However, when *carA* is mutated, CarS is not required for *carB* expression (15). These observations taken together have led to the following model for the light-regulated expression of P\(_{\text{B}}\). In the dark, CarA would prevent transcription from P\(_{\text{B}}\) by an as yet unknown mechanism, and this transcriptional blockage would somehow be counteracted by CarS in the light. Derepression of P\(_{\text{B}}\) is observed when CarS production is increased on illumination or when it is expressed from a constitutive heterologous promoter (13). Hence, the relative levels of CarA and CarS may be important for the latter to exert its antagonistic role. The interplay between CarS and CarA in regulating P\(_{\text{B}}\) is further manifested by the identification of a gain-of-function mutation in *crtS* (*crtSl*) that leads to constitutive expression of the *carB* gene cluster (16).

The predicted amino acid sequence for CarS does not reveal any significant sequence homology to other known proteins nor does it suggest the presence of a defined DNA binding domain. By contrast, the amino acid sequence of CarA predicts an N-terminal stretch with high sequence homology to the helix-turn-helix DNA-binding motif of the MerR family of gene regulators (17). These transcriptional factors, found in both Gram-negative and Gram-positive bacteria, regulate response to stress such as exposure to toxic compounds or oxygen radicals (18–21). MerR, the prototypical member of the family, regulates expression of the Tn21 mercury resistance operon *merTPC/AD* that confers resistance to inorganic mercury (Hg(II)). MerR binds to the *mer* operator to function as a transcriptional repressor in the absence of Hg(II) and as an activator in its

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1 M. Cervantes and F. J. Murillo, personal communication.
tion initiation site of the first gene in the carB operon), and as primers the oligonucleotides proB1 (5'-CTCTGGATCCAGCCTTCAT-GAGG-3') and proB2 (5'-CTTCTCCCGAAGAACCCTTGTCC-3'). PCR was performed using Pfu DNA polymerase to yield a 130-bp blunt-ended DNA fragment spanning positions -102 to +28 relative to the transcription start point. The amplified product was ligated to EcoRI adapters, and the 5' ends were phosphorylated with T4 polynucleotide kinase after removing unbound adapters. The phosphorylated DNA fragments were then cloned into EcoRI-digested pMAR240, which carries a 1.5-kb DNA fragment of _M. xanthus_ DNA, sufficiently long for plasmid integration by homologous recombination, and with no palindromes used to identify a plasmid with the 130-bp fragment inserted in the right (pMAR191) or wrong (pMAR192) orientation to produce a transcriptional fusion to the promoter-less locZ gene lying downstream of the EcoRI site in pMAR240. pMAR191 and pMAR192 were introduced into _M. xanthus_ by electroporation, and integration of the plasmid was selected for on CTT plates containing 40 μg/ml kanamycin. Expression of the reporter lacZ gene under the control of the 130-bp fragment was quantitatively monitored on CTT plates containing 40 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and quantified qualitatively by measurement of β-galactosidase activity as described previously (27).

**Construction of Overexpressing Plasmids, Protein Overexpression, and Purification**—To produce overexpressing His<sub>6</sub>-tagged CarA, CarS, and CarS1 (28). DNA fragments encoding these proteins were obtained by PCR using _M. xanthus_ genomic DNA as template and cloned into the NdeI-BamHI sites of the vector. To overexpress proteins, cells cultured in 50 ml of LB/ampicillin at 37 °C to an _A_<sub>600</sub> of 0.6–1.0 were harvested by centrifugation, resuspended in 50 ml of fresh LB/ampicillin, and inoculated into 1 liter of the same medium. After growth to _A_<sub>600</sub> of 0.6–0.8, protein expression was induced with 0.4 μm isopropyl-1-thio-β-D-galactopyranoside for 2 or 4 h (all at 37 °C). Expression and solubility of each protein were checked by SDS-PAGE of whole cell extracts or of the supernatant and pellet obtained by sonication and centrifugation of cells from 1-ml cell culture. His<sub>6</sub>-CarA was partly soluble at 50–200 mM NaCl but insoluble at higher salt concentrations.

Cells from 1-liter of induced culture were pelleted and suspended in 80 ml of buffer A (50 mM Tris, 5% glycerol, 5 mM β-mercaptoethanol, pH 7.5) containing 1 mM NaCl (unless otherwise stated) and 1 mM phenylmethylsulfonyl fluoride and benzamidine. Resuspended cells were lysed by sonication under ice-cold conditions and centrifuged (12,000 × g, Beckman JA-20 rotor, 30 min, 4 °C) to separate cell debris and the soluble fraction. Soluble His<sub>6</sub>-tagged protein was purified off TALON metal affinity resin following the accompanying native purification protocol at neutral pH, with imidazole elution and subsequent elimination by dialysis (CLONTECH, Palo Alto, CA). Native His<sub>6</sub>-CarS and His<sub>6</sub>-CarS1 purified in this fashion yielded >20 mg/liter cell culture. Thrombin cleavage (1:1000 molar ratio of thrombin:protein) in 150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM β-mercaptoethanol, 5 mM CaCl<sub>2</sub> incubated overnight at 20 °C) followed by dialysis. Some native CarA (~50 μg of protein/g cell pellet) could be similarly purified by resuspending the cell pellet in buffer A containing 50 mM NaCl and performing cell lysis and purification off TALON in this same buffer.

Purification of His<sub>6</sub>-CarA from the insoluble fraction was carried out at room temperature and denaturing solution conditions. Cells pelleted from a 1-liter culture were resuspended in 40 ml of binding buffer (500 mM NaCl, 20 mM Tris, pH 7.9, 5% glycerol, 2 mM β-mercaptoethanol) and sonicated. Inclusion bodies were isolated by centrifugation at 20,000 × g for 15 min and were solubilized in binding buffer containing 8 M urea. Insoluble material was eliminated by centrifugation at 20,000 × g for 20 min. The supernatant yielded ~2 mg of CarA per g of cell pellet when purified off TALON metal affinity resin under denaturing conditions. CarA was renaturated following protocols described by Burgess and Knuth (29): buffer A with 50 mM NaCl was added to dilute urea from 8 to 3 M and protein to ~0.3 mg/ml. Sarkosyl (N-laurylsarcosine) was then added to 0.2% and the sample dialyzed against buffer A containing 50 mM NaCl. After eliminating any precipitate formed during dialysis by centrifugation, renatured protein was used immediately or stored at ~20 °C in 50% glycerol. To determine protein concentrations, absorbance at 280 nm and the following extinction coefficients, ε<sub>280</sub> (M<sup>-1</sup> cm<sup>-1</sup>), were used: CarA (4 Trp, 6 Tyr), 30,940; CarS (1 Trp and 1 Tyr), 6990; CarS1 (1 Tyr), 1480 (30).

**Gel Mobility Shift and DNase I Footprinting Assays**—Radioabeled
wild-type probes were prepared by PCR as follows. Primer pro B1 (see construction of pMAR191 and pMAR192 above) was labeled at its 5’ end with [γ-32P]ATP and T4 polynucleotide kinase and then added to a PCR mix containing the second unlabeled amplification primer. The radio-labeled PCR-amplified fragment was purified off a 2% low-melting agarose gel. The indicated deleted mutant probes were generated employing PCR site-directed mutagenesis by overlap extension (31). Binding was performed in 20-μl reaction volume containing 100 mM KCl, 15 mM HEPES, 4 mM MgCl2, pH 7.9, 1 mM dithiothreitol, 10% glycerol, 200 ng/μl bovine serum albumin, 1 μg of sheared salmon sperm DNA as nonspecific competitor, 1.2 mM end-labeled double-stranded probe (~13000 cpmp), and the indicated amounts of proteins. After incubation for 30 min, the samples were loaded onto 4% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide 37.5:1) pre-run at 200 V, 10°C for 30 min in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA), and electrophoresed for 1–1.5 h at 200 V, 10°C. Gels were vacuum-dried and analyzed by autoradiography. Experimental conditions for DNase I footprinting matched those used for the gel shift assays except that 10 mM MgCl2 was included in the reaction mix. After the 30-min incubation at 20°C, the mix was treated with DNase I (0.07 units) for 2 min and then quenched with EDTA. DNA was ethanol-precipitated and run in 8% urea, 8% polyacrylamide gels against G + A and C + T chemical sequencing ladders of the 130-bp fragment (25).

**Yeast Two-hybrid Analysis—**Yeast two-hybrid analyses were performed on the yeast two-hybrid system (34). The LexA DNA-binding protein was constructed in plasmid pEG202, whereas those to the B42 transcriptional activation domain were in plasmid pJG4-5. Genes *carA*, *carS*, and *carS1* were PCR-amplified using genomic DNA as template and cloned into EcoRI-XhoI double-digested pEG202 or pJG4-5, and the respective constructs were designated pEG-X or pJG-X, X referring to the gene cloned. The recipient yeast strain EGY48 was transformed by electroporation or by the lithium acetate method. Prior to use in the analysis of protein-protein interactions, self-activation and entry into the nucleus of the LexA fusion proteins were tested. pEG202 and pJG4-5-based constructs were introduced in different pairwise combinations into EGY48 cells bearing a LexA fusion protein in plasmid pMAR191, which contains the 130-bp fragment-lacZ transcriptional fusion (see “Experimental Procedures”), was introduced into *M. xanthus* wild-type strain DK1050 by electroporation. Chromosomal integration of the plasmid via homologous recombination was selected for by growth in the presence of kanamycin. On plates containing X-gal, DK1050-derived electroporants showed the light-inducible phenotype expected for *Pb*. Quantitative analysis of β-galactosidase activity for dark- and light-grown cultures of several of these electroporants provided results directly comparable with those reported previously with the longer 1058-bp stretch of DNA upstream of *orf1* (Fig. 2B; see ref. 17). On the other hand, pMAR191 introduced into strain MR844, where part of the *carA* gene is deleted, yielded electroporants that showed the constitutive, light-independent expression of the *lacZ* reporter gene expected for *carA* lack-of-function mutants (Fig. 2C). As shown in Fig. 2, B and C, control electroporation experiments with pMAR192 (where the 130-bp fragment is fused to the *lacZ* gene in the opposite orientation relative to pMAR191) gave rise to electroporants expressing low levels of basal β-galactosidase activity, which remained the same irrespective of the genetic background (wild type or MR844) or growth conditions (dark or light). Thus, we conclude that the 130-bp DNA fragment includes all the cis-acting elements essential for the correct regulation and expression of the *carB* promoter.

**CarA Exhibits Specific DNA Binding at Pb**—The paring-down experiment discussed above provides a DNA fragment that is sufficiently short for use in DNA binding assays, yet fully functional in *vivo*. To verify binding of CarA to the *carB* promoter/operator region, purified His6-CarA was used in gel mobility shift assays using as probe the 130-bp DNA fragment (CCR, *carB* control region). His6-CarA was purified under native conditions or under denaturing conditions followed by renaturation (see “Experimental Procedures”). These and other proteins used in this work were ≥95% pure, and their mobilities in SDS-PAGE were those expected based on the molecular weights (Fig. 3A).

EMSA analysis of CarA binding to probe CCR was characterized by two retarded bands that appeared as a function of CarA concentration (Fig. 3B). Only the higher mobility species (lower band) was apparent at the lowest concentrations of CarA used in these assays (4–8 nM; lanes 2 and 3, Fig. 3B). The lower mobility complex (upper band) appeared with increasing CarA concentration and became the predominant species at the highest concentrations of CarA used (Fig. 3B, lanes 6 and 7). This concentration-dependent appearance of the lower mobility band may reflect different modes of CarA binding to CCR, as a...
**Fig. 2.** Cis-acting elements of the carB operon. A, promoter/operator regions for the operons carB (top) and merT/P/C/AD (bottom). DNA sequence features of the segment from position +1 (the transcriptional start point) to position −68 are shown for the carB operon; the merT/P/C/AD from position +1 to −42 is shown for comparison. The −35 and −10 promoter elements corresponding to consensus sequences are in boldface. Palindromic inverted repeats are shown boxed with oppositely facing arrows below the sequence. Direct repeats are underlined and indicated by two arrows pointing in the same direction. B and C show β-galactosidase-specific activity measurements (average of three independent experiments) of M. xanthus strains with pMAR191 or pMAR192 integrated and grown in the dark or light. B, wild-type strain DK1050. C, MR844 (a DK1050 derivative with a non-polar carA deletion).1 Open symbols refer to growth in the light and are connected by solid lines; filled symbols correspond to growth in the dark and are connected by dashed lines (the lines are shown to aid in visualization only). Strains with pMAR191 integrated are shown by circles and those with pMAR192 are represented by squares.

**Fig. 3.** Specific binding of CarA to DNA probe CCR. A, 15% SDS-PAGE of the purified His6-tagged proteins used in this study. Lanes are marked as follows: M, molecular mass standards; lane 1, His6-CarA purified under native conditions; lane 2, His6-CarA renatured after purification from inclusion bodies; lane 3, His6-CarS purified under native conditions. B, EMSA of His6-CarA binding to the 130-bp CCR probe. At the top are shown the increasing CarA concentrations used. A 10-fold excess of cold probe CCR was used in the competition assay (indicated by “+” shown in lane 10). Other solution conditions are described in the text.

A consequence of distinct binding sites on the DNA and cooperativity between these binding modes. It could also reflect specific DNA binding by higher oligomeric form(s) of CarA that could be increasingly populated as the protein concentration is raised. That the binding of CarA is probe-specific was demonstrated by the fact that addition of excess cold CCR probe effectively competed in EMSA. It may be noted that these results obtained with His6-CarA purified under native conditions were reproducible with purified, renatured His6-CarA. Hence, CarA manifests the DNA binding ability predicted from its sequence analysis, and moreover, it binds specifically within the region of the Pβ promoter shown to be essential in vivo.

**Dissection of the CarA DNA-binding Site**—The 130-bp CCR probe used above contains a palindromic DNA sequence upstream of the −35 region which, by analogy with MerR proteins, could be a potential binding site for CarA. It also includes two direct repeats (one overlapping with the palindrome and the other lying between the −35 and −10 regions, Fig. 2A), an arrangement reminiscent of the operator for the Bacillus subtilis DeoR repressor of the dra-nupC-pdp operon (37, 38). Therefore, we analyzed in further detail CarA binding around Pβ.

We first examined DNA binding by EMSA analysis with a 64-bp probe corresponding to the segment −39 to −102. This probe contains the palindromic sequence and the direct repeat that overlaps with the 3′-half of the palindrome but lacks the other direct repeat located between the −35 and −10 regions. In striking contrast to the behavior observed for CarA binding to the longer 130-bp probe (Fig. 3B), only a single retarded band was observed with this probe for an equivalent range of protein concentrations (Fig. 4B, lanes 2–7, probe a). The observed differences in the gel-shift mobility pattern suggest that elements downstream of the 64-bp segment are necessary for the formation of both CarA-DNA complexes detected with the longer probe. The presence of two retarded bands may therefore be the consequence of increasing occupation of two possible binding sites on the DNA around Pβ. The single retarded band detected with the 64-bp probe could then be the result of CarA binding to the intact palindrome still present in this probe. To verify this possibility, we mutated either one or both of the palindromic half-sites. The intensity of the single retarded band
was considerably lowered when either one of the inverted repeats in the 64-bp probe was mutated (Fig. 4B, lanes 8 and 9, probe b; lanes 10 and 11, probe c). No retarded band could be detected when both the inverted repeats were mutated (Fig. 4B, lanes 12 and 13, probe d). Thus, mutating either or both of the inverted repeats leads to a drastic reduction in the DNA binding affinity of CarA. The palindromic sequence is therefore a specific CarA DNA-binding site but, on its own, is not capable of promoting the formation of the two CarA-DNA complexes that could be observed with the 130-bp DNA probe. In other words, the participation of additional downstream elements may also be important. This inference is supported by the following observation: mutations in both halves of the palindrome in the 64-bp probe d that led to undetectable DNA binding (Fig. 4B, lanes 12 and 13) resulted in two retarded bands in the 130-bp probe e (Fig. 4C, lanes 5 and 6), but of considerably lower intensity relative to wt, the 130-bp CCR probe (Fig. 4C, lanes 2 and 3).

CarA-DNA binding was further analyzed by DNase I footprinting of probes wt and a labeled at the upper strand. Binding of CarA to the 64-bp probe a protected positions −70 to −41, spanning the inverted repeats, the DNA between them, and 6 bases flanking each side of the palindrome (Fig. 4D, lanes 8 and 9). Two hypersensitive sites were also observed, one lying at the 5'-end of the left inverted repeat (position −63) and the other between the two half-sites (position −55). These results additionally support the inference that the palindrome constitutes a CarA-binding site and define the footprint features that characterize its occupation by CarA. To determine whether CarA is capable of occupying additional sites downstream of the palindrome, as suggested by EMSA, we performed DNase I footprinting with the 130-bp probe wt (Fig. 4D, lanes 1–7). With increasing concentrations of CarA, a footprint that extended beyond that observed with the 64-bp probe became apparent.

At the highest CarA concentration used (where the low mobility species predominates in EMSA), at least an additional 22 bp (positions −42 to −19) were protected. Included in the expanded footprint are the −35 promoter element and part of the 3'-direct repeat. In sum, the DNase I footprinting results reinforce our conclusion that the low mobility species in EMSA corresponds to CarA bound to the palindrome and to additional downstream elements.

**CarS Shows no DNA Binding in Vitro but Abolishes Specific CarA-DNA Binding at P_B**—Having established that CarA exhibits specific DNA binding to CCR, we next determined whether, under similar experimental conditions, this applied also to CarS. As shown in Fig. 5 (lane 2), we did not detect any specific binding of CarS to probe CCR even at CarS concentrations over 2 orders of magnitude greater than those at which specific binding of CarA to CCR could be observed. These results strongly suggest that CarS cannot exert its antagonistic role by directly competing with CarA for binding to the region surrounding and including P_B. CarS, nevertheless, could accomplish its function through the inactivation of CarA so that it no longer binds DNA. Fig. 5 shows an order-of-addition gel shift assay performed with probe CCR at a fixed concentration of CarA (60 nM) and in the absence or presence of increasing concentrations of CarS (0.4–11 μM). It may be noted that the protein concentrations are expressed in terms of the monomer, and the protein stocks are assumed to be fully active. It is evident from Fig. 5 that increasing concentrations of CarS can effectively abolish the specific DNA binding of CarA (compare lane 3 with lanes 4–8 and 9–13). When CarA and CarS were added to the reaction simultaneously, about a 15-fold excess of CarS relative to CarA was more than sufficient to completely abrogate CarA DNA binding (lanes 4–8). However, when CarA was first allowed to bind CCR and CarS subsequently added, even a 180-fold excess of CarS could not completely disrupt the

CarA-DNA complexes (lanes 9–13). Interestingly, before complete neutralization of CarA-DNA binding by CarS was achieved, CarS promoted a shift in the relative distribution of the two CarA-retarded species, from the lower mobility species to the higher mobility one (compare lane 3 with lanes 4 or 9–13). This effect was particularly obvious in those reactions where CarS was added to pre-formed CarA-CCR complexes, where the lower mobility band was “converted” into the high mobility species before DNA binding was completely eliminated at higher CarS concentrations. Hence, it appears that CarS is more effective against the formation of the lower mobility complex. CarS therefore acts as an antirepressor by preventing free CarA from binding to its cognate site and, less efficiently, by “disrupting” pre-established CarA-DNA complexes. As we have also demonstrated, this antagonistic role of CarS does not involve any CarS-DNA binding.

**CarA-CarS Physical Interactions Mediate the Functional Interplay between Them**—Possible CarA-CarS interactions were scored in *vivo* using the yeast LexA-based two-hybrid system (24). In this system, the N terminus of one of the protein pair is fused to the LexA DNA-binding domain (the “bait”), whereas the N terminus of the other protein is fused to the B42 activation domain (the “prey”). Expression of the prey protein is controlled by the GAL1 promoter, which is repressed by glucose and strongly activated by galactose. When both fusion proteins are expressed in yeast strain EGY48 bearing plasmid pSH18-34 (which contains the lacZ gene), physical interaction between the bait and prey results in activation of the reporter genes LEU2 and lacZ. We found that yeast cells producing the LexA-CarA and B42-CarS fusion proteins were able to develop colonies on galactose plates lacking leucine; moreover, the colonies acquired an intense blue color 30–60 min after the plates were overlaid with X-gal. By contrast, control cells producing only the LexA-CarA fusion protein were unable to grow on plates lacking leucine, and colonies grown on plates containing leucine remained white even 24 h after addition of X-gal. These effects are illustrated by measurements of the level of expression of the lacZ reporter gene as shown in Fig. 6A, which demonstrates galactose-dependent induction of lacZ expression and the absence of any such effect in the control cells. Considering the values of β-galactosidase activity attained after just a 2-h induction in galactose, the data indicate that a strong physical interaction exists between CarA and CarS. The reverse experiment, where CarS was fused to the LexA protein and CarA to the activation domain, rendered qualitatively similar results. However, the LexA-CarS construct did not fully satisfy the criteria to pass the self-activation and entry into the nucleus controls. Consequently, we did not proceed with an actual quantitative estimation in this case.

We further probed the interaction between CarA and CarS using the purified proteins in pull-down assays. In these assays, interactions are probed by tethering one of the proteins to a solid matrix, and then checking its ability to pull down a possible interacting partner that is incapable of binding to the matrix. Given that the His₉ tag in purified His₉-CarS could be completely cleaved off by thrombin (this was less efficient with His₉-CarA), we examined the ability of TALON-bound His₉-CarA to pull down CarS lacking its His tag (“CarS”) (see “Experimental Procedures”). We observed that TALON-bound His₉-CarA was capable of pulling down “CarS” in amounts sufficient to be detected in Coomassie-stained SDS-PAGE (Fig. 6B, lane 3) relative to a control of CarS passed through TALON resin alone (Fig. 6B, lane 1). This demonstrates that the two proteins do interact physically and with significant strength, as suggested by the yeast two-hybrid analysis. Moreover, these data also suggest that CarS by itself does not proteolyze CarA, because there was no loss of CarA in the course of the experiment. An ~1:1 mix of His₉-CarA and CarS compares well with the relative intensities of the two proteins in the pull-down assay (compare lanes 3 and 4, Fig. 6B). This suggests, but does
CarA Interacts with Itself whereas CarS Is a Monomer—CarA may form oligomers, given the sequence characteristics of its DNA-binding site, and the observation in gel mobility shift assays of two retarded bands whose relative intensities varied with protein concentration. We therefore investigated whether CarA interacts with itself by using the yeast two-hybrid system. The results summarized in Fig. 7A show this to be the case; yeast cells producing the LexA-CarA and B42-CarA fusion proteins were able to develop colonies on galactose (but not glucose) plates lacking leucine, and the colonies acquired an intense blue color when incubated for at least 6 h after the X-gal overlay. This was not the case with control cells producing only the LexA-CarA fusion protein. Significant levels of LacZ accumulation in cells with the LexA-CarA/B42-CarA fusion constructs required overnight induction (≥12 h), in contrast to cells with LexA-CarA/B42-CarS fusion constructs where much shorter times (2 h) were sufficient. This suggests that CarA-CarA interactions exist but may be weaker than those between CarA and CarS.

A biophysical characterization of the oligomeric state of CarA was attempted using size-exclusion high performance liquid chromatography and analytical ultracentrifugation. This, however, has not been possible thus far because of the loss of material (signal) observed at the micromolar concentrations required and used in these experiments. Size-exclusion experiments in the presence of a mild detergent (1 mM CHAPS) and the use of a lower pH (6.5, about two units less than the theoretical pI) or the presence of a higher glycerol concentration (25%) have proved unsuccessful so far. We have no clear explanation for this other than that CarA may have a relatively low solubility. On the other hand, His_6-CarS and its thrombin-cleaved product CarS were characterized by both these biophysical methods. Size-exclusion analysis indicated that both these proteins are predominantly monomeric (Fig. 7B); the apparent molecular masses (in kDa) of His_6-CarS and CarS of 16.6 and 15.5, respectively, compared well with the corresponding calculated values of 14.4 and 12.5 (confirmed by mass spectrometry). This was also confirmed in analytical ultracentrifugation carried out at rotor speeds of 18,000 and 25,000 rpm (shown for His_6-CarS in Fig. 7C). Here the weight average molecular mass (in kDa) obtained by fitting the equilibrium radial distribution to the equation for a single ideal species was (20 ± 1) for His_6-CarS and (16 ± 1) for CarS, and the residuals of the fits (small and randomly scattered) were indicative of a single species. Thus, on current evidence, CarA is a dimer or higher order oligomer, whereas CarS is monomeric.

Protein-Protein Interactions Involving Truncated CarA and CarS—In a pilot attempt to localize the regions of CarS and CarA involved in the interactions, we examined truncated forms of each protein. A gain-of-function mutation in carS (carS1) has been identified which provokes light-independent expression from the normally light-inducible PB promoter (16). The carS1 gene product, CarS1, is a truncated form of CarS lacking the last 25 amino acids (13). CarS1 was purified as native His_6-tagged protein and was found to be monomeric by gel filtration (apparent molecular mass of 15.4 kDa relative to the calculated value of 11.5 kDa, Fig. 7B) as well as by analytical ultracentrifugation (weight average molecular weight of 17 ± 1 kDa). His_6-CarS1, like CarS, was capable of abolishing the specific DNA binding of CarA (data not shown). We also found that CarS1 matched CarS in physically interacting with CarA when probed by the yeast two-hybrid analysis (see Table I). These results demonstrate that the CarA-binding domain maps to the first 86 N-terminal residues of CarS.

Because we had observed that CarS was less effective against pre-formed complexes of CarA and the CCR probe, we reasoned that the same region(s) of CarA could be involved in the specific binding to DNA as well as to CarS. So we tested whether CarA truncated to its first 78 N-terminal residues, CarA(Nter), was involved in any protein-protein interactions. This fragment of CarA was chosen because the homologous stretch in MerR proteins includes the helix-turn-helix motif and the two “wings” implicated in DNA binding (39). We found that yeast cells producing the LexA-CarA(Nter) and B42-CarS fusion proteins developed colonies on galactose plates lacking leucine that acquired an intense blue color 30–60 min after the plates were overlaid with X-gal. This was not seen with control cells producing only the LexA-CarA(Nter) fusion protein. These results parallel those described earlier for CarA-CarS interactions. Yeast two-hybrid analysis also indicated that CarA(Nter)
**Experimental Procedures.**

Calibration curve using the molecular weight standards, around the promoter, and this is antagonized by CarS through that CarA acts by specifically binding to DNA in the region around the promoter, and this is antagonized by CarS through direct physical interaction with CarA. CarA and CarS thus constitute a repressor-antirepressor pair. Given the nature of the binding sites, and that CarA is an oligomer, it is reasonable to infer that the specific CarA-DNA binding must involve at least dimers. The two distinct types of specific CarA-DNA complexes that we observe with increasing protein concentration could then be attributed to a lower and a higher order oligomeric form of CarA. Or it may be that an increasing number of sites on the DNA are being occupied as the protein concentration is raised. Our EMSA and DNase I footprint analyses indicate that a palindromic upstream of the −35 promoter region is involved in binding to CarA. However, our data suggest that additional elements downstream of the palindrome are also involved. A conspicuous feature of the DNA used in our EMSA analysis is that its sequence also includes two direct repeats, one of which overlaps with the 3′-half of the palindrome, and the other is located between the −10 and −35 promoter elements. A similar arrangement of a palindrome and two direct repeats that occurs in the promoter region of the dra-nupC-pdp operon in *B. subtilis* has been shown to constitute the operator for the octameric DeoR repressor (37, 38).

We propose that binding to the palindrome may serve as a beacon for a more effective homing-in of CarA to additional site(s). This could account for the two distinct types of specific CarA-DNA complexes observed as the concentration of CarA increases. This proposal does not exclude the possibility that different oligomeric states of CarA may also be involved. If prior binding of CarA to the palindrome then fosters binding to the direct repeats, this would provide a simple and effective mechanism for the repression of transcription. Because one of the direct repeats lies in the spacer between the −35 and −10 regions, its complex with CarA could block promoter access to RNA polymerase to repress transcription in the manner of most classical repressors (40). DNase I footprinting does show protection by CarA of the −35 promoter element that extends to at least the 5′ end of the downstream direct repeat. Because this occurs at CarA concentrations where the lower mobility complex predominates, it is attractive to speculate that this would be the functionally operative CarA-DNA complex *in vivo*. Remarkably, the lower mobility complex is also the one that is more easily dismantled by CarS. A detailed analysis of these proposals is currently being pursued.

**Possible Mode of CarS-mediated Antirepression of CarA—**

Since the involvement of two contrasting elements in transcriptional regulation was first suggested by Oppenheim *et al.* (41), several antirepressor-repressor systems have been reported. Distinct mechanisms for how the antirepressor antagonizes repressor activity include the following: (a) direct protein-protein association without any DNA binding by the antirepressor (42–45); (b) exclusion of the repressor by DNA binding of the antirepressor (46); and (c) proteolysis of the repressor promoted by the antirepressor (47). Our data have revealed that the monomeric CarS, which does not itself bind DNA, physically interacts with CarA. We also have no evidence for any CarS-
mediated degradation of CarA. On a per molecule basis, CarA-CarS binding may involve a 1:1 stoichiometry. This, as well as the stoichiometry of CarA-DNA binding, needs to be corroborated by additional experiments currently underway. We observe that CarS relieves DNA binding by CarA, being more effective in abolishing the lower mobility CarA-DNA complex. When simultaneously added with CarA, CarS lowers the effective concentration of CarA available for DNA binding. Preformed CarA-DNA complexes are more refractory to the action of CarS, suggesting that CarS interacts more readily with CarA that is free in solution. Disruption of the pre-bound CarA-DNA complex by CarS would then be dictated by the kinetics of dissociation of the complex and the subsequent trapping of freed CarA by CarS. Thus, the primary mechanism for CarS-mediated inactivation of CarA appears to be in binding to and blocking its DNA-binding domain. Consistent with this are a number of other observations. CarS is acidic (theoretical pI = 4.76) and could conceivably be an effective competitor for DNA-binding regions on CarA. Significantly, CarS1 is even more acidic (theoretical pI = 4.09) than CarS; of the 8 Arg in CarS (which also has 2 Lys), 6 are located in the C-terminal stretch beyond the scope of the present study and would, among other things, be aided by a knowledge of the three-dimensional structures of the proteins involved.

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A Repressor-Antirepressor Pair Links Two Loci Controlling Light-induced Carotenogenesis in *Myxococcus xanthus*

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