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The High-Mobility Group A-Type Protein CarD of the Bacterium *Myxococcus xanthus* as a Transcription Factor for Several Distinct Vegetative Genes

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ABSTRACT

CarD is the only reported prokaryotic protein showing structural and functional features typical of eukaryotic high-mobility group A transcription factors. In prokaryotes, proteins similar to CarD appear to be confined primarily to myxobacteria. In *Myxococcus xanthus*, CarD has been previously shown to act as a positive element in two different regulatory networks: one for light-induced synthesis of carotenoids and the other for starvation-induced fruiting body formation. We have now tested the effect of a loss-of-function mutation in the *carD* gene (*carD1*) on the expression of a random collection of *lacZ*-tagged genes, which are normally expressed in the dark during vegetative growth in rich medium. Our results indicate that CarD plays a significant role in the transcriptional regulation of various indicated genes. The *carD1* mutation downregulates some genes and upregulates others. Also reported here is the isolation of several mutations that suppress the strong effect of *carD1* on the expression of a particular vegetative gene. One of them (*sud-2*) also suppresses the effect of *carD1* on other vegetative genes and on fruiting-body formation. Thus, CarD and the *sud-2* gene product appear to participate in a single mechanism, which underlies various apparently diverse regulatory phenomena ascribed to CarD.

CELLS of the gram-negative bacterium *Myxococcus xanthus* respond to diverse environmental stimuli either individually or as a group. Well-known examples are the individual response to blue light, which results in the accumulation of carotenoids, and the cooperative response to starvation, which results in the formation of fruiting bodies where thousands of cells cluster and subsequently differentiate into myxospores. Genetic and molecular analysis of these two responses have revealed two complex networks of regulatory actions that are mostly independent (for reviews, see Dworkin 1996; Hodgson and Berry 1998).

Several gene loci have been identified that participate in the response of *M. xanthus* to blue light. Two of them, the *carQRS* operon and the unlinked single gene *crtI*, are of particular interest here. They are both regulated by light-inducible promoters (from here on referred to as P_{QRS} and P_I). The single gene *crtI* codes for an enzyme involved in the dehydrogenation of phytoene, the first C_{40} carotene precursor (Fontes *et al.* 1993). The *carQRS* operon contains three regulatory genes. Gene *carQ* codes for a σ-factor of the extracytoplasmic function (ECF) subfamily of σ^{70} factors (Lonetto *et al.* 1994). This σ-factor is required for the activation of its own promoter, P_{QRS}, and the P_I promoter (Fontes *et al.* 1993; McGowan *et al.* 1993; Martínez-Argudo *et al.* 1998). Gene *carR* codes for a membrane-spanning protein. Several lines of evidence indicate that, in the dark, protein CarR acts as an anti-σ-factor, sequestering CarQ to the membrane. Illumination of the cells somehow results in loss of CarR, so CarQ is free to activate the P_{QRS} and P_I promoters (Gorham *et al.* 1996). Gene *carS* codes for a protein involved in the light activation of other carotenoid genes unrelated to this work.

Two additional proteins are directly required for the proper activation of the P_{QRS} promoter. The first one is the integration host factor (IHF), a DNA architectural protein (Moreno *et al.* 2001). The second protein is the product of the gene *carD*, which is also independently required for the activation of the P_I promoter (Nicolás *et al.* 1994; see below).

In response to starvation, *M. xanthus* cells initiate a complex developmental program that culminates in the construction of a fruiting body (Kaiser and Losick 1993; Dworkin 1996). Development of the fruiting bodies entails the transcriptional activation of specific genes, which is mediated by five intercellular signals, factors A, B, C, D, and E. These factors are needed in various combinations to activate different sets of devel-

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opment-specific genes (Kaiser and Kroos 1993). Cells affected in the aforementioned gene *carD* are also blocked at an early stage of fruiting-body formation, thus establishing the only known genetic link between the light and the starvation responses of *M. xanthus*. The indicated developmental phenotype is associated with the lack of expression in the *carD* mutant of two sets of developmentally activated genes: those that depend on intercellular signals A or C. By contrast, the σ-factor CarQ is not required for the expression of these developmentally activated genes (Nicolás *et al.* 1994).

Protein CarD contains DNA-binding and acidic regions that are characteristic of eukaryotic high-mobility group A (HMGA) proteins (Nicolás *et al.* 1996; Bustin 2001). Among the prokaryotes, proteins having similar HMGA domains appear to be exclusive for the Cystobacterineae suborder of myxobacteria (Cayuela *et al.* 2003). The best-known HMGA proteins, the mammalian HMGA isoforms, are abundant, nonhistone chromosomal proteins that, among other actions, serve as general transcription factors (Yie *et al.* 1997; Bustin 1999). Given the known participation of CarD in two, otherwise unrelated, cases of gene activation in *M. xanthus*, and the similarities of CarD with the HMGA proteins, we reasoned that CarD might also participate in the proper expression of other *M. xanthus* genes. To test this idea, we have screened a random collection of *M. xanthus* genes, which are expressed in the dark during vegetative growth, for their dependence on CarD. As reported here, a number of genes have been found that are either positively or negatively regulated by CarD. The newly identified CarD-dependent genes are not involved in the light- or the starvation-induced response of *M. xanthus*, nor are they regulated by the σ-factor CarQ. One of them is also regulated by IHF, but another is not. To identify gene products that may interact with CarD, a search for suppressors of a *carD* mutation has been carried out. The result of this search and some properties of one of the suppressor mutations are also reported here.

MATERIALS AND METHODS

Bacterial strains, transducing phages, and growth conditions: Most of the *M. xanthus* strains used in this study are listed in Table 1, together with their phenotype, genotype, and origin. Other strains are introduced in the text. The standard strains DK1050 and DK1622 show normal light-induced synthesis of carotenoids (Car$^+$ phenotype) and starvation-induced fruiting-body formation (Fru$^+$ phenotype). They both derive from the prototype *M. xanthus* strain FB (Kaiser 1979; Wall *et al.* 1998). MR522, derived from DK1050, and MR563, derived from DK1622, both contain the recessive mutation *carD1*. This is a Tn*5-132* insertion in the middle of the *carD* gene, which results in the lack of protein CarD (nondetectable with polyclonal anti-CarD antibodies) and causes a Car$^-$ Fru$^-$ phenotype (Nicolás *et al.* 1994; Padmanabhan *et al.* 2001; our unpublished results). 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 translational start signal preceded by stop codons in all three reading frames; therefore, it produced transcriptional but not translational fusions. Insertion of transposon Tn*5-lac* causes resistance to kanamycin (Km^R) and insertion of transposon Tn*5-132* causes resistance to tetracycline (Tc^R). For cloning purposes, *Escherichia coli* strains DH10B (GIBCO BRL, Gaithersburg, MD) and MC1061 (Casadaban and Cohen 1980) were used. For generalized transduction between *M. xanthus* strains, phage Mx4-LA27 (Mx4 from now on) was used. The phage and the conditions used for transduction of Km^R and Tc^R have been previously described (Campos *et al.* 1978; Avery and Kaiser 1983). To transfer plasmids from *E. coli* to *M. xanthus*, transduction by coliphage P1*clr100*Cm (Gill *et al.* 1988) or electroporation (Kashefi and Hartzell 1995) were used. The rich medium CTT [1% Bacto-casitone, 10 mm Tris-HCl (pH 7.5), 1 mm KH_2PO_4, 8 mm $MgSO_4$] (Bretscher and Kaiser 1978) and the exact culture conditions for growth of *M. xanthus* in the dark and in the light have been previously described (Fontes *et al.* 1993). All incubations were at 33°. Induction of fruiting-body formation and sporulation was conducted by washing exponentially growing cells with TPM [10 mm Tris-HCl (pH 7.5), 1 mm KH_2PO_4, 8 mm $MgSO_4$]. Cells were spotted on TPM agar (1.5%) plates and incubated for 4 days. The fruiting bodies were observed and counted under a Zeiss dissecting microscope.

Mutagenesis procedures: Tn*5-lac* mutagenesis was conducted by infecting strain DK1050 with phage P1::Tn*5-lac* and by selecting for Km^R as described by Kroos and Kaiser (1984). The Tn*5-lac* strains were separately picked on CTT + Km (40 μg/ml) + X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, 40 μg/ml) agar plates and incubated for 2 days. The colonies were then classified according to the intensity of their blue color (see results). For UV mutagenesis, different 3-ml aliquots of an exponentially growing culture (≈10^8 cells/ml) were exposed to irradiation for 2.5 min with a germicidal lamp (General Electric, GI5T8), a treatment that yielded ∼2% survival.

Making pools of independent Tn*5-lac* insertions: Tn*5-lac* colonies of the same class were separately picked on CTT + Km plates. After incubation for 2 days, 2.5 ml of liquid CTT was added to the plates. These were then shaken for 15 min at 40 rpm to allow suspension of the cells in the liquid medium. The liquid phase was collected, vigorously vortexed, and used for the preparation of Mx4 lysates.

Plasmids: Cloning vector pDAH160 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. These two elements are also present in plasmid pDAH250 (Hodgson 1993). Plasmids pDAH217 and pMAR206 contain a *lacZ* transcriptional probe fused to the light-inducible promoter P_{QRS} (Hodgson 1993) or P_I (Fontes *et al.* 1993), respectively. They cannot replicate in *M. xanthus* cells but can integrate into the *M. xanthus* chromosome by homologous recombination. Integration produces a tandem duplication of the cloned DNA. The plasmid pMAR932, constructed for this work, contains a *ddvA*::*lacZ* transcriptional fusion (see results). For standard cloning procedures, vectors pUC19 (Norrander *et al.* 1983) and pBluescript (Short *et al.* 1988) were used.

Nucleic acid manipulations: The use of plasmid pDAH160 to clone wild-type DNA around a transposon insertion site has been described in detail previously (Hodgson 1993). This procedure was used to obtain plasmid pMAR904, which carries a 3.85-kb DNA fragment from the *ddvA* locus. For cloning of the *ddmC*, *ddmA*, and *ddmB* loci, chromosomal DNA from *M. xanthus* strains MR1104, MR1100, and MR915 (each carrying a Tn*5-lac* insertion at the respective locus) was digested with

*Kpn*I and electrophoresed on 1% low-melting agarose gels. DNA fragments ranging from 9 to 23 kb were purified and ligated to *Kpn*I-digested pBluescript. After electroporation of *E. coli* cells, a number of plasmids from Amp^R Km^R colonies were analyzed. Different portions of the DNA present in the corresponding plasmids were recloned by using the appropriate restriction enzymes (Roche) or exonuclease III treatment (Pharmacia). Southern analysis, nucleic acid manipulations, and enzymatic assays were done according to standard procedures (Sambrook *et al.* 1989).

Nucleotide sequencing and sequence analysis: Automated sequencing of the two strands of *M. xanthus* DNA was performed on an ABI PRISM 310 sequencer. For sequence comparisons with databases, the BLAST programs provided by the European Bioinformatics Institute (http://www.ebi.ac.uk) were used.

Expression of β-galactosidase: Rapid determination of β-galactosidase production was carried out by examining colony color on plates containing 40 μg X-Gal/ml. Quantitative assays of β-galactosidase-specific activity on dark- or light-grown liquid cultures were performed as previously described (Balsalobre *et al.* 1987). Data are given in nanomoles of *o*-nitrophenol produced per minute per milligram of protein. In each case, two or more parallel cultures of parental and mutant strains were analyzed, all with very similar results. Every individual sample was subjected to two or more independent assays, and the average result was taken. Data shown in Figures 1 and 5 are representative examples of comparison sets of wild-type and mutant cultures. The intra-sample standard deviations never exceeded 15% of the average.

RESULTS

A random search for CarD-dependent promoters: Transposon Tn*5-lac* (Km^R) functions as a promoter probe, given that one of its inverted terminal repeats is interrupted by a promoter-less *lacZ* gene, preceded by translational stop codons in all reading frames (Kroos and Kaiser 1984). If Tn*5-lac* is inserted within a transcriptional unit in the correct orientation, *lacZ* expression is controlled by the promoter of the interrupted gene. Tn*5-lac* was used as a mutagen of the *M. xanthus* standard strain DK1050. In three different experiments, a total of 1643 independent Km^R colonies were obtained, of which 613 gave a clearly visible blue color on X-Gal. The rest should have integrated the transposon at an inactive site or in the incorrect orientation for transcription. The positive colonies were grouped into four classes, on the basis of the intensity of the blue color developed on X-Gal plates: "very strong" (V, 50 colonies), "strong" (S, 110 colonies), "medium" (M, 113 colonies), and "weak" (W, 340 colonies). Pools of 25–30 colonies of the same class were used to prepare stocks of the *M. xanthus* generalized transducing phage Mx4. Each stock was then used to transduce the Km^R marker into the *M. xanthus carD1* mutant strain MR522. As a control, each stock was also transduced back into the standard strain DK1050. In each case, the number of selected Km^R colonies was at least five times higher than the number of independent Tn*5-lac* insertions present in the original pool, to ensure that most of those insertions were represented among the transductant colonies. These were then individually transferred to X-Gal plates and visually inspected for *lacZ* expression. Thirty-seven *carD1*-derived transductants were selected because they appeared to develop either a more or a less intense blue color than the one developed by the corresponding class of *carD*$^+$-derived transductants. Individual phage transducing stocks were obtained from each of the selected colonies, and they were then used separately to transduce the Km^R marker back into DK1050 and MR-522. When tested on X-Gal plates, 11 of the selected Tn*5-lac* insertions gave clearly reproducible differences between the *carD1* and the *carD*$^+$ genetic background.

A *carD1*- and a *carD*$^+$-derived strain for each one of the 11 insertions mentioned above were used for quantitative assays of β-galactosidase in liquid culture from the early exponential to the late stationary phase. In five cases, the *carD* mutation caused a reproducible but small effect (less than twofold up or down) on the activity of the tagged promoters. In the other six cases, the *carD1* mutation caused a more significant effect on the tagged promoters. Of these, one came from the V class, one from the S class, and one from the W class. The corresponding gene loci were named, respectively, *ddvA*, *ddsA*, and *ddwA* (from Car*D*-*d*ependent *v*ery strong, *s*trong, or *w*eak promoters). The other 3 insertions came from the M class, and the corresponding gene loci were named *ddmA*, *ddmB*, and *ddmC*. The activities of the *ddvA* and *ddmB* promoters were negatively affected and those of the four other promoters were positively affected by the *carD1* mutation. The effect was particularly strong for the *ddvA*, *ddmB*, and *ddwA* promoters (Figure 1).

All the *carD*$^+$-derived strains carrying any of the six Tn*5-lac* insertions showed normal light-induced accumulation of carotenoids. It was therefore unlikely that the affected loci would correspond to the *carQRS* operon or the *crtI* gene, previously reported to be regulated by CarD (see Introduction). The other set of *M. xanthus* genes whose expression is known to be regulated by CarD is transcribed at a detectable level only during the starvation-induced developmental process. Therefore, these genes should also be different from the CarD-dependent loci identified here (Nicolás *et al.* 1994). Moreover, all the *carD*$^+$-derived strains carrying any of the six Tn*5-lac* insertions exhibited a normal developmental response to starvation (not shown). The six CarD-dependent loci differed in both their expression level and their response to the *carD1* mutation, suggesting that they represent six different loci. This was confirmed by Southern analysis of chromosomal DNA, using a labeled probe derived from the *lacZ* gene (results not shown), and by the cloning and sequence analysis of four of the loci: the two loci negatively affected by *carD1* (*ddvA* and *ddmB*) and two of those positively affected by that same mutation (*ddmA* and *ddmC*).

Sequence analysis of CarD-dependent loci: From the *ddvA* locus, a DNA stretch of ∼3.4 kb was sequenced

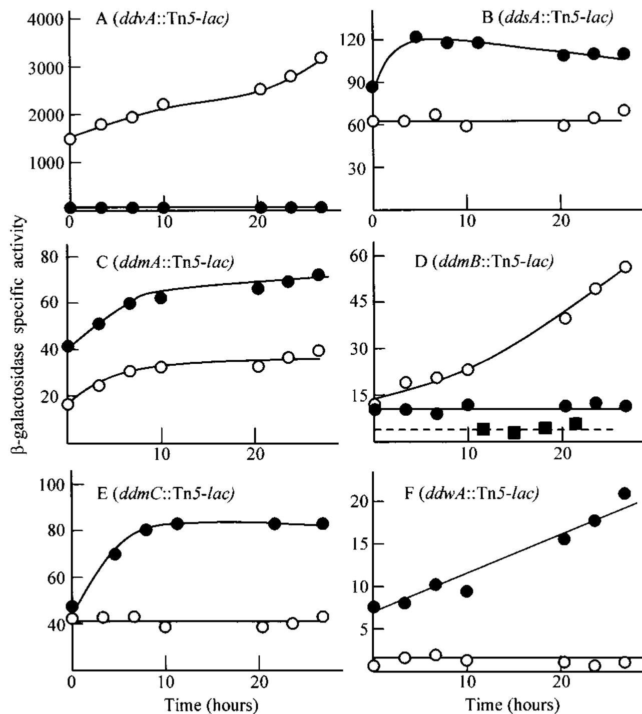

FIGURE 1.—Mutation *carD1* alters the normal expression of several *M. xanthus* vegetative genes. A–F represent the time course of β-galactosidase expression for a different Tn*5-lac* insertion in the wild-type (open circles) or *carD1* (solid circles) genetic background. The expression of *ddmB*::Tn*5-lac* in the *ihfA2* genetic background is also shown (D, solid squares). At time zero, cell samples at the late exponential phase were diluted 20-fold (to $\sim 5 \times 10^7$ cells/ml) in fresh medium. The stationary phase (2×10^9 cells/ml) was reached in all cases after 24 hr of growth. Specific β-galactosidase activities are given in nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

(GenBank accession no. AJ505753). A computer-aided search for coding regions detected a 2436-bp-long open reading frame (ORF) in the correct transcriptional orientation. Neither this ORF nor the various, much shorter ORFs that could be detected in other reading frames exhibited significant similarity to known proteins or DNA sequences.

A DNA stretch of ~2.2 kb from the *ddmA* locus was sequenced (GenBank accession no. AJ505849), and it was found to contain the 5′ end of a long ORF (2052 bp). The overall G + C content of this ORF is 71%, well in accord with the 68% G + C content of the whole genome of *M. xanthus*, but 93% of the codons contain G or C at their third positions. This bias at third codon positions is normally found in organisms with high G + C content (Bibb *et al.* 1984). The amino acid sequence predicted from the indicated ORF showed significant similarity with a family of outer membrane proteins that serve to import iron siderophores, vitamin B_{12} or colicins, in a process that requires the participation of protein TonB and other associated proteins at the inner membrane (Lazdunski *et al.* 1998; Moeck and Coulton 1998). Figure 2 shows the alignment of the protein predicted from the *ddmA* locus and the two proteins from the data bank showing the highest similarity. These were a hypothetical protein from *Chlorobium tepidum* (28.5% identity and 45% similarity over 75% of the predicted *ddmA* gene product) and CirA, the iron-regulated colicin I receptor from *E. coli* (31.2% identity and 49.5% similarity over 53% of the *ddmA* gene product). For economy of space, only part of the alignment is shown in Figure 2. This includes a region near the N terminus, which is highly conserved in TonB-dependent transport proteins.

Two ORFs were identified in the 2.25-kb DNA stretch sequenced from the *ddmB* locus (GenBank accession no. AJ505751). They both run in the correct transcriptional orientation, and they are separated by only 3 bp, so they are likely to be transcribed from the same promoter. Both of them showed a strong bias for G or C at third codon positions (>90%). The first one, the one interrupted by transposon Tn*5-lac*, would code for a protein (158 amino acids) showing high similarity to members of the AsnC subfamily of bacterial helix-turn-helix (HTH) transcription factors (Kyrpides and Ouzounis 1995; Newman and Lin 1995). Figure 3A shows the alignment of this protein with the most similar protein in the data bank (40% identity and 65% similarity over a 148-residue stretch) and with the consensus for the HTH domain of AsnC-like proteins (41.3% identity and 61.3% similarity over a 150-residue stretch; see legend of Figure 3). The protein predicted from the second ORF of the *ddmB* locus (406 amino acids) exhibited strong similarity to class I and II aminotransferases (identity >46% and similarity >63%; see Figure 3B).

Two ORFs, both running in the same direction, were detected in the sequenced DNA stretch (~3.25 kb) from the *ddmC* locus (GenBank accession no. AJ505752). The one interrupted by Tn*5-lac* (88% G + C at third codon positions) would code for a 583-residue-long peptide, which showed significant similarity to the family of outer membrane efflux proteins (OEP). A common feature of these proteins is the presence of a large tandem duplication. The duplicate elements show low intramolecular or intermolecular similarity, but they share some structural features. Each element contains two heptad repeats of nonpolar residues. The first one is followed by the consensus sequence θθ"Pro"*x*θ*x*θ (θ is a nonpolar amino acid, *x* is any amino acid), whereas the second one is followed by the consensus sequence "Gly"*xxxxx*"Asp" (Johnson and Church 1999). As shown in Figure 4, the product predicted from the indicated ORF of the *ddmC* locus contains a large tandem duplication. The C-terminal element of this duplication shows all the indicated features of the OEP duplicate elements, whereas its N-terminal element is missing the Gly residue that follows the second heptad repeat of nonpolar amino acids. The OEP are the outer membrane components of broad-specificity efflux pumps

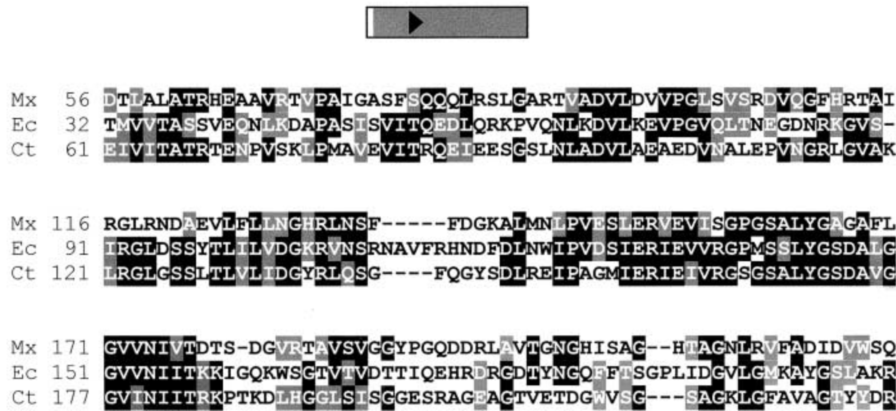

FIGURE 2.—The *ddmA* locus codes for a TonB-dependent outer membrane protein. The bar at the top represents the sequenced DNA stretch (2.2 kb) from the *ddmA* locus (DDBJ/EMBL/GenBank accession no. AJ505849). The approximate positions of the Tn*5*-*lac* insertion (solid triangle) and the single 2052-bp ORF found in this locus (solid portion) are indicated (similar representations are incorporated at the top of Figures 3 and 4). Shown below is a partial alignment of the predicted amino acid sequence of the *M. xanthus* protein (Mx, accession no. Q8KM34), the CirA protein from *E. coli* (Ec, accession no. Q8X633), and a putative TonB-dependent receptor from *C. tepidum* (Ct, accession no. Q8KBN2). Identical residues are indicated by a solid background, while similar residues are shaded (as in Figures 3 and 4).

that confer multidrug resistance to *Pseudomonas aeruginosa* and other gram-negative bacteria (Nikaido 1998). These pumps share a common, three-component organization, with the OEP being connected to an inner membrane efflux protein through a periplasmic efflux protein. The genes coding for the components of a particular efflux pump are often found in the same operon. This may also be the case here, as the predicted product of the second ORF from the *ddmC* locus showed significant sequence similarity with various bacterial periplasmic proteins (data not shown).

The new CarD-dependent promoters and the transcription factors CarQ and IhfA: As mentioned in the Introduction, two *M. xanthus* promoters previously known to be dependent on CarD, P_{QRS} and P_I, are controlled by the ECF σ-factor CarQ, P_{QRS} being also dependent on the DNA architectural protein IHF. The effect of the lack of CarQ or IHF on the newly identified, CarD-dependent promoters was tested. For this, the four Tn*5*-*lac* insertions were separately transduced (selection for Km^R) into *M. xanthus* strains MR132 and MR358 (see Table 1, section I). MR132 carries a loss-of-function mutation in *carQ* (*carQ1*), whereas MR358 carries a loss-of-function mutation in *ihfA* (*ihfA2*). The *ihfA* gene codes for one of the two subunits of the heterodimeric IHF protein. Fifty transductants from each cross were tested on X-Gal plates to check for homogeneity, and various, randomly picked colonies were then used for quantitative analysis of *lacZ* expression. None of the tested promoters were affected by mutation *carQ1* (data not shown). With respect to the *ihfA2* mutant, only the tagged genes *ddvA* and *ddmB* could be tested. The *ihfA2* mutation did not affect the activity of the *ddvA* promoter (data not shown), but it produced a strong negative effect on the *ddmB* promoter (Figure 1D). When the Tn*5*-*lac* insertions at *ddmA* or *ddmC* were transferred to MR358, very few, abnormally small transductant colonies were observed. These colonies could hardly be regrown on solid media, and they did not grow at all in liquid media. We have previously reported that the *ihfA2* mutation decreases the growth rate of the wild-type strain DK1050, and it is even lethal for wild-type DK1622 (Moreno *et al.* 2001). By themselves, neither the Tn*5*-*lac* insertion at *ddmA* nor that at *ddmC* caused a detectable growth defect, but somehow they may both enhance the growth impairment caused by the *ihfA2* mutation.

A search for suppressors of *carD1*: To probe for possible regulatory partners of CarD, and for possible regulatory connections between different CarD-dependent promoters, a search for suppressors of the *carD1* mutation was carried out. To facilitate subsequent strain manipulations, this search was initiated with a strain-bearing mutation *carD1* and a plasmid-based *lacZ* transcriptional fusion to the strong *ddvA* promoter. The *carD1* mutant used here was MR563 (Table 1, section I). It derives from the wild-type strain DK1622, which has been used as the standard strain in most studies on multicellular development in *M. xanthus*. The transcriptional fusion was generated by inserting a 2.2-kb DNA fragment from the *ddvA* locus upstream of the promoterless *lacZ* gene present in the plasmid vector pDAH274 (Km^R). The fragment was chosen because one of its ends corresponds to a site well upstream of the ORF detected in the *ddvA* locus, and the other end was situated within that ORF, near the original site of insertion of Tn*5*-*lac* in *ddvA*. The chimeric plasmid carrying the 2.2-kb fragment in the correct orientation for transcription of the *lacZ* probe, pMAR932, was transduced into wild-type DK1622 and the *carD1* mutant MR563 (selection for Km^R). Since the plasmid cannot replicate in *M. xanthus*, Km^R colonies arise from an integrative, homologous recombination event between the *M. xanthus* DNA in pMAR932 and the homologous chromosomal region in the recipient strains. Various of the wild-type- and *carD1*-

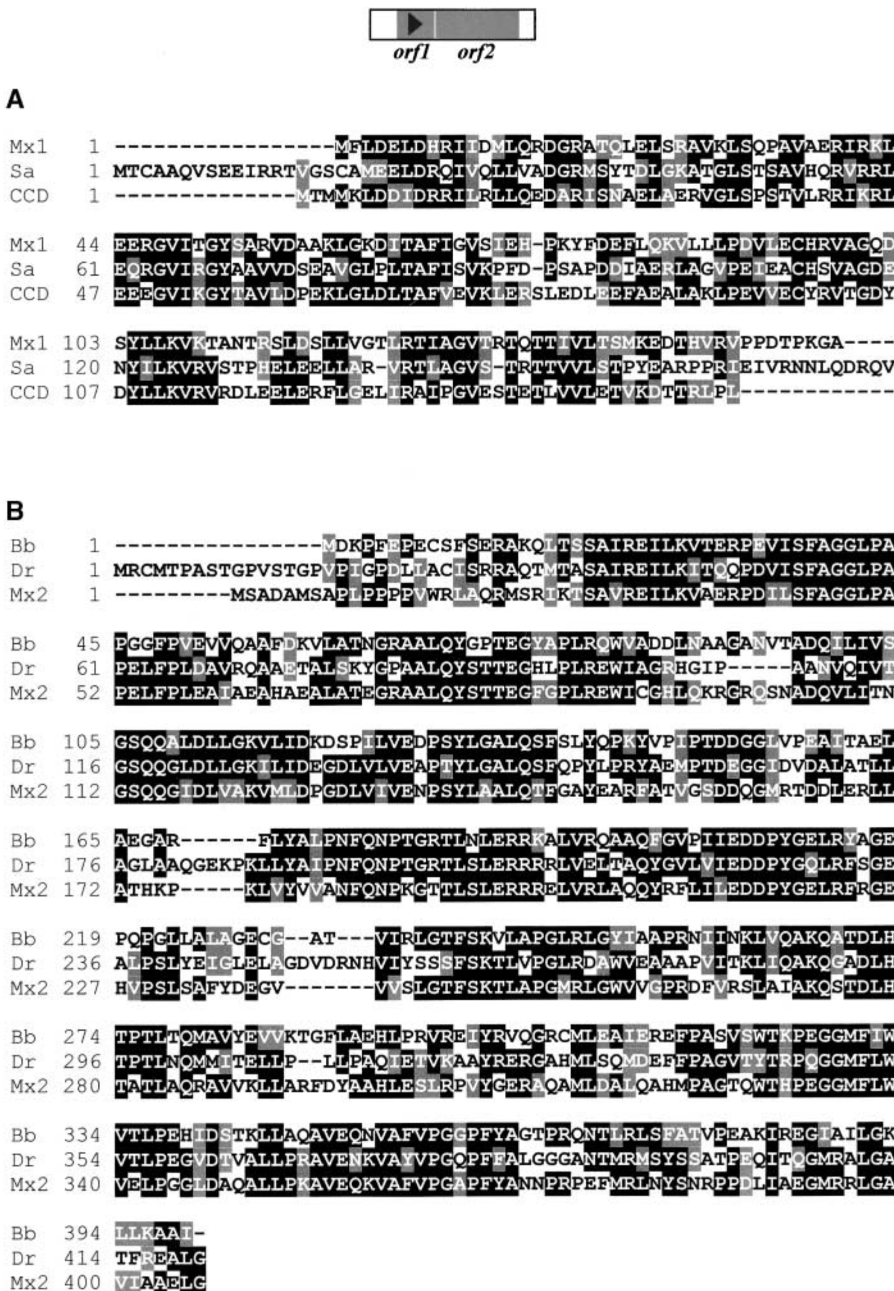

FIGURE 3.—Sequence analysis of the *ddmB* locus. Two open reading frames, *orf1* and *orf2*, have been detected in this locus (DDBJ/EMBL/GenBank accession no. AJ505751). (A) The protein predicted from *orf1* (Mx1, 158 amino acids) belongs to the AsnC subfamily of bacterial HTH transcription factors. Sequence alignment with a transcriptional regulator from *Streptomyces avermitilis* (Sa, accession no. Q827K3) and the HTH consensus for the indicated protein subfamily (conserved domain database number CDD3885) is shown. (B) Sequence alignment of the protein predicted from *orf2* (Mx2, 406 amino acids) with aminotransferases from *Bordetella bronchiseptica* (Bb, accession no. Q7W-R56) and *Deinococcus radiodurans* (Dr, accession no. Q9RU08).

derived merodiploids were checked on X-Gal plates. As expected, all the wild-type-derived transductants developed an intense blue color, whereas all the *carD1*-derived transductants did not, thus confirming the CarD dependence of the *lacZ* fusion. A wild-type- and a *carD1*-derived transductant were purified and named MR1141 and MR1142, respectively.

Various independent cultures of MR1142 were subjected to mutagenesis with UV light, plated on X-Gal, and screened for recovery of the blue color phenotype. Around 8000 mutagenized colonies were checked, and four independent candidate colonies were identified. They all appeared to retain the *carD1* allele, as they all showed the associated Tc^R Car^- phenotype. So, each of those four colonies were likely to contain a second mutation that would suppress the negative effect of *carD1* on the *ddvA* promoter, but not the known negative effect of *carD1* on the P_{QRS} and P_I promoters (see Introduction). The strains were named MR1157, MR1158, MR1197, and MR1198, and the corresponding suppressor mutations have been provisionally called *sud-1–4* (from **su**ppressor of *car**D***), respectively. As shown in Figure 5A, suppression was only partial in all cases, as none of the suppressed strains reached the β-galactosidase expression level generated when the *lacZ*-tagged *ddvA* promoter was introduced in wild-type DK1622 (compare also Figure 5A with Figure 1A).

The *sud-2* mutation suppresses the effect of *carD1* on other CarD-dependent promoters: As mentioned before, mutation *carD1* blocks the starvation-induced multi-

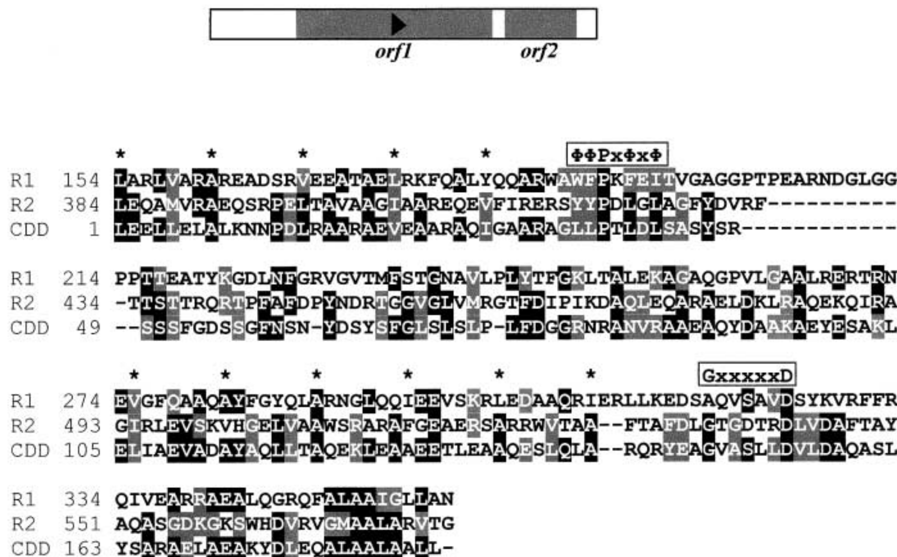

Figure 4.—The *ddmC* locus codes for an OEP. Two ORFs were detected in the 3.25-kb DNA stretch sequenced from the *ddmC* locus (DDBJ/EMBL/GenBank accession no. AJ505752). The one interrupted by Tn*5-lac* (*orf1*) contains an internal duplication of ∼200 residues, a feature characteristic of the OEP proteins. The alignment of the two elements of the Orf1 duplication (R1, residues 154–359, and R2, residues 384–576) and the consensus for the repeated element of OEP proteins (conserved domain database no. CDD25897) are shown. Two well-conserved motifs in the OEP consensus, both of which are preceded by heptad repeats of nonpolar amino acids, are shown in boxes above the alignment (Φ, nonpolar amino acid; x, any amino acid). The heptad repeats are marked by asterisks.

cellular process, which leads to the formation of fruiting bodies and sporulation. When starved for nutrients, the *carD1* mutant cells initiate the formation of loose multicellular aggregates, but fewer in number than the wild-type cells do. Furthermore, the aggregation process suffers an early stop in the mutant and does not culminate, as in the wild type, in the formation of compact, clearly defined fruiting bodies that turn dark as the cells inside develop into refringent spores (Nicolás *et al.* 1994). When checked for the starvation response, strain MR1158 (*ddvA*::*lacZ*, *carD1*, *sud-2*), but not the other suppressed strains, was observed to form well-defined, dark fruiting bodies (Figure 6). These were filled with mature, refringent spores, as observed under the microscope (not shown). The final number of fruiting bodies was ∼35% of the number of fruiting bodies formed by the wild type, suggesting that *sud-2* also partially suppresses the developmental defect caused by mutation *carD1*. This prompted us to further characterize strain MR1158.

First, the plasmid-based transcriptional probe inserted at the *ddvA* locus was removed from MR1158. For this, we relied on intramolecular recombination to revert the original plasmid integration event. Loss of the plasmid should result in loss of both *lacZ* expression and the Km^R phenotype. Strain MR1158 was grown for 120 generations in the absence of kanamycin and plated on X-Gal. Several colonies that did not develop a blue color were identified, all of which were sensitive to kanamycin. The loss of the plasmid was confirmed by Southern analysis (data not shown). The new strain, which should still carry the *carD1* and *sud-2* mutations, was named MR1165.

To check for linkage between the *carD1* and the *sud-2* mutations, the Tc^R marker (*carD1*) of MR1165 was transduced into strain MR1141, the *carD*$^+$ derivative bearing the plasmid-based transcriptional probe at *ddvA*. If there were a significant linkage between *carD1* and *sud-2*, many of the Tc^R transductant colonies would develop a blue color on X-Gal plates. More than 200 independent transductants from the indicated transductions were checked on X-Gal plates, and only 3 of them developed a blue color. These colonies could result from the translocation of the Tc^R transposon to a new site or might be indicative of a very loose linkage between *carD1* and *sud-2*.

Strain MR1165 was finally used to check if the *sud-2* mutation could also suppress the effect of *carD1* on other CarD-dependent promoters. The Tn*5-lac* transposon insertions at the *ddmA*, *ddmB*, and *ddmC* loci were transduced into MR1165 (selection for Km^R). As a control, the transposon inserted at the *ddvA* locus was also transduced into MR1165. Plasmid-based (Km^R) *lacZ* transcriptional fusions to the CarD-dependent promoters P_{QRS} and P_I were also separately transferred to MR1165 (Hodgson 1993; Fontes *et al.* 1993; see materials and methods). In all cases, ∼100 transductant colonies were tested on X-Gal plates to check for homogeneity (light and dark conditions were used for the transductants carrying the *lacZ* probe at P_{QRS} or P_I). In agreement with the Car^- phenotype of MR1165, none of the transductants carrying *lacZ* fused to the P_{QRS} or the P_I promoters developed a blue color in the dark or the light. Thus, *sud-2* does not suppress the negative effect of *carD1* on those two promoters. The intensity of the blue color was the same for all sister Km^R transductants from the rest of the transduction experiments, suggesting that none of the involved loci (*ddvA*, *ddmA*, *ddmB*, and *ddmC*) are linked to the *sud-2* mutation. On X-Gal plates *sud-2* also appears to suppress the effect of *carD1* on the *ddmA* and *ddmB* promoters and less so on the *ddmC* promoter. These conclusions were confirmed by quantitative analyses of β-galactosidase in liquid cultures of the wild type, the *carD1* single mutant, and the *carD1 sud-2* double mutant, all carrying the same *lacZ* transcriptional fusion (Figure 5, B–E; the results for the

TABLE 1

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

Strain[a]	Phenotype[b]	Relevant genotype	Reference or source
		I. Basic strains	
DK1050	Car^+ Fru^+		Ruiz-Vázquez and Murillo (1984)
DK1622	Car^+ Fru^+		Kaiser (1979)
MR132	Car^- Fru^+	*carR4 carQ1*	Martínez-Laborda and Murillo (1989)
MR358	Car^- Fru^-	*ihfA2*	Moreno *et al.* (2001)
MR522	Car^- Fru^- Tc^R	*carD1* (in DK1050)	Nicolás *et al.* (1994)
MR563	Car^- Fru^- Tc^R	*carD1* (in DK1622)	Nicolás *et al.* (1994)
		II. Strains carrying CarD-dependent Tn*5-lac* insertions	
MR900	Car^+ Fru^+ Km^R	*ddvA::*Tn*5-lac*	This work
MR902	Car^- Fru^- Km^R Tc^R	*ddvA::*Tn*5-lac carD1*	This work
MR915	Car^+ Fru^+ Km^R	*ddmB::*Tn*5-lac*	This work
MR1156	Car^- Fru^- Km^R Tc^R	*ddmB::*Tn*5-lac carD1*	This work
MR1100	Car^+ Fru^+ Km^R	*ddmA::*Tn*5-lac*	This work
MR1101	Car^- Fru^- Km^R Tc^R	*ddmA::*Tn*5-lac carD1*	This work
MR1104	Car^+ Fru^+ Km^R	*ddmC::*Tn*5-lac*	This work
MR1105	Car^- Fru^- Km^R Tc^R	*ddmC::*Tn*5-lac carD1*	This work
MR905	Car^+ Fru^+ Km^R	*ddsA::*Tn*5-lac*	This work
MR1196	Car^- Fru^- Km^R Tc^R	*ddsA*::Tn*5-lac carD1*	This work
MR1102	Car^+ Fru^+ Km^R	*ddwA::*Tn*5-lac*	This work
MR1103	Car^- Fru^- Km^R Tc^R	*ddwA*::Tn*5-lac carD1*	This work
		III. Strains related to the *carD1* supressors	
MR1141	Car^+ Fru^+ Km^R	*ddvA*::*lacZ*	This work
MR1142	Car^- Fru^- Km^R Tc^R	*ddvA*::*lacZ carD1*	This work
MR1157	Car^- Fru^- Km^R Tc^R	*ddvA*::*lacZ carD1 sud-1*	This work
MR1158	Car^- Fru^+ Km^R Tc^R	*ddvA*::*lacZ carD1 sud-2*	This work
MR1197	Car^- Fru^- Km^R Tc^R	*ddvA*::*lacZ carD1 sud-3*	This work
MR1198	Car^- Fru^- Km^R Tc^R	*ddvA*::*lacZ carD1 sud-4*	This work
MR1165	Car^- Fru^+ Tc^R	*carD1 sud-2*	This work

[a] Strains are classified into different groups for clarity. Group I includes standard strains, followed by strains carrying previously reported mutations, used here as probe strains. Group II includes strains carrying Tn*5-lac* insertions at one of the new CarD-dependent genes identified in this work. Group III includes the strains used to search for suppressors of *carD1* and the suppressor mutants.

[b] Car^+/Car^-, wild-type/mutant phenotype for light-induced synthesis of carotenoids; Fru^+/Fru^-, wild-type/mutant phenotype for starvation-induced fruiting formation.

P_I::*lacZ* fusion, similar to those obtained for the P_{QRS}::*lacZ* fusion, are not shown).

DISCUSSION

Gene *carD* was identified by a mutation, *carD1*, that simultaneously affected two different responses of the bacterium *M. xanthus* to external stimuli: the light-induced synthesis of carotenoids and the starvation-induced development of fruiting bodies. The normal *carD* gene product was shown to be required for the proper transcriptional activation of two unlinked, light-inducible gene loci and to be directly or indirectly required for the transcriptional activation of seven developmentally specific genes (Nicolás *et al.* 1994). We have now used a mobile transcriptional probe, transposon Tn*5-lac*, in a random search for CarD-regulated genes. A total of 613 properly oriented Tn*5-lac* insertions, which are normally transcribed in the dark during vegetative growth in rich medium, were screened. As shown here, mutation *carD1* alters the normal expression of six different tagged genes. A minor effect of *carD1* on five other tagged promoters was observed (Figure 1; see results). On a broad scale, the integration of Tn*5-lac* in the *M. xanthus* chromosome occurs at random (Kroos and Kaiser 1984). Assuming random insertion of Tn*5-lac* at least with respect to the CarD dependence of a particular gene, our results indicate that close to 1% of the *M. xanthus* genes expressed during vegetative growth might be controlled by CarD. This strengthens the idea that CarD acts as a global transcription factor. However, the normal growth rate and plating efficiency of the *carD1* mutant seem to suggest that CarD does not play an essential role during vegetative growth, at least under laboratory conditions.

The light- or starvation-induced, CarD-dependent

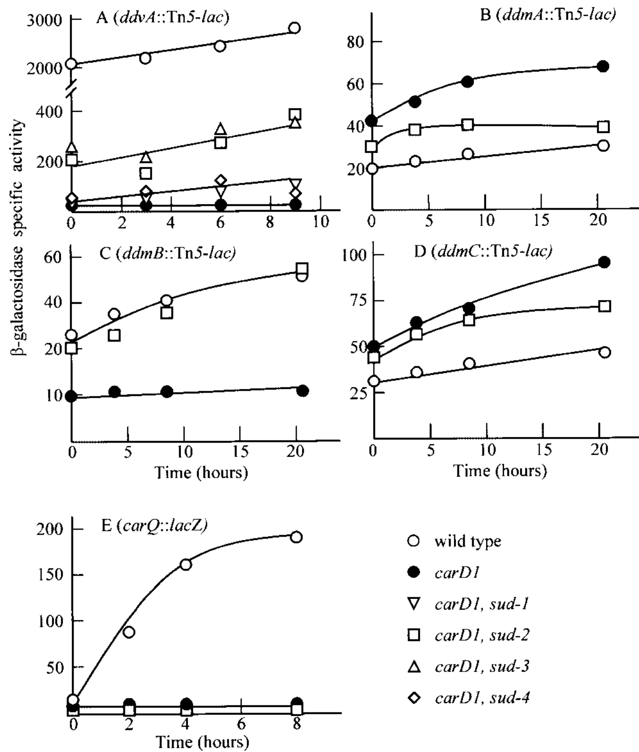

Figure 5.—The *carD1* suppresor mutations. (A) The time course of β-galactosidase expression from the same *ddvA*::*lacZ* transcriptional fusion in different genetic backgrounds: *carD*$^+$ (○), *carD1* (●), *carD1 sud-1* (▽), *carD1 sud-2* (□), *carD1 sud-3* (△), and *carD1 sud-4* (◇). Note the different scales in the bottom and top part of A. B–D show similar data from Tn*5*-*lacZ* insertions in *ddmA* (B), *ddmB* (C), and *ddmC* (D); symbols as in A. Experimental details for A–D as in Figure 1. (E) Time course of β-galactosidase expression from a *lacZ* transcriptional fusion to the light-inducible promoter P_{QRS} in different genetic backgrounds (symbols as before). At time 0, dark-grown cultures at late exponential phase were divided in two, one for the dark and the other for the light. For simplicity, only the results of the light-grown samples are shown (none of the samples from the dark-grown cultures gave β-galactosidase-specific activities above 10 units).

genes mentioned above were all negatively affected by mutation *carD1* (Nicolás *et al.* 1994). As shown in Figure 1, four of the newly identified CarD-dependent genes are transcribed at a higher level in the *carD1* mutant. Therefore, protein CarD can somehow participate in mechanisms for either up- or downregulation of gene expression, as shown for other bacterial proteins that act as global transcription factors. A well-known case is the IHF, a DNA architectural protein for many processes that involve higher-order protein-DNA complexes, including site-specific recombination, transcriptional regulation, and replication (Nash 1996; Colland *et al.* 2000; Moreno *et al.* 2001; and references therein).

The nucleotide sequences of four of the vegetative, CarD-dependent genes are also reported here. Their analysis does not reveal any obvious physiological connection among all of them. However, two loci, *ddmA*

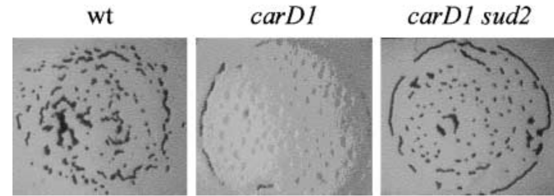

Figure 6.—Developmental behavior of *M. xanthus* strains. Photographs were taken 4 days after the samples (5 μl, 10^9 cells/ml) were spotted on starvation plates. Samples are from the wild-type strain DK1622 (left) and its derivatives, MR563 (middle) and MR1165 (right). As shown, DK1622 and MR1165 formed well-defined, spore-filled (dark) fruiting bodies. As previously reported (Nicolás *et al.* 1994), MR563 exhibited only weak signs of cell aggregation.

and *ddmC*, seem to code for membrane-associated proteins that in other bacterial species are involved in transport phenomena (Figures 2 and 4). Both *ddmA* and *ddmC* are negatively regulated by CarD (Figure 1, C and E), which may suggest the presence of a CarD-dependent regulatory network that is common to at least a subset of the *M. xanthus* transport systems. A third CarD-dependent locus, *ddvA*, codes for a product of unknown function, whereas the fourth one, *ddmB*, codes for a DNA-binding protein that likely acts as a transcription factor (Figure 3). The apparent CarD dependence of certain genes may then be an indirect consequence of CarD controlling the expression of other regulatory proteins. In fact, this has been experimentally proved in the case of the *M. xanthus* light regulon. In this regulon, the transcriptional activation of the *carQRS* operon by blue light requires direct binding of CarD to a particular site at the P_{QRS} promoter. As the *carQRS* operon includes a gene, *carS*, whose product is required for the transcriptional activation of a different, unlinked operon, the latter also appears to be regulated by CarD (Nicolás *et al.* 1994, 1996).

The σ-factor CarQ controls the activities of the CarD-dependent, light-inducible promoters P_{QRS} and P_I (see Introduction). Lack of CarQ does not affect the expression of any of the other CarD-dependent genes—neither the expression of those that are developmentally activated (Nicolás *et al.* 1994) nor the expression of those that are expressed during vegetative growth (this work). This is consistent with previous evidence indicating that σ-factor CarQ is sequestered in the dark by an anti-σ-factor and that it is released in an active form only upon illumination (Gorham *et al.* 1996; Fontes *et al.* 2003). The light activation of P_{QRS}, but not that of P_I, also depends on the *M. xanthus* IHF protein (Moreno *et al.* 2001). As shown here, the normal expression of the CarD-dependent gene *ddmB* also depends on IHF, but that of gene *ddvA* does not. This suggests that the direct or indirect collaboration of CarD and IHF in gene regulation, although not mandatory, may not be uncommon in *M. xanthus*. Altogether, these observations strengthen

the versatility of CarD function, in the sense that it is recruited for the proper operation of different transcriptional regulatory complexes.

Hints on the mechanism of action of CarD came from the striking similarity of its DNA-binding domain to that of eukaryotic HMGA proteins and from studies on the binding of CarD to the P_{QRS} promoter. HMGA proteins are abundant, chromatin-associated proteins that serve as DNA architectural factors for a variety of cellular functions. Its DNA-binding domain is formed by multiple repeats of a conserved Arg-Gly-Arg-Pro sequence (the "AT-hook" motif) embedded in a less-conserved cluster of basic and proline residues (Reeves and Nissen 1990; Aravind and Landsman 1998). The most extensively studied HMGA proteins, the mammalian HMGA isoforms, are small proteins ($\leq$107 residues) with three AT-hooks lying between a highly acidic C-terminal stretch and a short N-terminal region. The AT-hooks bind specifically to the narrow minor groove of appropriately spaced AT-rich tracts. Mammalian HMGA proteins act as general transcription factors because they facilitate the binding of specific transcription factors to their cognate promoter regions. This is done by the HMGA-mediated modulation of local DNA conformation and/or by direct protein-protein interactions. Both HMGA-dependent activator or repressor complexes have been reported (Falvo *et al.* 1995; Bagga and Emerson 1997; Yie *et al.* 1997; Aravind and Landsman 1998; Bustin 1999). The *M. xanthus* protein CarD (316 amino acids) has four AT-hooks, and its acidic region is situated toward the N rather than toward the C terminus of the AT-hook region. The only well-characterized DNA-binding site of CarD, that of the P_{QRS} promoter, is also formed by appropriately spaced AT-rich tracts, and the binding is also to the narrow minor groove (Nicolás *et al.* 1994; Padmanabhan *et al.* 2001). Furthermore, like HMGA proteins, CarD can be phosphorylated *in vitro* at the acidic region, and this dramatically decreases the DNA-binding affinity of CarD, although the possible functional role of this phosphorylation is not known (Cayuela *et al.* 2003). Given all these similarities to mammalian HMGA proteins, one may speculate that the function of CarD is to facilitate the building of distinct nucleoprotein complexes through changes in DNA topology and/or binding to specific transcription activators or repressors. Unlike the mammalian HMGA proteins, the acidic and AT-hook portions of CarD are preceded by a large N-terminal stretch of ~180 amino acids. Although the function of this domain is not yet known, it is also present and highly conserved in the CarD protein of *Stigmatella aurantiaca*, a relative of *M. xanthus* (Cayuela *et al.* 2003).

As a first step in the identification of other gene products that might participate in the CarD-dependent regulatory networks, a search for *carD1* suppressor mutations has been carried out. For this, we took advantage of the strong negative effect of *carD1* on the very active *ddvA* promoter (Figure 1A). Mutagenized *ddvA*::*lacZ carD1* cells were plated and screened for a significant increase in the response to X-Gal. In this way, four independent mutations (*sud-1–4*) were identified that partially suppress the effect of *carD1*. Three of them do not suppress the effect of mutation *carD1* on the light- or the starvation-induced responses. The fourth mutation, *sud-2*, is also ineffective in suppressing the effect of *carD1* on the light response. Quite interestingly, however, *sud-2*, which is not linked to *carD* or to any of the studied *lacZ*-tagged genes, partially suppresses the *carD1* effect on multicellular development (Figure 6) and on the expression of at least two other vegetative genes. These include both down- and upregulated genes (Figure 5). This result suggests that the gene product affected by *sud-2* is somehow associated with CarD in the operation of a single mechanism, which underlies many of the apparently diverse regulatory phenomena that we have ascribed to CarD in this and previous works (the light induction of gene expression would be one exception). The *sud-2* mutant strain should be instrumental in the identification of the corresponding gene product. This in turn may reveal some clues about the proposed molecular operation in which it would participate together with CarD.

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