Light-dependent gene regulation by a coenzyme B_{12}-based photoreceptor

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Cobalamin (B_{12}) typically functions as an enzyme cofactor but can also regulate gene expression via RNA-based riboswitches. B_{12}-directed gene regulatory mechanisms via protein factors have, however, remained elusive. Recently, we reported down-regulation of a light-inducible promoter in the bacterium *Myxococcus xanthus* by two paralogous transcriptional repressors, of which one, CarH, but not the other, CarA, absolutely requires B_{12} for activity even though both have a canonical B_{12}-binding motif. Unanswered were what underlies this striking difference, what is the specific cobalamin used, and how it acts. Here, we show that coenzyme B_{12} (5′-deoxyadenosylcobalamin, $AdoB_{12}$), specifically dictates CarH function in the dark and on exposure to light. In the dark, $AdoB_{12}$-binding to the autonomous domain containing the B_{12}-binding motif foments repressor oligomerization, enhances operator binding, and blocks transcription. Light, at various wavelengths at which $AdoB_{12}$ absorbs, dismantles active repressor oligomers by photolysing the bound $AdoB_{12}$ and weakens repressor–operator binding to allow transcription. By contrast, $AdoB_{12}$ alters neither CarA oligomerization nor operator binding, thus accounting for its B_{12}-independent activity. Our findings unveil a functional facet of $AdoB_{12}$ whereby it serves as the chromophore of a unique photoreceptor protein class acting in light-dependent gene regulation. The prevalence of similar proteins of unknown function in microbial genomes suggests that this distinct B_{12}-based molecular mechanism for photoregulation may be widespread in bacteria.

carotenogenesis | *Thermus thermophilus* | antirepressor | MerR | TtCarH

B_{12} is an essential vitamin in humans and other animals, and the only vitamin synthesized exclusively by microorganisms (1–5). It is also the largest and most complex of nonpolymeric biomolecules, whose central feature is a tetrapyrrole-derived cobalt-containing corrin moiety. Two distinct B_{12} forms are used in vivo (4, 6): one ($AdoB_{12}$) has 5′-deoxyadenosine and the other (methylcobalamin, MeB_{12}) has a methyl coordinated to the corrin cobalt. The stable but nonfunctional vitamin B_{12} or cyanocobalamin (CNB_{12}), with a cyano–cobalt bond, must be converted to $AdoB_{12}$ or MeB_{12} for intracellular use. Typically, B_{12} functions as an essential cofactor of enzymes such as isomerases, methyltransferases, and reductases (6, 7). B_{12} has also been implicated in directly regulating gene expression via the B_{12} riboswitch, an RNA-based mechanism widespread in bacteria (8–11). Here, specific binding of $AdoB_{12}$ to the 5′-untranslated messenger RNA leader sequence of a target gene or operon (usually involved in B_{12} synthesis or transport) allosterically modulates RNA structure to regulate gene expression. In contrast to this RNA-based switch, B_{12}-directed gene regulation via a protein factor is virtually uncharted. We recently reported that B_{12} is absolutely required by a transcriptional repressor to down-regulate a light-inducible promoter in the bacterium *Myxococcus xanthus* (12). However, the molecular mechanism for this B_{12}-dependent gene regulation has remained enigmatic.

Blue light is known to trigger a well studied regulatory cascade in *M. xanthus* that culminates in the synthesis of carotenoids, which protect cells against photooxidative damage (13). Cells usually sense light, a key environmental factor, via photoreceptor proteins containing small-molecule chromophores, and six such photoreceptor families have been cataloged in bacteria (14–16). However, none of these have been implicated in *M. xanthus* light-induced carotenogenesis, in which available data suggest that light is sensed by a photosensitizer molecule, protoporphyrin IX, rather than by a dedicated photoreceptor protein (13, 17). This essentially leads to the release of the extracytoplasmic function σ-factor CarQ from its membrane-bound anti–σ-factor CarR. Freed CarQ then associates with core RNA polymerase and, together with a number of other accessory factors, drives the expression of one carotenogenic gene, *crtIb,* as well as of the regulatory *carQRS* operon, which encodes CarS besides CarQ and CarR (Fig. S1*A*) (13). CarS is an antirepressor with an SH3-domain topology that mimics operator DNA to specifically target the DNA recognition helix of the MerR-type DNA-binding winged-helix domain of two paralogous repressors, CarA and CarH, to counteract their binding to operator (18, 19). In the dark, these two repressors down-regulate the promoter P_B that drives expression of the *carB–carA* gene cluster, where the remaining carotenogenic genes are grouped (Fig. S1*A*). CarA and CarH have a similar two-domain architecture (Fig. 1*A* and Fig. S1*B*). Besides their autonomously stable, N-terminal DNA-binding domains (CarANt and CarHNt, respectively) that recognize the same operator (12, 19, 20), CarA and CarH have C-terminal domains (CarACt and CatHCt, respectively) with a B_{12}-binding motif of the type found in enzymes like methionine synthase (6, 12, 21–23). However, only CarH requires B_{12} for its repressor activity (12).

Why CarH, and not CarA, depends on B_{12}, what is the B_{12} form involved, and how it is linked to a light response are studied here. We show that it is $AdoB_{12}$ that specifically directs CarH activity. Our studies in vivo and in vitro uncover an ability of $AdoB_{12}$ to induce repressor oligomerization and markedly enhance operator DNA binding to bring about gene repression in the dark. Furthermore, the photosensitivity of $AdoB_{12}$ (24–26) is exploited in light-induced disassembly of active repressor oligomers to diminish operator binding and relieve P_B repression in vivo, thus linking B_{12} to the light response. $AdoB_{12}$ did not produce similar effects with CarA, thereby providing a rationale for its B_{12}-

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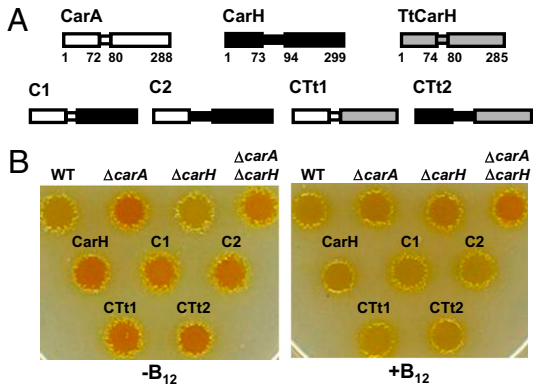

Fig. 1. A single module confers $AdoB_{12}$-dependent activity in vivo. (*A*) Schematic representations of CarA, CarH, TtCarH, and the chimeras derived from them. Wider rectangles represent N- and C-terminal domains, the narrower one the linker between them, with regions in white, black, and gray corresponding to CarA, CarH, and TtCarH, respectively. Numbers correspond to residues delimiting the indicated segment. (*B*) Color phenotype in the presence or absence of 1 μM CNB_{12} ("B_{12}") in the dark of *M. xanthus* WT strain and those with the genotype indicated above the cell spots. C1, C2, CTt1, CTt2, or CarH (as positive control) are expressed from a heterologous site in the Δ*carA* Δ*carH* strain.

independent action. The $AdoB_{12}$-based mechanism serves as a direct route to P_B photoregulation by CarH, distinct from the one involving CarS described previously. More importantly, our findings reveal a functional role of $AdoB_{12}$: that as a chromophore of broad spectral sensitivity in a unique type of photoreceptor protein. Given the numerous CarA/CarH-like proteins of unknown functions being identified in other bacterial species from genome data (Table S1), it is likely that the $AdoB_{12}$-based photoregulation mechanism we describe here is widely used in bacteria.

Results

A Single Autonomous Module Confers B_{12}-Dependent Activity in Vivo. To address why CarH but not CarA requires B_{12} for activity, we first asked if replacing CarACt by CarHCt can confer B_{12}-dependent repressor activity. For this, we constructed two chimeric proteins, C1 and C2, in which CarANt is linked to CarHCt by the shorter CarA "linker" (C1) or the longer CarH one (C2; Fig. 1*A* and Fig. S1*B*). C1 and C2 were then introduced into a strain lacking both CarA and CarH, so that neither of the endogenous proteins can mask the function of the introduced chimera, which was directly assessed by the colony color phenotype.

WT *M. xanthus* cells are yellow in the dark and turn red in the light from the carotenoids produced by expressing all of the structural genes (Car^+ phenotype; Fig. 1*B* and Fig. S1 *A* and *C*). In the dark, cells lacking CarH exhibit the Car^+ phenotype (yellow) because CarA represses P_B. By contrast, those lacking CarA are yellow when B_{12} (CNB_{12}, $AdoB_{12}$, or MeB_{12}) is present in the growth medium and CarH can repress P_B, but are orange when B_{12} is absent, as *carB*, though not *crtIb*, is expressed [$Car^{(+)}$ phenotype] (12). Cells lacking CarA and CarH are always orange in the dark (Car^C) because *carB* is not repressed (Fig. 1*B*). In the light, all three mutant strains become red, like the WT, as both *carB* and *crtIb* are turned on (Fig. S1*C*). A plasmid construct with the gene for C1 or C2 (or CarH, as a control) expressed from a constitutive promoter (12, 20) was introduced into the *M. xanthus* strain lacking endogenous CarA and CarH, where it integrates at an unlinked site in the chromosome. Unlike the Car^C recipient strain, the resulting transformants were $Car^{(+)}$: yellow in the dark only in the presence of B_{12} and red in the light, just like the control strain with CarH (Fig. 1*B* and Fig. S1*C*). Thus, C1 and C2 behave like CarH, indicating that swapping CarACt for CarHCt (regardless of the linker) confers B_{12}-dependent repressor activity.

Although native CarA, CarANt, and CarACt have been purified, as has CarHNt, the impossibility of obtaining active CarH or CarHCt has thwarted in vitro studies aimed at unraveling its B_{12} dependence. As noted earlier, CarA/CarH homologues of unknown functions are present in many bacterial species. That one such homologue exists in *Thermus thermophilus* (whose function is unknown) is particularly advantageous as thermophilic proteins are usually soluble, easily purified, and amenable to biochemical/structural analysis. Also, *M. xanthus* and *T. thermophilus* genomes have similar GC contents (69–70%). Hence, we constructed two more chimeras, CTt1 and CTt2, in which the C-terminal domain of the *T. thermophilus* homologue was fused to CarANt and CarHNt, respectively, via their corresponding linkers (Fig. 1*A*). As with C1 and C2 earlier, CTt1 or CTt2 were introduced into the *M. xanthus* strain lacking endogenous CarA and CarH, and again the resulting transformants were yellow in the dark only when B_{12} was present and became red in the light (Fig. 1*B* and Fig. S1*C*). Hence, CTt1 and CTt2 also function like CarH. This indicates that, as with CarHCt, the C-terminal domain of the *T. thermophilus* homolog (henceforth TtCarH) can confer B_{12}-dependent activity.

$AdoB_{12}$ Specifically Enhances Operator DNA Binding in Vitro. We could purify native CTt1 and CTt2 and thus examine their binding to the CarA/CarH operator in vitro and their dependence on a specific B_{12} form. CarA binds cooperatively to its operator, first to a high-affinity, palindromic site upstream of P_B and then to a low-affinity site straddling the −35 region of P_B to block access to RNA polymerase (Fig. S2*A*) (27). This is observed in EMSA by the progressive appearance of a lower band followed by an upper band at higher CarA concentrations (Fig. S2*B*). Although CTt1 has the same DNA-binding domain as CarA, it appeared to bind operator less tightly: even at concentrations 15-fold higher than CarA, two diffuse bands were formed rather than the single, well defined low-mobility complex seen with CarA (Fig. 2*A*). This difference must therefore stem from the distinct C-terminal domains in CTt1 and CarA, and probably results from CarA, but not CTt1, binding cooperatively to DNA. Operator affinity for CTt2 was even lower, with hardly perceptible binding even at high protein concentrations (Fig. 2*A*). That CTt2 binds less tightly than CTt1 is likely because CarANt has a greater affinity for operator DNA than CarHNt (12).

Both CTt1 and CTt2 required B_{12} to function in vivo. Hence, we probed if any of the different B_{12} forms affected DNA binding in vitro. Given the light sensitivity of cobalamins (24), special care was taken to carry out these experiments in the dark. Neither MeB_{12} nor CNB_{12} affected operator binding by CTt1 or CTt2 (Fig. 2*B*). Also, no effect was seen with hemin (Fig. S2*C*), which has been reported to bind to some proteins involved in redox-light sensing that have a B_{12}-binding motif (28–30). By contrast, $AdoB_{12}$ enhanced DNA binding by CTt1 and, even more dramatically, by CTt2: a single, well defined, low-mobility retarded complex was now formed, with little or no free probe detected (Fig. 2*B*). Consistent with its action being B_{12}-independent in vivo, CarA DNA-binding in vitro was unaffected by $AdoB_{12}$, MeB_{12}, or CNB_{12} (Fig. S2*D*). In DNase I footprinting, CarA protects a stretch extending from the −19 to −70 positions relative to the transcription start site, with hypersensitivities at −55 and −63 (27). $AdoB_{12}$ enabled CTt1 and, more markedly, CTt2 to protect against DNase I the same operator stretch mapped for CarA (Fig. 2*C*). DNase I hypersensitive sites indicate DNA distortion on repressor binding. The two additional hypersensitive sites (−34 and −45) seen for CTt2 possibly reflect variations in DNA binding between CarHNt and CarANt. Altogether, these data demonstrate that $AdoB_{12}$ specifically enhances binding by CTt1 or CTt2 to the CarA/CarH operator in vitro.

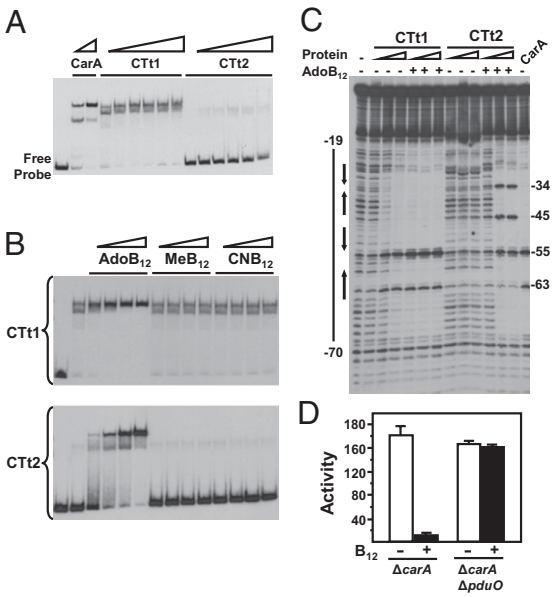

Fig. 2. $AdoB_{12}$ enhances DNA binding in vitro and is required for CarH-dependent P_B repression in vivo. (*A*) EMSA with CarA, CTt1, or CTt2 and a 130-bp probe containing the CarA/CarH operator (CCR130). Protein concentrations increase from left to right in order: 30, 60, 110, 220, 450, and 900 nM for CTt1 and CTt2, and 20 and 40 nM for CarA. (*B*) EMSA with CCR130 and 30 nM of CTt1 (*Top*) or CTt2 (*Bottom*) in the presence of increasing concentrations (16, 31, 63, and 125 nM from left to right) of $AdoB_{12}$, MeB_{12}, or CNB_{12}, as indicated. (*C*) DNase I footprinting with CCR130 and CTt1 or CTt2 in the presence or absence of $AdoB_{12}$. Protein concentrations for CTt1 and CTt2 increase from left to right in order: 60, 110, and 220; for CarA, 60 nM was used. $AdoB_{12}$ was added at a fivefold molar excess relative to protein. The solid line on the left (labeled −19 to −70) is the segment protected against DNase I by CarA (rightmost lane), which is also observed with CTt1 or CTt2 when $AdoB_{12}$ is present. pI and pII, the two operator subsites, are each shown by a pair of arrows facing one another, and the hypersensitive sites are numbered on the right. (*D*) Reporter P_B-*lacZ* expression levels in the absence and in the presence of 1 μM CNB_{12} for the Δ*carA* and Δ*carA* Δ*pduO* strains. The mean of three independent measurements and the SEM for the specific β-gal activities ("Activity") are shown.

$AdoB_{12}$ Is Essential for P_B Repression by CarH in Vivo. Eliminating $AdoB_{12}$ should abolish CarH-mediated P_B repression in vivo if, as suggested by the in vitro analysis described here, $AdoB_{12}$ is required to enhance operator binding. $AdoB_{12}$ biosynthesis or its generation in vivo from inactive forms like CNB_{12} requires ATP:corrinoid adenosyltransferase (ATR) activity (4, 31). Hence, knocking out ATR would eliminate intracellular $AdoB_{12}$. Three ATR classes (CobA, PduO, and EutT) are implicated in converting inactive cobalamins to $AdoB_{12}$ (31–33). The *M. xanthus* genome lacks genes for de novo $AdoB_{12}$ biosynthesis and has one ATR gene, whose product is similar to the PduO-type human ATR (Fig. S3). We therefore deleted this *pduO* gene in the Δ*carA M. xanthus* strain (to avoid CarA masking CarH activity). Despite endogenous CarH being available, reporter P_B-*lacZ* expression in a strain lacking both *pduO* and *carA* remained high with or without B_{12}, unlike in the Δ*carA* control (Fig. 2*D*). Thus, CarH cannot repress P_B expression if $AdoB_{12}$ is not generated in vivo.

CarH and TtCarH, but Not CarA, Require $AdoB_{12}$ for Oligomerization. The results described here demonstrate that $AdoB_{12}$ enhances operator binding in vitro and determines P_B repression by CarH in vivo. However, how exactly does $AdoB_{12}$ modulate repressor function? CarA oligomerization via CarACt is crucial for efficient DNA binding in vitro and repression of P_B in vivo (20). We therefore examined if $AdoB_{12}$ acts at the level of repressor oligomerization by using different protein–protein interaction assays. To study such interactions in vivo, we chose an *Escherichia coli*–based two-hybrid system (34), as this organism is incapable of de novo B_{12} synthesis but can generate $AdoB_{12}$ from exogenously supplied B_{12} (2, 5). Two-hybrid analysis revealed that CarHCt or the TtCarH C-terminal domain (TtCarHCt) self-interact only in the presence of B_{12}, in clear contrast to CarACt that self-interacts whether or not B_{12} is present (Fig. 3*A* and Fig. S4*A*). Moreover, mutating to A the conserved H of the B_{12}-binding motif (Fig. S1*B*) in CarHCt (H193) or TtCarHCt (H177) eliminated their B_{12}-dependent oligomerization, but an equivalent change in CarA (H175A) had no effect (Fig. 3*A* and Fig. S4*A*). This is consistent with our previous observation that the CarH H193A mutation abolished B_{12}-dependent repression of P_B in vivo, whereas the H175A mutation in CarA had no effect on its function (12). Thus, CarH and TtCarH, although not CarA, require B_{12} and an intact B_{12}-binding motif to oligomerize via their C-terminal domains.

Concordant results were obtained in size-exclusion chromatography (SEC) performed in the dark. TtCarHCt alone, with CNB_{12}, or MeB_{12}, eluted with an apparent molecular weight (M_r) of 24.4 ± 0.7 kDa, close to the measurement of 22.6 kDa calculated for a monomer (Fig. 3*B*). A coincident peak at 361 nm, where only B_{12} absorbs (35), when CNB_{12} or MeB_{12} was present indicated that these can bind TtCarHCt (Fig. 3*B*, *Bottom*). Remarkably, with $AdoB_{12}$, a tetrameric species was observed (M_r = 99.2 ± 2 kDa). The absorption spectrum of this peak fraction was similar to that of free $AdoB_{12}$ (Fig. S4*B*, *Top*), and estimates of its protein and $AdoB_{12}$ contents (*SI Materials and Methods*) indicated a 1:1 stoichiometry. Similarly, CTt2 alone, with CNB_{12}, or MeB_{12}, was a monomer but formed an $AdoB_{12}$-bound tetramer (Fig. S4*C*). By comparison, CarACt with or without $AdoB_{12}$ was a stable dimer (M_r = 41.3 ± 1 kDa; calculated monomer value = 22.9 kDa; Fig. 3*C*, *Top*), and the TtCarHCt H177A mutant was a monomer with no detectable B_{12} binding (TtCarHCt*; Fig. 3*C*, *Bottom*). The photosensitivity of the complex (as described later) precluded use of light-based methods such as analytical ultracentrifugation or dynamic light scattering to independently check $AdoB_{12}$-driven tetramer formation. Nonetheless, and in agreement with SEC, chemical cross-linking experiments revealed a band that would correspond to a TtCarHCt tetramer only when $AdoB_{12}$ was present (Fig. 3*D*). $AdoB_{12}$ also markedly increased the intensity of the band for the TtCarHCt dimer, presumably because it is more probable to simultaneously cross-link two subunits than four. Isothermal titration calorimetry (ITC) detects heat changes associated with binding and provides values for the thermodynamic parameters describing this binding. We detected no heat change on titrating TtCarHCt with CNB_{12} or MeB_{12} at 25 °C. By contrast, for the same conditions, with $AdoB_{12}$ there was net heat uptake. These ITC data, when fit to a single-site binding model, yielded a binding enthalpy (ΔH) of 15.2 ± 0.9 kcal/mol, entropy ($T\Delta S$) of 23.5 ± 1.4 kcal/mol, and equilibrium K_d of 832 ± 240 nM, but underestimated the stoichiometry (N) as 0.34 ± 0.10 (Fig. 3*E*), possibly because the single-site binding model does not consider cooperativity effects from $AdoB_{12}$-induced tetramerization. A more realistic treatment of these complexities must await future work, but ITC does reveal tight, entropically favored binding of $AdoB_{12}$ to TtCarHCt. In sum, specific binding of $AdoB_{12}$ to the C-terminal domain of CarH or TtCarH, but not of CarA, drives their higher-order assembly, thus providing a molecular rationale for why only the first two depend on B_{12} for function.

Light Impairs $AdoB_{12}$-Driven Oligomerization and DNA Binding. Light-induced homolytic cleavage of the $AdoB_{12}$ Co-C bond is very rapid (24–26). Hence, extreme care was taken to avoid light in the aforementioned experiments. However, given the implication of $AdoB_{12}$ in a cellular response to light, its photosensitivity could have direct physiological relevance. This prompted us to

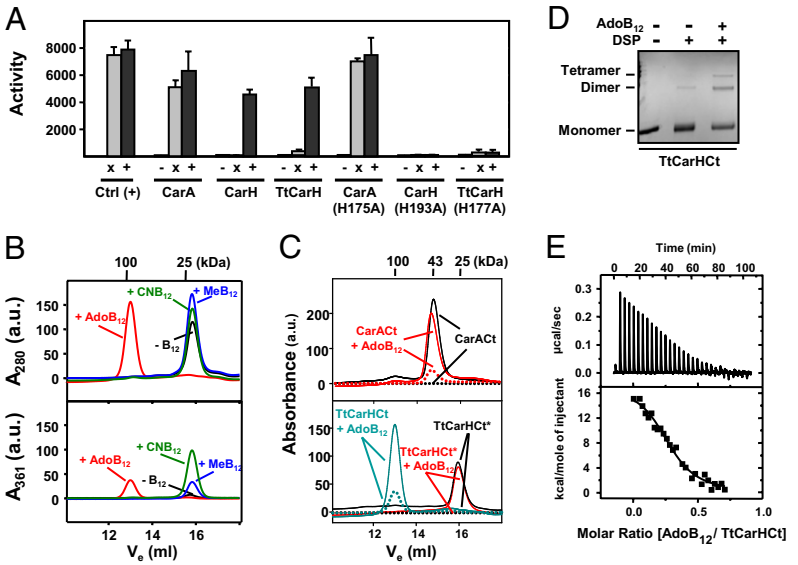

Fig. 3. $AdoB_{12}$ binding drives CarHCt and TtCarHCt oligomerization. (*A*) Two-hybrid analysis in *E. coli* for self-interactions in the dark of the CarA, CarH, or TtCarH C-terminal domain and their variants with the conserved H of the B_{12}-binding motif mutated to A. Interactions correlate with β-gal specific activities ("Activity"; mean and SEM from three independent measurements are shown) from reporter *lacZ* expression. Ctrl (+), positive control using the GCN4 leucine zipper; –, negative control expressing only one fusion protein. Cells expressing both fusion proteins were grown in the absence (x; gray bars) or presence (+; black bars) of 1 μM CNB_{12}. (*B*) Elution profiles off a Superdex200 analytical gel filtration column tracked by the absorbance (in arbitrary units) at 280 nm (*Top*) or 361 nm (*Bottom*) for 45 μM TtCarHCt alone (black lines) or with fivefold excess of $AdoB_{12}$ (red lines), CNB_{12} (green lines), or MeB_{12} (blue lines). M_r (in kDa) for each peak maximum is marked at the top. (*C*) Elution profile in the presence (red lines) or absence (black lines) of fivefold excess $AdoB_{12}$ tracked by the absorbance (in arbitrary units) at 280 nm (solid lines) or at 361 nm (dotted lines) for CarACt (*Top*) and the TtCarHCt H177A mutant, TtCarHCt* (*Bottom*). The latter includes, for comparison, the elution profile for TtCarHCt in the presence of $AdoB_{12}$ tracked at 280 and 361 nm (solid and dotted lines in cyan, respectively). (*D*) Dithiobis(succinimidylpropionate) cross-linking of TtCarHCt in the presence and absence of $AdoB_{12}$. (*E*) ITC of 18 μM TtCarHCt titrated with 78 μM $AdoB_{12}$ at 25 °C. *Top*: Heat change with each injection. *Bottom*: Corresponding integrated heat normalized and corrected for the heat of dilution versus molar ratio, the line being the best fit of the data to a single-site binding model. Parameters from four independent experiments are indicated in the text.

check how light affects $AdoB_{12}$-induced oligomerization, DNA binding in vitro, and P_B repression in vivo. In two-hybrid analysis, light impaired B_{12}-mediated oligomerization of CarHCt (Fig. 4*A*). Furthermore, $AdoB_{12}$-bound TtCarHCt or CTt2 exposed to light (white or at wavelengths centered at 360, 438, or 540 nm, at which $AdoB_{12}$ absorbs) eluted in SEC as monomers, not as the tetramers observed in the dark (Fig. 4*B*). Moreover, the absorption spectrum of the eluted peak had a maximum at 358 nm (Fig. S4*B*, *Bottom*), which is characteristic of hydroxocobalamin (OHB_{12}) produced on aerobic $AdoB_{12}$ photolysis (26). Also, no heat change was detected in ITC titrations of TtCarHCt with light-exposed $AdoB_{12}$. In EMSA, irradiating $AdoB_{12}$-bound CTt1 or CTt2 samples at the three wavelengths mentioned earlier caused the well defined retarded complex formed in the dark to revert to that observed with no $AdoB_{12}$ (Fig. 4*C*). This did not occur with red light, at a wavelength at which $AdoB_{12}$ does not absorb (Fig. S5*A*). Thus, light over a wide spectral range (UV, blue, and green, but not red) antagonizes $AdoB_{12}$-induced oligomerization and DNA binding that occurs in the dark.

These data strongly suggest that TtCarH activity in *T. thermophilus* would be $AdoB_{12}$-dependent and light-sensitive. Interestingly, *TtcarH* is adjacent to genes encoding a carotenogenic enzyme (*crtB*) and a photolyase (*phr*), but is transcribed in the opposite sense (36) (Fig. S6*A*). If, like CarH, TtCarH regulates carotenogenesis, it may recognize a site upstream of *crtB*. To address this, we purified TtCarH and examined its binding in vitro to a probe containing the *crtB–TtcarH* intergenic region (Fig. S6*A*). In EMSA, significant levels of free probe were observed even at high protein concentrations in the absence of $AdoB_{12}$ (Fig. S6*B*). Including $AdoB_{12}$ considerably enhanced DNA binding and, moreover, led to the formation of a well defined lower-mobility complex that was not observed even at high TtCarH concentrations when $AdoB_{12}$ was absent. Consistent with this, TtCarH yielded a DNase I footprint only in the presence of $AdoB_{12}$ (Fig. S6*C*). Interestingly, inspection of the sequence corresponding to the DNase I footprint indicated elements akin to the CarA/CarH operator (Fig. S6*A*). Moreover, light at wavelengths at which $AdoB_{12}$ absorbs counteracted this binding (Fig. S6*D*). As the identified DNA-binding site lies in the intergenic region, which would contain promoter elements for both *crtB* and *TtcarH*, it is conceivable that TtCarH regulates *crtB* as well as its own expression and, more importantly, that it employs the $AdoB_{12}$-based photoregulatory mechanism.

$AdoB_{12}$–CarH Complex Provides a Distinct Photosensory Pathway for Light-Induced Carotenogenesis in *M. xanthus*. We next examined the functional relevance of $AdoB_{12}$ photosensitivity on P_B expression. For this, we used a Δ*carS M. xanthus* genetic background, to separate the direct effect of light on $AdoB_{12}$-CarH from antirepression by CarS (12, 18). Consistent with CarA being B_{12}-independent and with the need for CarS to counteract its activity, the strain in which P_B is repressed only by CarA (Δ*carS* Δ*carH*) remained yellow in the light with or without B_{12} (Fig. S5*B*), and P_B-*lacZ* expression was always low, as in the Δ*carS* strain (Fig. 4*D*). In contrast, the strain with P_B repressed solely by $AdoB_{12}$-CarH (Δ*carS* Δ*carA*) turned from yellow to red when exposed to white light, and P_B-*lacZ* expression was induced approximately 10-fold, to levels comparable to those for WT under the same conditions or for the Δ*carS* Δ*carA* Δ*carH* strain (which expresses P_B constitutively). By contrast, and consistent with the data in vitro, red light did not relieve P_B repression by CarH in vivo (Fig. S5 *B* and *C*). Therefore, its intrinsic photosensitivity enables $AdoB_{12}$ to serve, via CarH, as both an "off" and an "on" switch that couples gene regulation to the light signal. This provides a distinct photosensory pathway, which is independent of CarS, for light-induced carotenogenesis in *M. xanthus*. Most impor-

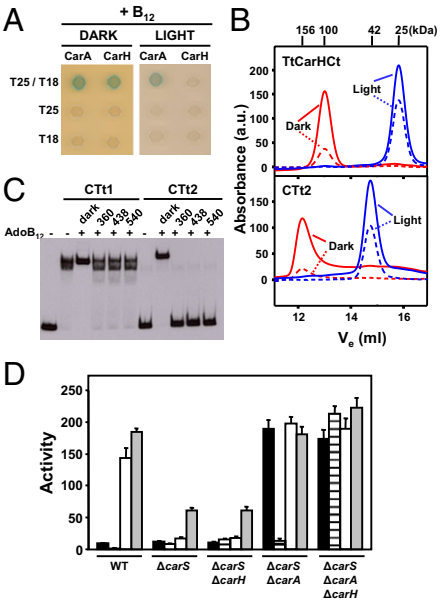

Fig. 4. Light disrupts $AdoB_{12}$-mediated oligomerization, DNA binding in vitro, and P_B repression in vivo. (*A*) Two-hybrid cell spot assay for CarACt and CarHCt in the dark and in the light in the presence of CNB_{12}. Cells expressing the indicated C-terminal domain fused to T25 and to T18 (T25/T18) or to just one of these as negative controls (T25 or T18 in the figure) were spotted on plates containing X-gal. Interaction correlates with the blue color intensity of the spot. (*B*) Elution profiles off a Superdex200 analytical gel filtration column for 45 μM TtCarHCt (*Top*) or CTt2 (*Bottom*) with $AdoB_{12}$ tracked by absorbance (in arbitrary units) at 280 nm (solid lines) or at 361 nm (dashed lines) in the dark (red lines), and after exposure to light (blue lines). M_r (in kDa) for each peak maximum is marked at the top. (*C*) Effect of light on DNA binding in vitro. EMSA with samples containing CCR130 and 30 nM CTt1 or CTt2, and with or without fivefold molar excess $AdoB_{12}$ relative to protein that were incubated in the dark (35 min) or irradiated with light (5 min) at the wavelength (in nm) indicated at the top after 30 min incubation in the dark. (*D*) Reporter P_B-*lacZ* expression (in terms of specific β-gal activity) in the dark (black bars), in the dark with 1 μM CNB_{12} (striped bars), in the light (white bars), and in the light with 1 μM CNB_{12} (gray bars) for each of the strains indicated. The mean of three independent measurements and SEM for the specific β-gal activities ("Activity") are shown.

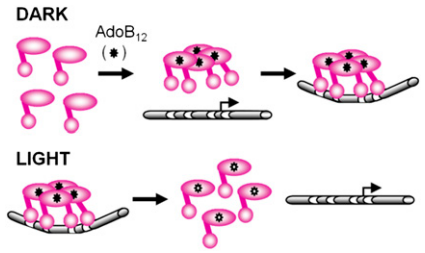

Fig. 5. Model for the light-responsive $AdoB_{12}$-based regulatory switch. The schematic depicts how specific-binding of $AdoB_{12}$ (filled asterisk) to CarH favors formation of tetramers that can then bind to the operator and repress P_B. Light activates P_B expression in a CarS-independent pathway by disrupting the photosensitive $AdoB_{12}$-repressor complex to diminish operator-binding and allow RNA polymerase access to P_B. The two-domain repressor organization is shown by the small sphere for the N-terminal DNA-binding domain, a large oval for the C-terminal $AdoB_{12}$-binding domain, and a line for the linker between the two domains. DNA, represented as a tube with each consecutive pair of white strips representing one site of the bipartite operator, is shown bent when bound by active repressor. $AdoB_{12}$ photolysis to OHB_{12} is represented by the unfilled asterisk.

tantly, it reveals a use of $AdoB_{12}$ in a unique photoreceptor design to achieve light-dependent gene regulation.

Discussion

The principal discovery of this study is the finding that $AdoB_{12}$, best known as an enzyme cofactor, determines a light-responsive switch that modulates gene expression via a transcriptional factor (Fig. 5). In the only other B_{12}-based mechanism for gene regulation, the B_{12} riboswitch, RNA and not protein binds tightly (K_d of approximately 300 nM) and specifically to $AdoB_{12}$ to adopt a distinct structure that down-regulates gene expression (10). Our studies indicate that $AdoB_{12}$ is also the form that specifically modulates CarH (and TtCarH) activity by fomenting higher-order assembly to enhance specific binding to operator DNA and bring about repression of target genes. Association of $AdoB_{12}$ to a repressor to induce tetramer formation for functionally effective binding to a two-site operator echoes a frequently exploited strategy by several other bacterial transcriptional factors. In these, binding of a specific ligand is allosterically coupled to cooperative binding to operator (usually also bipartite) to achieve tightly regulated function (37). That the CarH paralogue, CarA, despite having a canonical B_{12}-binding site, does not require the cofactor for oligomerization nor for effective operator binding, thus underlies the striking difference between the two proteins. Our domain-swap and mutational analyses show that the $AdoB_{12}$ dependence is conferred by CarHCt or TtCarHCt, and that the conserved H of the consensus B_{12}-binding motif in this domain is crucial for $AdoB_{12}$-induced oligomerization. Future high-resolution studies could provide further insights into the fine details of how $AdoB_{12}$ directs the oligomerization and activity of CarH and TtCarH, yet not of CarA.

A major finding in this work is that, besides fomenting repressor assembly, $AdoB_{12}$ enables the repressor to serve a photosensory role in a more direct signaling route for light-induced carotenogenesis in *M. xanthus*, an alternative to the CarS-mediated pathway that is also light-activated. This ability is based on photolysis of the repressor-bound $AdoB_{12}$, which can be evoked by light over a broad wavelength range. As a consequence, repressor oligomers are disassembled and operator binding is impaired. $AdoB_{12}$ is therefore one more in the list of chromophores such as flavin mononucleotide, flavin adenine dinucleotide, retinal, p-coumaric acid, and linear tetrapyrroles (bilins and biliverdins) that confer light sensing ability to photoactive proteins (14–16). The predominant trigger for carotenogenesis in *M. xanthus* is blue light, as this alone can activate the *carQRS* operon (via the distinct protoporphyrin IX-mediated pathway) to produce CarS and neutralize P_B repression by CarA and CarH (13). Blue light is also within the broad wavelength range in which the $AdoB_{12}$–CarH switch can operate. However, although the CarA-CarS–mediated pathway is B_{12}-independent, $AdoB_{12}$ must be available for CarH to operate. This suggests that the CarA-CarS mechanism may have evolved to bypass conditions of limited B_{12} availability, as *M. xanthus*, unable to synthesize B_{12} de novo, can only gain access to this cofactor from other soil microorganisms upon which it preys. Retention of the $AdoB_{12}$–CarH switch in *M. xanthus* may be an evolutionary vestige given the very ancient origins of B_{12} and B_{12}-mediated pathways (2). It may also reflect a selective advantage that could be related to the ability of this switch to sense wavelengths distinct from blue light and/or to control the expression of yet-unknown genes not subject to CarA-CarS regulation.

Our discovery of an $AdoB_{12}$-based photosensor has wider implications. It could underlie the unknown functions of many proteins with a CarA/CarH-like domain architecture that have come to light as a result of large-scale genome sequencing, TtCarH being one example. A database search for B_{12}-binding, MerR family proteins identifies more than 100 nonredundant entries in various bacterial genomes (Table S1). Most of these (except for *M. xanthus* and two others) contain only one version of the protein. Strikingly, the corresponding gene is often located

MICROBIOLOGY

among those encoding putative photolyases or carotenogenic enzymes, typically linked to a light response. Some of these homologues may, like CarH, depend on B_{12} for function, whereas others, like CarA, may not. It is worth noting that, other than in *Stigmatella aurantiaca* (a myxobacterium very closely related to *M. xanthus*), CarS homologues are not found (12, 18). Although factors resembling CarS in function, but not in sequence, may exist for on/off control of some of the newly identified CarA/CarH homologues, a more straightforward possibility is that the activities of many of these homologues are modulated solely by a light-responsive $AdoB_{12}$ switch, like the one identified in this study.

Known photoreceptor proteins are typically of a modular design, with light-sensing entrusted to an independent domain (14–16). As a result, the light-sensing input module can be found linked to various types of effector or output domains for use in diverse processes (14–16). Our finding that B_{12}-dependent photosensing is also housed in a single, autonomous module suggests that, other than in MerR-type DNA-binding factors or conventional B_{12}-dependent enzymes, it may also occur in association with other effector domains. A search in the genome database produced hits in which the B_{12}-binding module is found associated with sensor histidine kinases, response regulators and, curiously, even as stand-alone proteins, all with functions as yet undefined (Table S1). It is conceivable that the $AdoB_{12}$-driven higher-order assembly and light-sensing mechanism described in this study is an underlying feature of the functions of a number of these newly identified proteins. Also, the finding that the $AdoB_{12}$-dependent light-sensing module is autonomous and can thus be readily transplanted would be advantageous for designing novel, synthetic light-responsive factors.

Materials and Methods

Strains, Plasmids, and Growth Conditions. Table S2 lists the bacterial strains and plasmids used in this study. Growth conditions and strain constructions are detailed in *SI Materials and Methods*.

Protein Purification and Protein–Protein, Protein–DNA, and Protein–Cobalamin Interactions. Procedures for protein purification and their analysis are described in *SI Materials and Methods*.

Database Analysis. Proteins with a CarA/CarH-like B_{12}-binding domain associated to a MerR-type DNA-binding domain or other effector domains, or present as standalone modules in the nonredundant protein and microbial genome databases, were identified by using the BLAST suite of programs (38).

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1. Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. *Nature* 438:90–93.
2. Roth JR, Lawrence JG, Bobik TA (1996) Cobalamin (coenzyme B_{12}): Synthesis and biological significance. *Annu Rev Microbiol* 50:137–181.
3. Taga ME, Larsen NA, Howard-Jones AR, Walsh CT, Walker GC (2007) BluB cannibalizes flavin to form the lower ligand of vitamin B_{12}. *Nature* 446:449–453.
4. Warren MJ, Raux E, Schubert HL, Escalante-Semerena JC (2002) The biosynthesis of adenosylcobalamin (vitamin B_{12}). *Nat Prod Rep* 19:390–412.
5. Zhang Y, Rodionov DA, Gelfand MS, Gladyshev VN (2009) Comparative genomic analyses of nickel, cobalt and vitamin B_{12} utilization. *BMC Genomics* 10:78.
6. Ludwig ML, Matthews RG (1997) Structure-based perspectives on B_{12}-dependent enzymes. *Annu Rev Biochem* 66:269–313.
7. Banerjee R, Ragsdale SW (2003) The many faces of vitamin B_{12}: Catalysis by cobalamin-dependent enzymes. *Annu Rev Biochem* 72:209–247.
8. Mandal M, Breaker RR (2004) Gene regulation by riboswitches. *Nat Rev Mol Cell Biol* 5:451–463.
9. Nahvi A, Barrick JE, Breaker RR (2004) Coenzyme B_{12} riboswitches are widespread genetic control elements in prokaryotes. *Nucleic Acids Res* 32:143–150.
10. Nahvi A, et al. (2002) Genetic control by a metabolite binding mRNA. *Chem Biol* 9: 1043–1049.
11. Vitreschak AG, Rodionov DA, Mironov AA, Gelfand MS (2003) Regulation of the vitamin B_{12} metabolism and transport in bacteria by a conserved RNA structural element. *RNA* 9:1084–1097.
12. Pérez-Marín MC, Padmanabhan S, Polanco MC, Murillo FJ, Elías-Arnanz M (2008) Vitamin B_{12} partners the CarH repressor to downregulate a photoinducible promoter in *Myxococcus xanthus*. *Mol Microbiol* 67:804–819.
13. Elías-Arnanz M, Fontes M, Padmanabhan S (2008) Carotenogenesis in *Myxococcus xanthus*: A complex regulatory network. *Myxobacteria: Multicellularity and Differentiation*, ed Whitworth DE (ASM Press, Washington, DC), pp 211–225.
14. Losi A, Gärtner W (2008) Bacterial bilin- and flavin-binding photoreceptors. *Photochem Photobiol Sci* 7:1168–1178.
15. Purcell EB, Crosson S (2008) Photoregulation in prokaryotes. *Curr Opin Microbiol* 11: 168–178.
16. van der Horst MA, Key J, Hellingwerf KJ (2007) Photosensing in chemotrophic, non-phototrophic bacteria: Let there be light sensing too. *Trends Microbiol* 15:554–562.
17. Burchard RP, Dworkin M (1966) Light-induced lysis and carotenogenesis in *Myxococcus xanthus*. *J Bacteriol* 91:535–545.
18. León E, et al. (2010) A bacterial antirepressor with SH3 domain topology mimics operator DNA in sequestering the repressor DNA recognition helix. *Nucleic Acids Res* 38:5226–5241.
19. Navarro-Avilés G, et al. (2007) Structural basis for operator and antirepressor recognition by *Myxococcus xanthus* CarA repressor. *Mol Microbiol* 63:980–994.
20. Pérez-Marín MC, López-Rubio JJ, Murillo FJ, Elías-Arnanz M, Padmanabhan S (2004) The N terminus of *Myxococcus xanthus* CarA repressor is an autonomously folding domain that mediates physical and functional interactions with both operator DNA and antirepressor protein. *J Biol Chem* 279:33093–33103.
21. Cervantes M, Murillo FJ (2002) Role for vitamin B_{12} in light induction of gene expression in the bacterium *Myxococcus xanthus*. *J Bacteriol* 184:2215–2224.
22. Drennan CL, Huang S, Drummond JT, Matthews RG, Ludwig ML (1994) How a protein binds B_{12}: A 3.0 A X-ray structure of B_{12}-binding domains of methionine synthase. *Science* 266:1669–1674.
23. Ludwig ML, Drennan CL, Matthews RG (1996) The reactivity of B_{12} cofactors: The proteins make a difference. *Structure* 4:505–512.
24. Bond CM, Lees KA, Enever RP (1972) Photolytic decomposition of 3 cobalamins: A quantitative study. *J Pharm Pharmacol* 24(suppl):143.
25. Chen E, Chance MR (1990) Nanosecond transient absorption spectroscopy of coenzyme B_{12}. Quantum yields and spectral dynamics. *J Biol Chem* 265:12987–12994.
26. Schwartz PA, Frey PA (2007) 5′-Peroxyadenosine and 5′-peroxyadenosylcobalamin as intermediates in the aerobic photolysis of adenosylcobalamin. *Biochemistry* 46: 7284–7292.
27. López-Rubio JJ, et al. (2004) Operator design and mechanism for CarA repressor-mediated down-regulation of the photoinducible *carB* operon in *Myxococcus xanthus*. *J Biol Chem* 279:28945–28953.
28. Han Y, Meyer MH, Keusgen M, Klug G (2007) A haem cofactor is required for redox and light signalling by the AppA protein of *Rhodobacter sphaeroides*. *Mol Microbiol* 64:1090–1104.
29. Moskvin OV, Gilles-Gonzalez MA, Gomelsky M (2010) The PpaA/AerR regulators of photosynthesis gene expression from anoxygenic phototrophic proteobacteria contain heme-binding SCHIC domains. *J Bacteriol* 192:5253–5256.
30. Moskvin OV, Kaplan S, Gilles-Gonzalez MA, Gomelsky M (2007) Novel heme-based oxygen sensor with a revealing evolutionary history. *J Biol Chem* 282:28740–28748.
31. Yamanishi M, Vlasie M, Banerjee R (2005) Adenosyltransferase: An enzyme and an escort for coenzyme B_{12}? *Trends Biochem Sci* 30:304–308.
32. Johnson CL, et al. (2001) Functional genomic, biochemical, and genetic characterization of the *Salmonella pduO* gene, an ATP:cob(I)alamin adenosyltransferase gene. *J Bacteriol* 183:1577–1584.
33. Mera PE, Escalante-Semerena JC (2010) Multiple roles of ATP:cob(I)alamin adenosyltransferases in the conversion of B_{12} to coenzyme B_{12}. *Appl Microbiol Biotechnol* 88:41–48.
34. Karimova G, Ullmann A, Ladant D (2000) A bacterial two-hybrid system that exploits a cAMP signaling cascade in *Escherichia coli*. *Methods Enzymol* 328:59–73.
35. Firth RA, Hill HA, Pratt JM, Williams RJ, Jackson WR (1967) The circular dichroism and absorption spectra of some vitamin B_{12} derivatives. *Biochemistry* 6:2178–2189.
36. Henne A, et al. (2004) The genome sequence of the extreme thermophile *Thermus thermophilus*. *Nat Biotechnol* 22:547–553.
37. Lu D, et al. (2010) Crystal structure of TtgV in complex with its DNA operator reveals a general model for cooperative DNA binding of tetrameric gene regulators. *Genes Dev* 24:2556–2565.
38. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.