

Involvement of CarA/LitR and CRP/FNR Family Transcriptional Regulators in Light-Induced Carotenoid Production in *Thermus thermophilus*^{∇†}

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Members of the CarA/LitR family are MerR-type transcriptional regulators that contain a C-terminal cobalamin-binding domain. They are thought to be involved in light-induced transcriptional regulation in a wide variety of nonphototrophic bacteria. Based on the distribution of this kind of regulator, the current study examined carotenoid production in *Thermus thermophilus*, and it was found to occur in a light-induced manner. *litR* and carotenoid and cobalamin biosynthesis genes were all located on the large plasmid of this organism. *litR* or cobalamin biosynthesis gene knockout mutants were unable to switch off carotenoid production under dark conditions, while a mutant with a mutation in the downstream gene adjacent to *litR* (TT_P0055), which encodes a CRP/FNR family transcriptional regulator, was unable to produce carotenoids, irrespective of light conditions. Overall, genetic and biochemical evidence indicates that LitR is bound by cobalamin and associates with the intergenic promoter region between *litR* and *crtB* (phytoene synthase gene), repressing the bidirectional transcription of *litR* and *crtB*. It is probable that derepression of LitR caused by some photodependent mechanism induces the expression of TT_P0055 protein, which serves as a transcriptional activator for the *crtB* operon and hence causes the expression of carotenoid biosynthesis and the DNA repair system under light condition.

Light is an environmental stimulus that affects many living organisms, including prokaryotes. In recent years, various kinds of photoreceptors have been discovered across a wide range of bacterial taxa (24). Interestingly, genome sequencing studies have revealed the presence of many putative light-sensing proteins in nonphotosynthetic bacteria (30). Such findings provide new insight into the physiology and ecology of bacteria, although photodependent bacterial phenotypes have not yet been extensively characterized.

A well-known photodependent physiological feature of non-phototrophic bacteria is their production of carotenoids, the yellow, orange, or red pigments that protect cells from harmful oxygen radicals, including photooxidative stress. It has been widely observed that bacterial cells accumulate this kind of pigment when they are illuminated. This means that many bacteria have retained a certain genetic regulatory system that transmits an illumination signal to the genes that govern the expression of carotenoid biosynthesis, but the details of such a regulatory system are poorly known. The only exception to this is the knowledge of light-induced carotenoid production in *Myxococcus xanthus*, a Gram-negative gliding bacterium. The

complex signaling network of this organism involves the function of multiple regulators, including the MerR-type transcriptional regulators CarA and CarH (see reference 23 and references cited therein).

Recently, we discovered that carotenoid production in *Streptomyces coelicolor* A3(2), a Gram-positive filamentous bacterium, also occurred in a light-induced manner, and we identified the regulatory proteins responsible for its control (29). We also studied the transcriptional regulation of its carotenoid biosynthetic gene (*crt*) cluster and found that *litR* (light-induced transcription, regulator) and *litS* (light-induced transcription, sigma factor) were responsible for the photodependent transcription of the *crt* cluster. Evidence indicated that the LitR protein, a MerR-type transcriptional regulator, is involved in the light-induced transcription of *litS*, which encodes an extracytoplasmic function (ECF) sigma factor that directs the transcription of *crt* operons (29). A subsequent study involving a heterologous host expression experiment raised the possibility that LitR is used exclusively in the light-dependent transcriptional control of the *litS* promoter (28).

LitR belongs to the MerR family, whose regulatory activity depends on the binding of a ligand (3, 12). Usually, the ligand has a direct correlation with the role of genes whose expression is controlled by the MerR family regulator. For example, the MerR of Gram-negative bacteria is bound by mercury ions and induces the transcription of the mercury resistance (*mer*) operon; the operator-bound MerR represses the transcription of the *mer* operon in the absence of mercury ions but in turn

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activates transcription when mercury ions bind to the C-terminal ligand-binding site of MerR (3).

LitR, as well as CarA and CarH, of *M. xanthus* contains a cobalamin (Cbl) (vitamin B₁₂)-binding motif in its C-terminal region. Our previous observations suggest that blue-light absorption by Cbl plays a crucial role in the LitR function (28). Blue light is responsible for light-induced carotenoid production in *S. coelicolor*. The LitR of *S. coelicolor* A3(2), however, could not be obtained as a soluble recombinant protein in an *Escherichia coli* expression system; in addition, its coding sequence could not be disrupted in our repeated experiments (29). These difficulties have hampered our detailed characterization of the roles of LitR in *S. coelicolor*.

Interestingly, CarA/LitR homologs are found in a wide variety of nonphototrophic bacteria, including not only *Actinobacteria* but also Gram-negative bacteria such as *Thermus*, *Pseudomonas*, *Shewanella*, and *Vibrio* spp. (28). In these divergent organisms, the *litR* homologs commonly cluster with illumination-related genes, such as those encoding carotenoid biosynthesis enzymes, DNA photolyase, and PAS domain proteins. These findings have prompted us to speculate that these genes are expressed in a light-dependent manner, based on the function of the CarA/LitR homologs.

In the current study, we examined the role of the CarA/LitR homolog of *Thermus thermophilus*, an extremely thermophilic bacterium. The chemical structure of the carotenoids of this organism has been studied (31, 32), but its light dependence has not been elucidated. The evidence obtained via this study indicates that LitR and an additional transcriptional regulator are crucial for the light-dependent transcriptional control of carotenoid biosynthesis in this organism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The wild-type (WT) strain of *T. thermophilus* used in this study was HB27 TH104 (*proC*, pTT8) (14). *Escherichia coli* JM109 and BL21(DE3)/pLysS (Takara-Shuzo, Kyoto, Japan) were used as hosts for DNA manipulation and protein expression, respectively. pUC19 (Takara-Shuzo) was used for general DNA manipulation. pT7Blue (Takara-Shuzo) was used for TA cloning of PCR-generated DNA fragments. pGEX-6P-2 (GE Healthcare Bio-Sciences KK, Tokyo, Japan) was used for the expression of LitR and TT_P0055 (here designated TTP55) protein in *E. coli*. Enzymes used for DNA manipulation were purchased from Takara-Shuzo. The conditions for the culture and genetic manipulation for *E. coli* and *Thermus* were as described by Maniatis et al. (19) and Koyama et al. (18), respectively. The *E. coli*-*Thermus* shuttle vector pTEV (carrying hygromycin B resistance) and pUC18-pJHK3 (carrying ampicillin and kanamycin resistance) (13) were used for the genetic manipulation of *T. thermophilus*. These plasmids had a copy number of eight per genome (11). *T. thermophilus* was grown at 60°C in TM medium (containing, per liter, 2 g yeast extract [Difco Laboratories, Detroit, MI], 4 g polypeptone, 1 g NaCl, and 10 ml Castenholz basal salt solution) (all chemicals were purchased from Wako Pure Chemicals, Osaka, Japan, unless otherwise indicated). Castenholz basal salt solution contained, per liter, 1 g nitrilotriacetic acid, 0.6 g CaSO₄ · 2H₂O, 1 g MgSO₄ · 7H₂O, 0.08 g NaCl, 1.03 g KNO₃, 6.89 g NaNO₃, 1.11 g Na₂HPO₄, 10 ml aqueous 0.03% FeCl₃ solution, and 10 ml Nitsch's trace elements (pH 8.2) (containing, per liter, 5 ml H₂SO₄, 2.2 g MgSO₄ · 5H₂O, 0.5 g ZnSO₄ · 7H₂O, 0.5 g H₃BO₃, 0.016 g CuSO₄, 0.025 g Na₂MoO₄ · 2H₂O, and 0.046 g CoCl₂ · 6H₂O). *E. coli* was grown in Luria-Bertani (LB) medium (19), and 1.0 to 1.5% agar (Kokusan, Tokyo, Japan) was added to prepare solid media. To enable the selection of transformants of *E. coli* and *T. thermophilus*, ampicillin, kanamycin, and hygromycin B were added at 50 µg/ml.

Carotenoid production. To observe light-dependent carotenoid production, *T. thermophilus* HB27 was cultured at 60°C for 2 days on solid TM medium under dark and light conditions, using an illuminating incubator (BR-180LF; Taitech, Saitama, Japan) equipped with white-light fluorescent lamps (20 W; Toshiba, Tokyo, Japan). Under light conditions, the solid culture was illuminated with

white light at approximately 2.4 µmol s⁻¹ m⁻². The same lamp, covered with a blue or red light filter, was used to generate blue (400- to 460-nm) or red (600- to 700-nm) light, respectively. The method of extracting carotenoids was described previously (31). An absorption spectrum of the carotenoid fraction was recorded by using a UV spectrometer (UVmini-1240; Shimadzu, Kyoto, Japan).

Gene disruption. Kanamycin-resistant mutants of *T. thermophilus* were generated by the standard homologous recombination technique, using disruption plasmids. Each disruption plasmid contained a thermostable kanamycin resistance gene (*htk*) cassette (13). To construct disruption plasmids for *litR*, TTP55, and the *cob* cluster, two flanking fragments were amplified by PCR using the primer sets R-F/R-MR and R-MF/R-R (*litR*), 55F/55MR and 55MF/55R (TTP55), and *cobF*/*cobMR* and *cobMF*/*cobR* (*cob* cluster) (oligonucleotide primers used in this study are shown in Table S1 in the supplemental material) and cloned onto pUC19 by three-fragment ligation. Each resulting plasmid was digested with BglII or BamHI and ligated with a promoterless *htk* cassette amplified by PCR using the primer sets HTKF1/HTKR to generate the disruption plasmid. Disruption plasmids were linearized by digestion with DraI and introduced into *T. thermophilus* WT cells (9). Subsequently, kanamycin-resistant mutants were screened and assessed for true recombination by performing PCR with appropriate primers. For genetic complementation, the coding sequence cassette for *litR* and TTP55 was prepared by PCR using the primer sets RcomF/RcomR and 55comF/55comR, respectively, and cloned between the NdeI and SphI sites of pTEV. The resultant plasmid carried each coding sequence downstream from the *slp* promoter (7), which directed the constitutive expression of each coding sequence.

RNA isolation. To isolate total RNA, *T. thermophilus* strains were cultured in TM liquid medium. A preculture (60°C, 17 h, with rotary shaking at 135 rpm) was inoculated at 1% into 100 ml of TM medium prepared in a 500-ml baffled Erlenmeyer flask and incubated at 60°C with rotary shaking at 135 rpm, using the same illuminating incubator as described above. Cells were harvested by centrifugation and suspended in modified Kirby mix (containing 1% [wt/vol] *N*-lauroyl-sarcosine, 6% [wt/vol] *p*-aminosalicylic acid sodium salt, and 6% [vol/vol] phenol in 50 mM Tris-HCl, pH 8.3) (17); 2-mm glass beads were added, and the mixture was vigorously beaten using a Shake Master (Biomedical Science, Tokyo, Japan). Contaminating proteins were removed by a three-iteration extraction with acid-phenol-chloroform. The total nucleic acid volume thus obtained was precipitated first by 2-propanol treatment and then via a sodium acetate treatment. The precipitant was then treated with DNase I, extracted with acid-phenol-chloroform, and ethanol precipitated to obtain the RNA fraction. Approximately 200 to 300 µg of total RNA was obtained from the 40-ml culture.

S1 nuclease mapping. The transcriptional activities of promoters preceding TTP55 (P55), *litR* (*PlitR*), and *crtB* (*PcrtB*) were studied by S1 protection analysis. Hybridization probes were first generated by PCR, using the primers 55SF/55SR (P55) and RSF/RSR (*PlitR* and *PcrtB*) (see Table S1 in the supplemental material), and cloned onto pT7Blue by TA cloning. The probes were then reamplified by PCR using the primers M13-RV/55SR* (P55), M13-RV/RSR* (*PlitR*), and M13-M4/RSF* (*PcrtB*) (primers labeled at their 5' ends with [γ -³²P]ATP using T4 polynucleotide kinase are denoted with asterisks). The primers M13-M4 and M13-RV (M13 sequencing primers) were purchased from Takara-Shuzo. The resulting probes contained a 5'-terminal mismatch region that distinguished the full-size mRNA-DNA hybrid (371, 395, and 355 bp for P55, *PlitR*, and *PcrtB*, respectively) from the unhybridized probe DNA (264, 288, and 288 bp for P55, *PlitR*, and *PcrtB*, respectively). For the high-resolution analysis of *PlitR* and *PcrtB*, probes were prepared by PCR using the primer sets RSFH/RSRH* and RSFH*/RSRH, respectively.

The ³²P-labeled probe (30,000 cpm) was mixed with 40 µg of total RNA extracted from *T. thermophilus* in 20 µl of sodium trichloroacetate (NaTCA) hybridization buffer (containing 3 M NaTCA, 50 mM PIPES, and 5 mM EDTA, pH 7.0), incubated at 65°C for 15 min, and gradually cooled to 45°C within 12 to 15 h. To this mixture was added 300 µl of chilled 5× S1 digestion buffer containing 100 units S1 nuclease; the mixture was then incubated at 37°C for 1 h. The S1 reaction was terminated by adding 75 µl of stop solution (containing 2.5 M ammonium acetate and 50 mM EDTA). After ethanol precipitation using 10 µg yeast tRNA as a carrier, the DNA-RNA hybrid was dissolved in 4 µl of formamide dye and applied to a 6% polyacrylamide gel. Radioactivity detection was carried out by exposing dried gels to a Fuji imaging plate (Fuji Film, Tokyo, Japan) and scanning with a Typhoon 9410 image analyzer (GE Healthcare, Tokyo, Japan). Marker 10 (Nippon Gene, Tokyo, Japan) labeled with [γ -³²P]ATP using T4 polynucleotide kinase was used as a standard to estimate the transcript sizes in the low-resolution assay. To determine the transcription start sites in the high-resolution analysis, Maxam-Gilbert sequencing ladders (G+A and T+C reactions) derived from the ³²P-labeled probe DNA were used as a reference. The quality of the RNA was verified via a control assay for *sigA*,

encoding the major sigma factor (22). The probe for *sigA* was amplified by PCR using the primers ASF/ASR* (see Table S1 in the supplemental material).

Preparation of recombinant proteins. LitR and TTP55 were expressed and purified via a standard system, using *E. coli* as a host. The coding sequences of *litR* and TTP55 were amplified by PCR using the primers RexF/RexR (WT LitR), RexF/RexR2 (C-terminally truncated mutant; Δ LitR), and 55exF/55exR (TTP55) and cloned between the BamHI and EcoRI sites of pGEX-6P-2 to generate the expression plasmid. A preculture (28°C, 16 h, LB medium) of *E. coli* harboring this plasmid was inoculated at 1% into 150 ml LB medium prepared in a 500-ml baffled Erlenmeyer flask and cultured for 6 h (1 mM isopropyl- β -D-thiogalactopyranoside [IPTG] was added at 3 h) at 28°C with rotary shaking (135 rpm). Cells were harvested by centrifugation, suspended in phosphate-buffered saline (PBS) (19), and disrupted with a mechanical cell presser. The cell extract was centrifuged at $70,000 \times g$ for 30 min; the resulting supernatant was used in glutathione *S*-transferase (GST) affinity chromatography. The supernatant was applied to a 5-ml GSTrap FF column, using an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare). The GST tag was removed by treatment with Precision protease (GE Healthcare). The protein concentration was measured with a protein assay kit (Bio-Rad). The molecular size of LitR was estimated by gel filtration of 0.3 mg of the purified recombinant protein. Gel filtration was performed by using a Superdex 200 HR 10/30 column on an ÄKTA FPLC system (GE Healthcare) according to the manufacturer's recommendation. The column was developed with PBS (containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4 , and 1.8 mM KH_2PO_4) at a flow rate of 0.25 ml/min. Molecular size standards (aldolase, albumin, ovalbumin, RNase A, and blue dextran, indicating 158, 67, 43, 13.7, and 2 kDa, respectively) were contained in a gel filtration calibration kit (GE Healthcare). To prepare Cbl-treated recombinant protein, the GST-tagged LitR (WT LitR and Δ LitR) was added to a 10-fold molar excess of methylcobalamin (MeCbl) (Sigma-Aldrich, Tokyo, Japan) and incubated for 1 h at 37°C. The mixture was then applied to a GSTrap FF column and eluted by treatment with Precision protease as described above. Absorption spectra of the resultant LitR recombinants were recorded by using a UVmini-1240 spectrometer (Shimadzu).

DNA-binding assay. The DNA-binding activity of LitR was first studied with a gel mobility shift assay. Probe DNA was generated by PCR using primer sets RSE/RSRH (*PlitR*) and ASF/ASR (*PsigA*) (control) (see Table S1 in the supplemental material) and labeled at the 5' end with [γ - ^{32}P]ATP, using T4 polynucleotide kinase. A total of 0.5 to 5.0 ng of ^{32}P -labeled probe (10,000 to 20,000 cpm) was mixed with 30 to 120 pmol of recombinant LitR, prepared as discussed above; it was then incubated at 30°C for 30 min in 50 μl of binding buffer [containing 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 1 μg poly(dI-dC), and 50 $\mu\text{g}/\text{ml}$ of bovine serum albumin]. Following incubation, the DNA-protein complex and free DNA were resolved on nondenaturing polyacrylamide gels containing 6% acrylamide.

To determine the binding site of LitR, a DNase I footprint analysis was carried out. ^{32}P -labeled DNA fragments were prepared by PCR using the primers FPA/FPB* for the antisense strand and FPA*/FPB for the sense strand. The reaction mixture (50 μl) contained 10 kcpm ^{32}P -labeled DNA probe, 10 to 320 pmol LitR, 25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA-NaOH (pH 8.0), 50 mM KCl, and 10% glycerol. After incubation at 55°C for 30 min, DNase I was added at a final concentration of 20 $\mu\text{g}/\text{ml}$, and the mixture was further incubated for 1 min at 25°C. The reaction was terminated by adding 100 μl of stop solution (containing 100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% sodium *N*-lauroyl sarcosinate, 10 mM EDTA-NaOH [pH 8.0], and 25 mg/ml salmon sperm DNA) and 300 μl of phenol-chloroform (1:1). After ethanol precipitation, the pellet was washed with 80% ethanol, dissolved in a 6- μl formamide-dye mixture, and run on a 6% polyacrylamide gel. To determine the LitR-binding site in the high-resolution analysis, Maxam-Gilbert sequencing ladders (G+A and T+C reactions) generated from the ^{32}P -labeled probe DNA fragment were used as a reference.

In vitro runoff transcription. The *in vitro* runoff transcription assay was performed using a previously described method (26). DNA templates containing the transcriptional start sites of *PlitR* and *PcrtB* were generated by PCR using the primers PLA/PLR for template A (313 bp), PLB/PLR for template B (213 bp), PLC/PLR for template C (172 bp), PLD/PLR for template D (173 bp), and PLE/PLR for template E (174 bp) (see Table S1 in the supplemental material). The mutated template (template A') was prepared by PCR using primers PLA/PLR from the mixture of two amplicons generated by PCR with primers PLA/PMR and PMF/PLR. A total of 0.5 pmol of template DNA was mixed with 2 pmol RNA polymerase holoenzyme of *T. thermophilus* (AR Brown, Tokyo, Japan), 100 nmol of ribonucleotides (including [α - ^{32}P]CTP), 0 to 20 pmol of LitR, and 0 to 10 pmol TTP55. Transcripts were analyzed by polyacrylamide gel electrophoresis. Marker 10 (Nippon Gene, Tokyo, Japan) labeled with [γ - ^{32}P]ATP was used as a standard.

RESULTS

Gene organization of the *litR-crt* locus on the large plasmid of *T. thermophilus*. The complete genome sequence of *T. thermophilus* is available for two strains, HB8 and HB27, which exhibit marked similarity to each other (5, 10). Each genome consists of a 1.85-Mb chromosome and a large (0.26-Mb) plasmid; HB8, but not HB27, harbors a small (9.32-kb) plasmid (10). We used the HB27 strain for genetic characterization; the numbering used here is for that strain.

The locus containing the coding sequence for CarA/LitR homolog (here the coding sequence will be referred as *litR* based on its involvement in the light-induced transcription of not only carotenoid biosynthesis genes but other protective genes) was located on the large plasmid, pTT27 (Fig. 1). The corresponding region was known to be a locus involved in DNA damage repair (5). *litR* (TT_P0056) comprised a possible operon structure with two downstream coding sequences that encode a cyclic AMP (cAMP) receptor protein (CRP)/fumarate and nitrate reduction regulator (FNR) family transcriptional regulator (TT_P0055; designated TTP55 here) and a subunit of NADH:ubiquinone oxidoreductase (TT_P0054). The *litR* operon was flanked by the *crtB* operon, which contained coding sequences for phytoene synthase (*crtB*) (TT_P0057), DNA photolyase (*phr*) (TT_P0058), and cytochrome P450 monooxygenase. The P450 monooxygenase is known for its involvement in the hydroxylation of β -carotene (2) (TT_P0059). Additional genes involved in carotenoid biosynthesis (TT_P0066 and TT_P0067) existed downstream of the *crtB* operon. pTT27 also contained a Cbl biosynthetic gene cluster (TT_P0001-0023) (Fig. 1). No *litR* homolog or Cbl biosynthetic genes were found on the chromosome.

Light-induced carotenoid production in *T. thermophilus*. Although carotenoid production in *T. thermophilus* has not been known to be affected by illumination, the proximity of *litR* and *crtB* suggested that it occurs in a photodependent manner. To assess this possibility, *T. thermophilus* was grown under light and dark conditions. As shown in Fig. 2A (panels corresponding to WT/pTEV), the WT strain produced a marked yellow pigment under light conditions; in contrast, the colonies of this strain appeared white or pale yellow under dark conditions. The induction of pigment production was observed when white or blue light was used and was not observed when red light was used (data not shown). The UV-visible absorption spectrum of a methanol extract of the illuminated cells showed a typical carotenoid profile, exhibiting multiple absorption peaks around 450 nm (Fig. 3). It is known that *T. thermophilus* produces zeaxanthin derivatives, including a novel glucosylated form termed thermozeaxanthin (λ_{max} , 452 and 477 nm) (32). The yellow pigments produced under light conditions are likely to contain these known carotenoids.

To study the role of *litR* and the downstream regulatory gene that encodes a CRP/FNR family protein (TTP55), kanamycin-resistant knockout mutants were generated through a homologous recombination technique (see Materials and Methods). The resulting *litR* mutant produced carotenoids under both light and dark conditions (Fig. 2A, Δ *litR*/pTEV); in contrast, the TTP55 mutant was defective in carotenoid production (Fig. 2A, Δ TTP55/pTEV). This suggests that *litR* and TTP55 encode

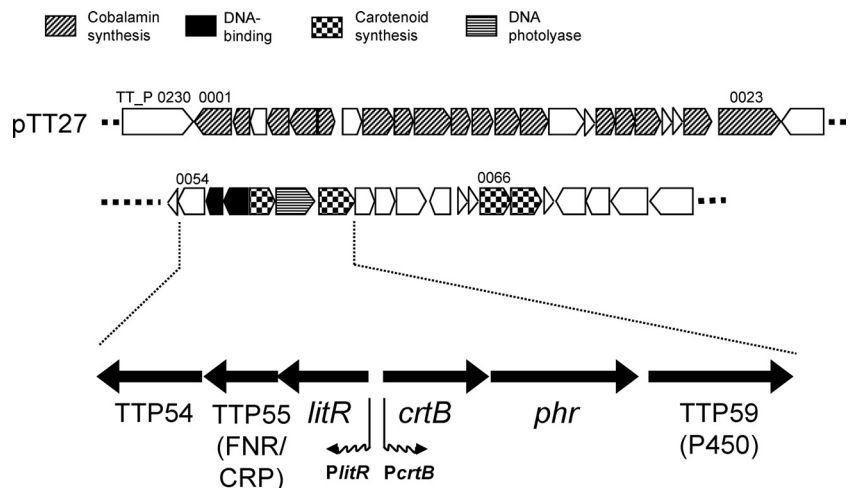


FIG. 1. Schematic representation of the *litR* locus of *T. thermophilus*. This locus was carried by the large plasmid pTT27 in a region known to contain genes involved in DNA damage repair (5). The genes flanking *litR* (TT_P0056) encode a cAMP receptor protein homolog (TT_P0055), NADH-ubiquinone oxidoreductase (TT_P0054), phytoene synthase (*crtB*) (TT_P0057), DNA photolyase (*phr*) (TT_P0058), and cytochrome P450 monooxygenase (TT_P0059). The two downstream carotenoid biosynthesis genes, TT_P0066 and TT_P0067, encode phytoene dehydrogenase and isopentenyl pyrophosphate isomerase, respectively. pTT27 also carries a Cbl biosynthesis gene (*cob*) cluster that encompasses the 20-kb region corresponding to TT_P0001 to TT_P0023.

negative and positive regulators for carotenoid production, respectively.

The introduction of a plasmid carrying an intact *litR* gene (pTEV-*litR*) restored the light-dependent carotenoid production in the *litR* mutant (Fig. 2A, $\Delta litR$ /pTEV-*litR*) but did not affect the carotenoid-deficient phenotype of the TTP55 mutant ($\Delta TTP55$ /pTEV-*litR*). On the other hand, the introduction of a

plasmid carrying an intact TTP55 gene (pTEV-55) caused constitutive carotenoid production in both mutants as well as in the WT strain, probably due to the constitutive expression of the plasmid-borne TTP55 (Fig. 2A, pTEV-55).

Transcriptional analysis by S1 nuclease mapping. To study the details of light-dependent genetic control, transcription activities were studied by low-resolution S1 nuclease protection analysis (Fig. 4A). Preliminary analyses with respect to the intergenic regions indicated that the two divergent promoters, *PlitR* and *PcertB* (Fig. 1), were responsible for the major transcription of the *litR* and *crtB* operons, respectively (data not shown). In the WT strain, the activity of *PcertB* was induced by light, while the activity of *PlitR* was largely not affected by illumination (Fig. 4A). In the *litR* mutant, *PcertB* was active under both light and dark conditions; *PlitR* activity was remarkably upregulated compared to that in the WT strain under

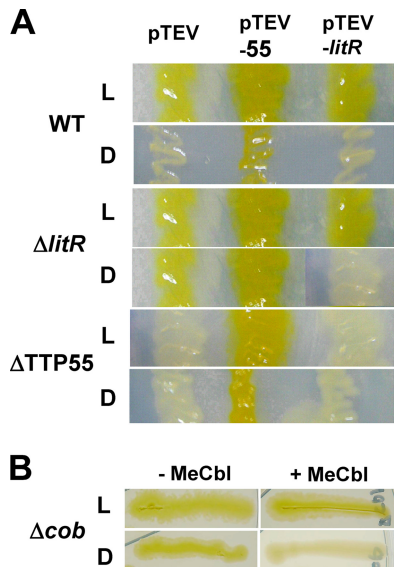


FIG. 2. Light-induced carotenoid production in *T. thermophilus*. (A) Colonies of the wild-type (WT) strain, the *litR* mutant ($\Delta litR$), and the CRP/FNR family transcriptional regulator gene mutant ($\Delta TTP55$), each harboring the plasmids pTEV (empty vector; control), pTEV-55 (pTEV carrying TTP55), and pTEV-*litR* (pTEV carrying *litR*). (B) Colonies of the mutant lacking Cbl biosynthesis genes (Δcob) grown in the absence (–MeCbl) and presence (+MeCbl) of 0.1 mM MeCbl. All colonies were developed under light (L) and dark (D) conditions at 60°C for 2 days on solid TM medium.

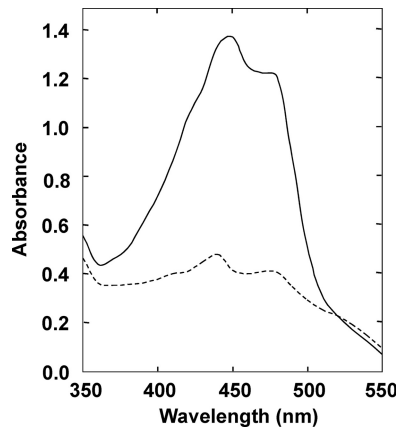


FIG. 3. UV-visible absorption spectrum of carotenoid extracted from illuminated cells of *T. thermophilus* (solid line). The spectrum for cells grown under dark conditions is also shown (dashed line).

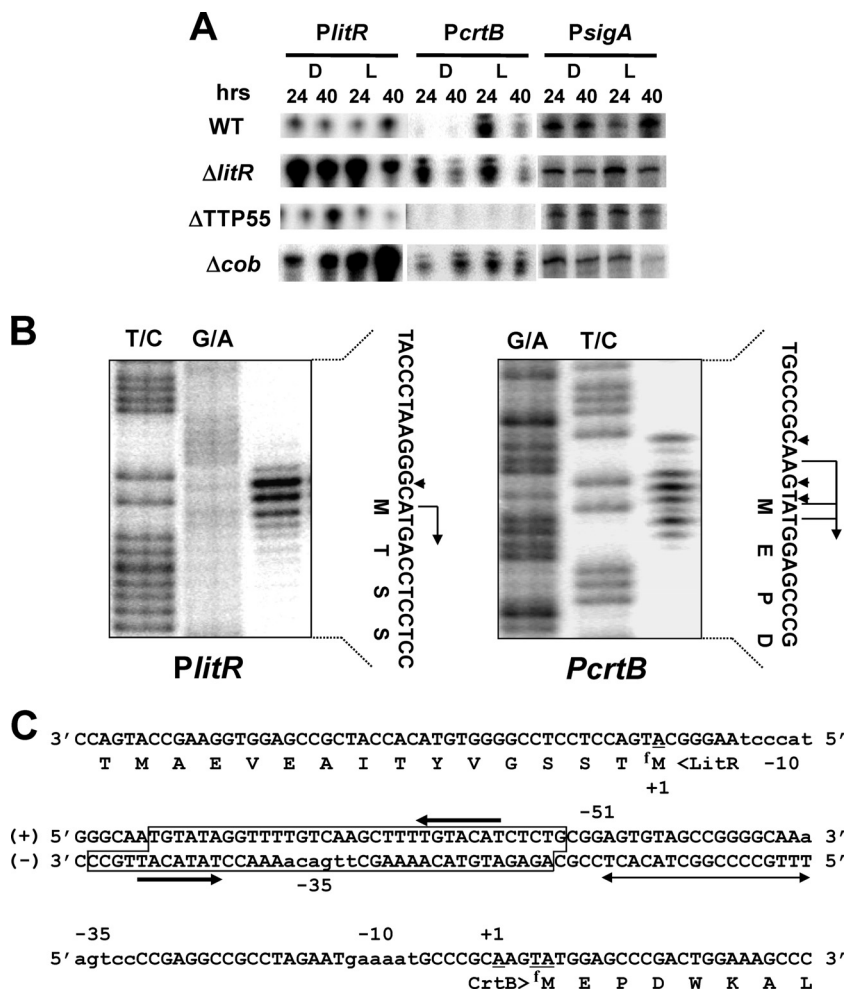


FIG. 4. S1 protection analyses of the bidirectional promoter. (A) Low-resolution analysis. Activities of promoters preceding *litR* (*PlitR*), *crtB* (*PcrB*), and *sigA* (the housekeeping sigma factor gene; control) (*PsigA*) were estimated by the signal intensities of protected fragments. Since the *litR* mutant ($\Delta litR$) retains the N-terminal portion of the coding sequence, the same S1 probe was used for both the WT and $\Delta litR$ strains. RNA was isolated from cells grown for 24 and 40 h at 60°C. Strain designations are the same as those in Fig. 2. (B) High-resolution analysis for the determination of transcriptional start sites of *PlitR* and *PcrB*. Maxam-Gilbert sequencing ladders (T+C and G+A reactions) were generated using the same 32 P-labeled fragment as the probe DNA. The positions of the S1-protected fragments are denoted by arrowheads, and the transcriptional start sites assigned to the residues are denoted by bent arrows. It is known that the fragments generated by the chemical-sequencing reactions migrate 1.5 nucleotides (nt) further than the corresponding fragments generated by the S1 nuclease digestion of the DNA-RNA hybrids (half a residue from the presence of the 3'-terminal phosphate group and one residue from the elimination of the 3'-terminal nucleotide) (27). The RNA prepared from WT cells grown under light conditions for 40 h on solid TM medium was used for hybridization. (C) Nucleotide sequence of the intergenic promoter region between *litR* and *crtB*; partial amino acid sequences of LitR and CrtB are also shown. The translation initiation codon of CrtB is located 18 bp downstream from that assigned in the genome sequence database (<http://www.genome.ad.jp>). The transcriptional start positions determined by high-resolution analysis are designated +1. The potential -35 and -10 sequences are shown in lowercase. The region protected in the DNase I footprint experiment (Fig. 7) and an inverted repeat contained in this region are denoted by a box and convergent arrows, respectively. The region essential for transcriptional activation by TTP55 is indicated by a divergent arrow.

either condition. In the TTP55 mutant, *PcrB* activity was abolished, irrespective of illumination, while *PlitR* activity was at the same level as in the WT strain.

The transcriptional start sites of *PlitR* and *PcrB* were determined by high-resolution S1 analysis (Fig. 4B and C). The results showed that transcription in *PlitR* started at the A nucleotide that is the first nucleotide of the translational initiation codon (ATG) of LitR and that transcription in *PcrB* started at the A nucleotide 4 bp upstream from the translational initiation codon (ATG) of CrtB. The evidence indicates that both LitR and CrtB are translated by a leaderless mech-

anism (20). We previously observed a similar situation with respect to the transcriptional start site of *litR* in *S. coelicolor* A3(2) (29). The putative -35 and -10 sequences were TTGACA...TACCTT (*PlitR*) and AAGTCC...GAAAAT (*PcrB*); the former was identical to the consensus of σ^A -dependent promoters of *T. thermophilus* (TTGACA...TANCTT) described by Sevostyanova et al. (25).

Binding of Cbl to LitR and of LitR to DNA. LitR belongs to the MerR family, whose regulatory activity depends on the binding of a ligand (3, 12). The C-terminal region of LitR exhibits similarity with a Cbl-binding motif (corresponding to

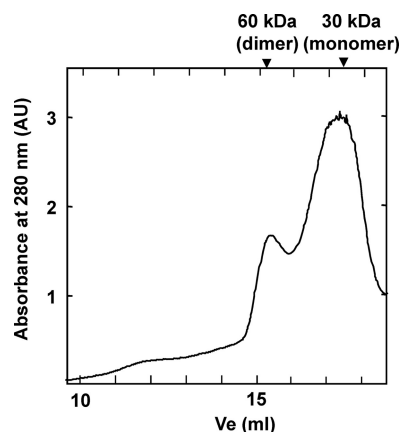


FIG. 5. Gel filtration chromatogram of LitR recombinant protein.

amino acids [aa]165 to 273 of *T. thermophilus* LitR). Cbl is a cobalt (Co)-containing tetrapyrrole that absorbs blue light; hence, it seems likely that Cbl serves as a ligand of LitR and plays some role in photosensing. To study the actual binding of Cbl, recombinant LitR was prepared by the standard method, using an *E. coli* expression system (see Materials and Methods). Gel filtration analysis of the recombinant LitR, purified by standard affinity chromatography, showed the presence of its dimer form (Fig. 5). The LitR recombinant protein was then incubated with MeCbl, and its absorption spectrum was studied (see Materials and Methods). The MeCbl-treated LitR recombinant exhibited a spectrum similar to that of free MeCbl (Fig. 6). On the other hand, the similarly treated LitR mutant lacking the C-terminal Cbl-binding domain (corresponding to aa 151 to 285, containing the whole Cbl-binding domain) did not exhibit the characteristic absorption profile. These results indicate that Cbl binds the C-terminal region of LitR.

Furthermore, to study the involvement of Cbl in photodependent regulation, a mutant with a knockout of the Cbl biosynthesis gene (*cob*) cluster was generated. The null mutant for the coding sequences corresponding to TT_P0001-0005 (Fig. 1A) was viable and produced carotenoids under both light and dark conditions (Fig. 2B, left panels). The ability of this mutant to repress carotenoid production under dark conditions was restored by supplying MeCbl (Fig. 2B, right panels) or hydroxycobalamin or cyanocobalamin (data not shown). The supply of MeCbl affected neither the constitutive carotenoid production in the *litR* mutant nor the carotenoid-deficient phenotype of the TTP55 mutant (data not shown). The transcriptional activities of *PcrtB* and *PlitR* in the *cob* mutant exhibited a profile similar to that in the *litR* mutant (Fig. 4A, bottom); *PcrtB* was active under both light and dark conditions, and *PlitR* activity was markedly upregulated compared to that in the WT strain. These results indicate that Cbl is essential for the light dependence of the transcription at *PcrtB* and hence that of carotenoid production.

The LitR recombinant was then studied for its DNA-binding activity. First, it was subjected to a gel mobility shift assay; the result showed that LitR caused a specific mobility shift of a probe DNA that contained the intergenic region between *litR*

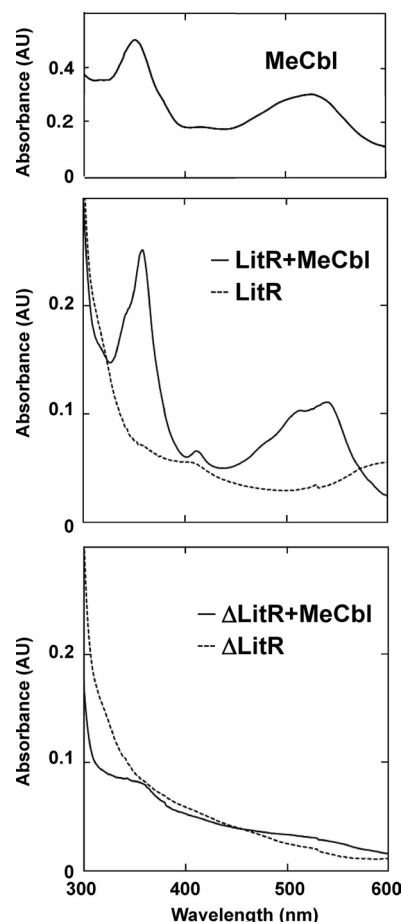


FIG. 6. Absorption spectrum of MeCbl-treated LitR recombinant. Spectra of free MeCbl (upper panel) and the MeCbl-treated WT LitR (LitR+MeCbl) (solid line in the middle panel) and truncated mutant LitR (Δ LitR+MeCbl) (solid line in the lower panel) are shown. Spectra of untreated recombinants are also shown (dashed lines in the middle and lower panels).

and *crtB* (see Fig. S1 in the supplemental material). This DNA-binding was observed with LitR in both monomeric and dimeric fractions in the gel filtration (Fig. 5) irrespectively of the treatment with illumination or MeCbl (data not shown).

We then carried out a DNase I footprint analysis to determine the binding site. As shown in Fig. 7, the region corresponding to positions -55 to -94 with respect to the transcriptional start point of *crtB* was protected from digestion by DNase I, due to the binding of LitR (see also Fig. 4C). The protected region contained the -35 region of *PlitR* and an inverted repeat, ATGTATA...(16 bp)...TGTACAT. The protection profile was not affected by treatment of LitR protein with illumination or MeCbl (data not shown).

In vitro transcription. Overall, the results supported the view that the LitR protein negatively controls *PlitR* activity via its binding to the intergenic region and that the TTP55 protein serves as a positive regulator for *PcrtB*. To verify this, an *in vitro* transcription analysis was carried out (Fig. 8). The commercial RNA polymerase holo complex successfully generated LitR mRNA (89 bases for template A), but the transcription was not affected by the addition of LitR or TTP55 recombinant

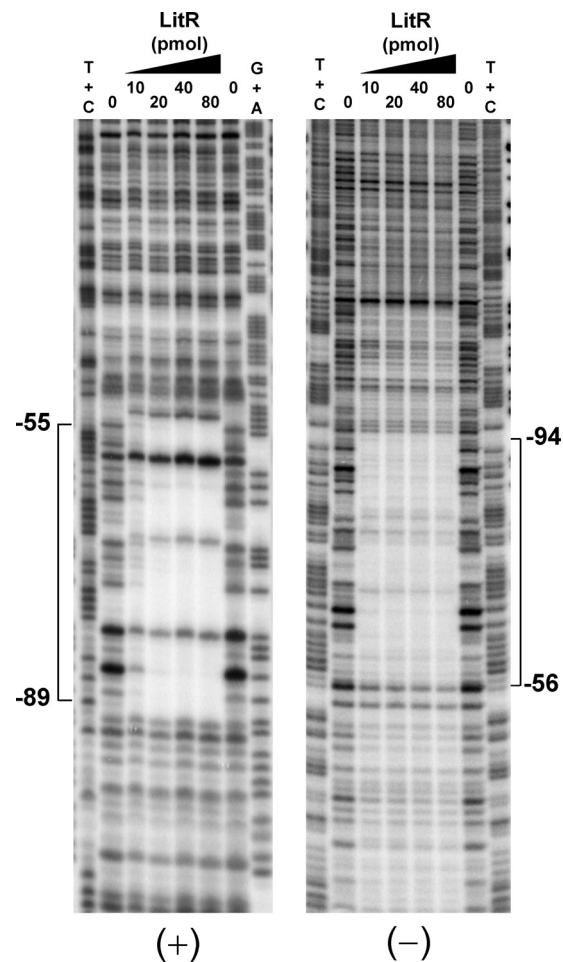


FIG. 7. DNase I footprinting for determining the LitR binding site. The assay was performed on the sense (+) and antisense (–) strands. The amounts of recombinant LitR protein added to the reaction are shown. The position numbering is based on that for *PcrB* (the transcriptional start point of *PcrB* is numbered +1). The DNase I digests were run with the same probes that were chemically cleaved (G+A and T+C lanes).

protein. We tested various conditions but could not observe repression of *PlitR* activity by LitR.

In contrast, the formation of *CrtB* mRNA (117 bases for templates A and B) (the results for template B are shown in Fig. S2 in the supplemental material) depended on the supply of TTP55 protein. This TTP55-dependent transcription for *crtB* was inhibited by the dose of LitR recombinant. The assay using the trimmed templates (templates C to E) showed that TTP55-dependent transcription occurs on templates D and E but not on template C, indicating that the transcription at *PcrB* based on the function of TTP55 requires the region encompassing positions –51 to +1 (see also Fig. 4C). This region contains a potential binding sequence (AGTGT[N7]G CAAAA) that exhibits similarity with the consensus [WWGT GA(N5-7)ACACWW] for the cAMP-independent CRP/FNR regulator of *T. thermophilus* (1). Based on this observation, a mutant template (A′) was generated by introducing transversion mutations (from T to G) at positions –47 and –49 (corresponding to the underlined bases in the potential binding

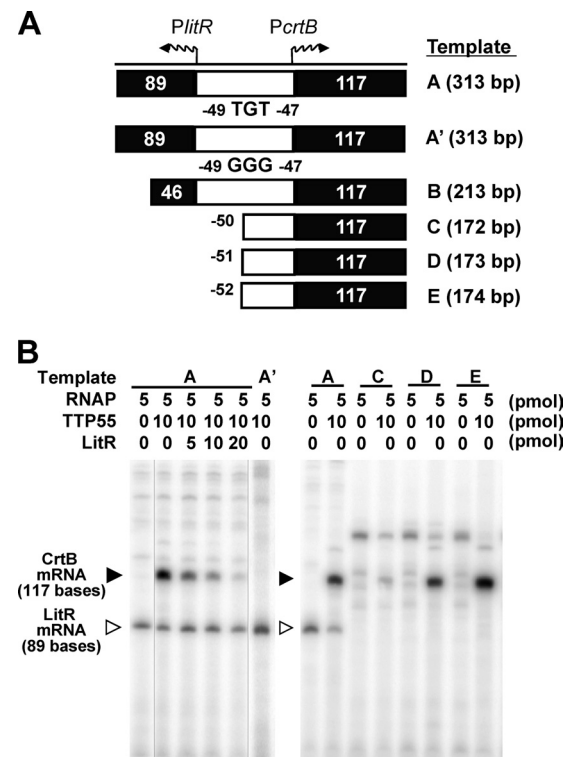


FIG. 8. *In vitro* runoff transcription assay. A schematic representation of the locations and sizes of the template DNA fragments (A) and a representative result of the assay (B) are shown. The commercial RNA polymerase holoenzyme (RNAP) of *T. thermophilus*, the purified TTP55 recombinant, and the LitR recombinant were added to the reaction mixture in the indicated amounts. Closed and open triangles denote the positions of the expected transcripts for *PcrB* and *PlitR*, respectively.

sequence given above) and used as a template for *in vitro* runoff analysis. The result showed that the reaction using this mutated fragment did not form *CrtB* mRNA (Fig. 8B). This supported the view that the corresponding region is involved in the recognition by TTP55; however, our gel shift analysis has not yet successfully detected the binding of TTP55 protein to any DNA fragment containing this region. The transcription efficiency in any assay was not affected by exposure to light or supply of Cbl and cAMP (data not shown).

DISCUSSION

As inferred from the distribution of the CarA/LitR homolog (28), *T. thermophilus* was found to produce carotenoids in a light-dependent manner. Although some observations have suggested a certain correlation between illuminated environments and the occurrence of carotenoid production in this bacterial genus (6), the existence of a light-dependent regulatory mechanism has not been known. Perhaps the ability to switch off the expression of protective functions under dark conditions benefits the organism by allowing it to save energy and prevent DNA repair errors. We have also studied pigment production in related organisms and found that carotenoids also occur in a photodependent manner in several other species, including *Thermus aquaticus*, *Thermus oshimai*, *Thermus*

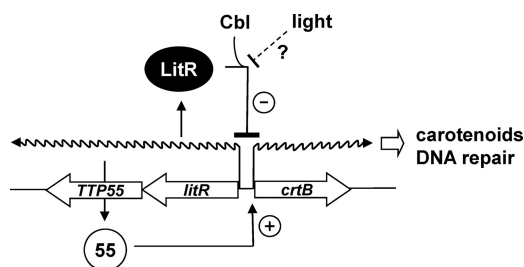


FIG. 9. Hypothetical model for light-dependent transcriptional control at the intergenic region between *litR* and *crtB* of *T. thermophilus*. LitR binds the unique site overlapping the -35 region of *PlitR*, and it negatively regulates the *PlitR* activity. Derepression of LitR due to an unknown mechanism causes the expression of TTP55, which in turn activates *PcrtB*. *PcrtB* directs the transcription of genes that encode carotenoid biosynthesis enzymes and DNA photolyase. Illumination may induce *PcrtB* activity via the inactivation of LitR.

igniterrae, and *Thermus filiformis* (our unpublished observations).

Figure 9 shows the current working hypothesis for the transcriptional control of the *crtB* promoter. The genetic evidence indicates that the two tandem transcriptional regulators LitR and TTP55 are essential for the light-dependent transcriptional control of carotenoid production in *T. thermophilus*. A simple explanation for the roles of the two regulators is that LitR represses *PlitR* and *PcrtB* activities through its binding at a unique site located close to these promoters, and its derepression allows the production of TTP55, which in turn activates *PcrtB* to direct the expression of carotenoid biosynthesis and photolyase. This model is largely supported by the results obtained in this study, except that the repression of *PlitR* activity by LitR was not reproduced in the *in vitro* transcription assay (Fig. 8). This makes us think of the possibility that an additional element is required in order to fully reproduce the transcriptional control at *PlitR in vitro*. We can also find an inconsistency in the negative feedback loop formed by LitR; the regulatory circuit in the current model keeps just a low transcription level of the *litR* operon. Some posttranscriptional inactivation mechanism for LitR should be involved in the effective expression of TTP55 and hence that of the *crtB* operon.

The absorption spectrum of the MeCbl-treated LitR recombinant (Fig. 6) demonstrated that LitR is bound by MeCbl. The localization of Cbl biosynthesis on the large plasmid also suggests its correlation with LitR function. Further, the characteristics of the *cob* mutant indicated that Cbl is essential to the photodependence of the transcription of *crt* genes. Although *in vitro* experiments have not yet shown the dependence of LitR function on Cbl, the evidence strongly suggests that Cbl binding is crucial for the function of LitR.

LitR belongs to the MerR family, but it differs from that kind of regulator in terms of the structure of the promoter controlled. The typical MerR family regulator binds a palindrome structure localized between the -10 and -35 regions. It is known that the unusual distance between -10 and -35 regions of the MerR family-dependent promoter (19 bp) is longer than the normal promoter (17 bp) and prevents the RNA polymerase holo complex from initiating transcription. The binding of the ligand-free MerR regulator to the operator

site also inhibits the initiation of transcription. However, the ligand-bound form, in turn, causes DNA to unwind through its conformation change and allows RNA polymerase to reach the -10 region and initiate transcription (3). Unlike this feature of the MerR-type promoter, the *PlitR* region bound by LitR exhibited a normal structure (i.e., a 17-bp spacer between the -10 and -35 regions); this suggests that the function of LitR has diverged from that of the MerR family regulator. A similar feature is known for the CarA-dependent promoter of *M. xanthus* (21).

TTP55 is one of the four CRP/FNR family proteins of *T. thermophilus*. Although TTP55 has a putative cAMP-binding domain in its N-terminal region, cAMP was not required for its activity to induce *PcrtB*. Agari et al. (1) recently reported that SdrP, another CRP/FNR family protein of *T. thermophilus*, does not require cAMP binding for its activity; in fact, the three-dimensional (3D) structure of this protein does not afford sufficient space for the incorporation of cAMP. A similar feature has been observed with respect to the 3D structure of TTP55 (Y. Agari and A. Shinkai, personal communication). Therefore, currently we assume that the *crtB* operon is not included in the cAMP regulon of *T. thermophilus*.

The major question now is how the illumination signal is transmitted to the activity of *PcrtB*. There is the simple hypothesis that blue-light absorption by Cbl causes the aforementioned derepression of LitR and induces the expression of TTP55 to activate *PcrtB*. It is known that blue-light absorption affects the activity of MetH, a Cbl-dependent methionine synthase; the catalytic reaction of this enzyme, involving methyl transfer from MeCbl to homocysteine, is known to suffer a photolytic conversion of cob(I)alamin to cob(II)alamin, which in turn prompts the inactivation of the enzyme (15, 16). Although the hypothesis is attractive, *in vitro* study has not yet reproduced the photodependent activation of *PcrtB* or shown any effect of Cbl on LitR function. This again makes us think of the involvement of a regulatory element(s) other than LitR and TTP55 in the light-dependent transcriptional control in *T. thermophilus*. In *M. xanthus*, light-induced carotenoid production is regulated by a complex system involving two Cbl-binding MerR family proteins (i.e., CarA and CarH), as well as related signal transducers, including a specific sigma factor and antagonists. It is assumed that a membrane-bound protoporphyrin and CarF are involved in photosensing (4, 8), but the exact mechanism is not yet known. Currently, we believe the photodependent regulatory mechanism in *T. thermophilus* to be different from that in *M. xanthus*, since the regulators identified in *M. xanthus* do not have distinct homologs in *T. thermophilus*; however, it is still possible that an illumination-dependent element affects the transcriptional control by LitR and TTP55. A detailed biochemical characterization of the function of the LitR-Cbl complex will help in understanding the exact mechanism of light-dependent transcriptional control in *T. thermophilus*.

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