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Running title: LitR of *B. megaterium*

The role and function of LitR, an AdoB$_{12}$-bound light-sensitive regulator of *Bacillus megaterium* QM B1551, in the regulation of carotenoid production

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ABSTRACT

The LitR/CarH family of proteins is a light-sensitive MerR family of transcriptional regulators that contain an adenosyl B_{12} (coenzyme B_{12}, $AdoB_{12}$)-binding domain at the C-terminus. The genes encoding these proteins are found in phylogenetically diverse bacterial genera; however, the biochemical properties of these proteins from Gram-positive bacteria remain poorly understood. We performed genetic and biochemical analysis of a homolog of the LitR protein from *Bacillus megaterium* QM B1551, a Gram-positive endospore-forming soil bacterium. Carotenoid production was induced by illumination in this bacterium. *In vivo* analysis demonstrated that LitR plays a central role in light-inducible carotenoid production and serves as a negative regulator of the light-inducible transcription of *crt* and *litR* itself. Biochemical evidence showed that LitR in complex with $AdoB_{12}$ binds to the promoter region of *litR* and the *crt* operon in a light-sensitive manner. *In vitro* transcription experiments demonstrated that $AdoB_{12}$-LitR inhibited the specific transcription of the *crt* promoter generated by an σ^A-containing RNA polymerase holoenzyme in the dark condition. Collectively, these data indicate that the $AdoB_{12}$-LitR complex serves as a photoreceptor with DNA-binding activity in *B. megaterium* QM B1551, and that its function as a transcriptional repressor is fundamental to the light-induced carotenoid production.

IMPORTANCE

Members of the LitR/CarH family are $AdoB_{12}$-based photosensors involved in light-inducible carotenoid production in non-phototrophic Gram-negative bacteria. Our study revealed that *Bacillus* LitR in complex with $AdoB_{12}$ also serves as transcriptional regulator with photosensory function, which indicates that LitR/CarH family is generally

involved in the light-inducible carotenoid production of non-phototrophic bacteria.

58 **INTRODUCTION**

59 LitR (light-induced transcription regulator)/CarH family is a MerR-family
60 transcriptional regulatory protein that contains a cobalamin (Cbl; synonym, vitamin
61 B_{12})-binding domain at its C-terminus (1-4). The first observation regarding the
62 involvement of vitamin B_{12} in the light-inducible carotenoid (Crt) production of the
63 Gram-negative gliding bacterium *Myxococcus xanthus* was presented by Cervantes *et*
64 *al.* (5). In non-phototrophic bacteria, the main function of Crt is to protect the cells from
65 photo-oxidative damage by scavenging harmful agents such as singlet and triplet
66 molecular species produced upon illumination (6, 7). Induction of Crt production by light
67 reasonably saves energy under dark. The genetic and biochemical studies
68 demonstrated that CarH is a central regulator of light-inducible Crt production in *M.*
69 *xanthus* (3).

70 Our previous study revealed that the Crt production of *Streptomyces coelicolor*
71 A(3)2, a high-GC Gram-positive bacterium, is markedly increased by illumination, and a
72 genetic study of its regulatory mechanism revealed that LitR of *S. coelicolor*, a homolog
73 of CarH, is a central regulator of *crt* biosynthesis gene expression (8). The phenotype
74 conferred by the knockout of *litR* suggests that LitR serves as a positive regulator of the
75 photo-dependent expression of extracytoplasmic function (ECF)-type sigma factor, LitS,
76 which is adjacently located in the *litR*. The precise function of LitS was demonstrated
77 using an *in vitro* transcription assay; RNA polymerase containing σ^{LitS} directs the
78 transcription of *crt* biosynthesis genes. We recently reported that cobalamin
79 biosynthesis genes are required for light-inducible Crt production (9).

80 Our previous study also showed that a LitR homolog encoded in the genome of
81 *Thermus thermophilus* HB27, a high-GC Gram-negative thermophilic bacterium, plays a

central role as a negative regulator for light-inducible carotenoid production (4). *litR* comprises an operon with *ldrP* whose product belongs to the CRP (cAMP-receptor protein)/FNR family (10). *In vivo* and *in vitro* studies showed that LitR directly binds to the intergenic promoter region of *litR* and *crtB* to repress the bidirectional transcription under dark conditions. Light irradiation causes the inactivation of LitR, which in turn allows the expression of LdrP that directly activates the transcription of the *crt* biosynthesis gene cluster and the adjacent genes to *litR* (2). Interestingly, *litR*, cobalamin biosynthesis, and other light-inducible genes, including the *crt* genes, were all found to be located on the large plasmid of this organism (2).

Ortiz-Guerrero *et al.* (1) showed that CTt2, a chimeric recombinant protein composed of the N-terminal DNA-binding domain of CarH and the C-terminal Cbl-binding domain of *T. thermophilus* HB8 LitR is an adenosyl B_{12} ($AdoB_{12}$)-binding protein with light-sensitive DNA-binding activity. The Co-C bond of $AdoB_{12}$ is chemically cleaved by light, which converts $AdoB_{12}$ to hydroxocobalamin (OHB_{12}). Therefore, light causes photolysis of $AdoB_{12}$ bound to CTt2, causing the conversion of $AdoB_{12}$ to OHB_{12}, which abolishes its DNA-binding activity. The photolysis of $AdoB_{12}$ also induces a dramatic conformational change: subunit dissociation of CTt2 from tetramer to monomer. A full-length native TtCarH of *T. thermophilus* HB8, an ortholog of LitR with a single residue substitution, has been reported to exhibit light-dependent DNA-binding behavior (11).

One of the intriguing features of the LitR is the wide distribution of its homologs in phylogenetically diverse genera of non-phototrophic bacteria (1, 12, 13); they are frequently flanked by genes for *crt* biosynthesis and DNA photolyase. In the current study, we examine the role and function of a LitR homolog of *Bacillus megaterium* QM

B1551, a low-GC, Gram-positive, endospore-forming soil bacterium. Although members of the LitR/CarH family are widely distributed in both Gram-negative and Gram-positive bacteria, biochemical analyses of this family have been limited to proteins from Gram-negative bacteria (1, 4). Therefore, we believe that a biochemical study of a LitR protein derived from Gram-positive bacteria provides an insight into the general role of LitR/CarH family members. The results obtained in this study indicate that *B. megaterium* LitR, in complex with $AdoB_{12}$, serves as a photoreceptor protein directly regulating carotenoid production in a light-inducible manner.

115 **MATERIALS AND METHODS**

116 **Bacterial strains, plasmids, and culture media.** The wild-type strain of *B. megaterium*
117 QM B1551, DSM319, and NBRC15308 used in this study were obtained from the
118 Bacillus Genetic Stock Center (BGSC, Ohio State University; http://www.bgsc.org/),
119 DSMZ (Braunschweig, Germany), and the Biological Resource Center, NITE (NBRC),
120 Kazusa, Japan. *B. subtilis* 168, Δ*yvqK*, Δ*yvrA*, Δ*yvrB*, and Δ*yvrC* were obtained from the
121 National BioResource Project (NIG, Japan): *B. subtilis*. *Escherichia coli* HST08 and
122 Rosetta2(DE3)pLysS (Takara Bio Inc., Shiga, Japan) were used as hosts for DNA
123 manipulation and protein expression, respectively. pUC19 and pUC118 (Takara Bio)
124 were used for general DNA manipulation of *E. coli*. pT7Blue and pMD19 (Takara Bio)
125 were used for TA cloning of PCR-generated DNA fragments. pGEX-6P-2 (GE
126 Healthcare UK Ltd, Buckinghamshire, England) and pET51-b(+) (Takara Bio) were used
127 for the overexpression of *B. megaterium* QM B1551 LitR and SigA, respectively.
128 pUCTV2, an *E. coli-Bacillus* temperature-sensitive plasmid shuttle vector (14) carrying
129 tetracycline resistance was obtained from Dr. F. Meinhardt, and used for a gene
130 disruption and complementation analysis of *B. megaterium*. pDG1661 (carrying
131 chloramphenicol resistance and *amyE* for homologous recombination) was obtained
132 from BGSC and used as a chromosome-integration plasmid for *B. subtilis*. Enzymes
133 used for DNA manipulation were purchased from Takara Bio and New England Biolabs
134 (Ipswich, MA). The conditions for culture and genetic manipulation of *E. coli* have been
135 described (15). *B. megaterium, B. subtilis,* and *E. coli* were grown at 37 °C in
136 Luria-Bertani (LB) medium (15), 1.0–1.5% agar (Kokusan, Tokyo, Japan) was added to
137 prepare solid media. Cells were illuminated by an illuminating incubator (BR-180LF;
138 Taitech; Saitama, Japan) equipped with a white light fluorescent lamp (20W; Toshiba;

Tokyo, Japan) if required. To enable the selection of *E. coli* transformants, 50 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin, and 10 μg mL^{-1} tetracycline were added. For selection of *Bacillus*, 10 μg mL^{-1} tetracycline, 5 μg mL^{-1} chloramphenicol, and 1 μg mL^{-1} erythromycin were added.

Taxonomic characterization of light-dependent isolates. Eighty strains that exhibited photo-dependent phenotypes were isolated from soil by cultivating at 28 °C or 37 °C on LB solid medium. An illuminating incubator (BR-180LF) equipped with white fluorescent lamps was used. To taxonomically characterize the isolates, total DNA was extracted from the isolates by using a PurElute Bacterial Genomic Kit (Edge BioSystems; Gaithersburg, MD), and used for the PCR amplification of 16S rRNA gene using B8F/B1500R primer pair (the oligonucleotide primers used are shown in Supplementary Table S1 in the Supplemental Materials) (16). The purified PCR amplicon was TA-cloned into pMD19. The nucleotide sequence of the 16S rRNA gene clone was determined with a BigDye Terminator v3.1 cycle sequencing kit on an ABI3100 automated DNA sequencer (Thermo Fisher Scientific Inc., Waltham, MA). The BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/) and the SEQUENCE_MATCH program from the Ribosomal Database Project database (17) were used to compare the obtained nucleotide sequences of the 16S rRNA gene with those in the GenBank/EMBL/DDBJ nucleotide sequence databases. The analysis revealed that 24 isolates exhibit > 99.5% sequence identity with *Bacillus* spp. Of these, 5 strains affiliating with *B. megaterium* (Nos. 164 and 423), *B. pumilis* (No. 393) and *B. sphaericus* (Nos. 294 and 319) were used in this study.

Extraction of carotenoids. To examine light-dependent carotenoid production, *B. megaterium* were cultured at 28 °C for 2 d on liquid LB medium under light and dark conditions. Extraction of carotenoids was performed as described previously (4). An illuminating incubator BR-180LF equipped with white, blue, green and red fluorescent lamps (20W; Toshiba, Tokyo, Japan) was used. Light intensity was measured using a Model LI-250 Light Meter (LI-COR Inc., Lincoln, NE). A UV spectrometer U-2800A (Hitachi High-Tech Science Corp., Tokyo, Japan) and a NanoDrop 2000 (Thermo Fisher Scientific) were used to record the absorption spectra of the carotenoid fractions.

Quantification of Cbl. The Cbl content was determined by cultivating *Lactobacillus leichmannii* ATCC 7830 (ATCC, Manassas, VA) as an indicator strain with Difco^{TM}B$_{12}$ Assay Medium (Becton, Dickinson and Co.) according to the instructions supplied by manufacturer.

Gene disruption. The temperature-sensitive *E. coli-Bacillus* shuttle vector pUCTV2 was used for construction of a markerless mutant of the *litR*, *cobS* (*BMQ_1998*), *crt* operon (*BMQ_0654-0656*) of *B. megaterium* QM B1551. pUCTV2 is replicated stably in *Bacillus* spp. at a temperature of 30 °C; at the non-permissive temperature of 42 °C plasmid replication is disturbed, eventually leading to loss of the vector (14). To construct each disruption plasmid, the upstream region and the downstream region were amplified by PCR using the primer sets DRF/DRMR and DRMF/DRDR (*litR*), DSF/DSMR and DSMF/DSR (*cobS*), and DCF/DCMR and DCMF/DCR (*crt* operon) (Supplementary Table S1). The two amplified fragments were digested by *Sph*I for *litR* and *BMQ_0654-0656*, and *Kpn*I for *cobS*, and after purification the digestion products

were ligated. The ligated products were then amplified by PCR with the primer sets DRF/DRDR (*litR*), DSF/DSR (*BMQ_1998*), and DCF/DCR (*BMQ_0654-0656*). The amplified fragment was cloned onto pUC118 and the nucleotide sequence was verified by sequencing using an ABI3100 Genetic Analyzer or sequencing was performed by Eurofins Genomics K.K. (Tokyo, Japan). Each resulting plasmid was digested with *Bam*HI and inserted into the same site of pUCTV2 to generate the disruption plasmids. The resultant plasmid was introduced into *B. megaterium* QM B1551 by PEG-mediated protoplast transformation (18). Subsequently, tetracycline-resistant transformants were grown at 42 °C in LB medium containing tetracycline to select the single-crossover (plasmid-integrated) colonies. Among these transformants, single-crossover strains were selected by PCR using the appropriate primers. A single selected strain was serially grown in LB without tetracycline to induce second-crossover recombination. Finally, tetracycline-sensitive colonies were selected as double-crossover mutants to yield Δ*litR*, Δ*crt*, and Δ*BMQ_1998*. The expected double crossover-mediated homologous recombination of such derivatives was verified by PCR using the appropriate primer sets.

Plasmids for genetic complementation. For genetic complementation, pUCTV2thrC, an integration vector directing the *thrC* region of *B. megaterium* was constructed as follows. The *thrC* locus is generally used as integration site for integrative vectors of *B. subtilis*. The 1.1 kb internal region of *thrC* was amplified by PCR using the primers thrCF and thrCR (Supplementary Table S1), recovered as an *Mfe*I-digested fragment and inserted between the *Eco*RI sites of pUCTV2, giving rise to pUCTV2thr. The intact *litR* with its own promoter region was amplified by PCR using the primer sets RcF/RcR

(Supplementary Table S1), and cloned into the *Bam*HI site of pUCTV2thrC. The resulting plasmid was introduced into Δ*litR*, and the whole plasmid-integrated strains with single-crossing over were selected, and designated Δ*litR*/*litR*. These integrated strains were obtained using gene disruption methods similar to those described above. In all cases, proper integration was verified by PCR.

Functional analysis of LitR in *B. subtilis* 168 as a heterologous host. Because the *B. subtilis* 168 chromosome appeared not to contain a *litR* homolog, we used this strain as a heterologous host to investigate the function of *litR*. An *amyE*-integrative reporter assay vector, pDG1661 (19) was used to measure the activity of the light-induced promoter and related promoters in *B. subtilis in vivo* via β-galactosidase activity. The DNA fragments containing the promoter regions of *litR* and *rpoB* (*BMQ_0128*) were amplified by PCR using the primers RZF/RZR for *litR*, RZF/RZR for *litR*, and BZF/BZR for *rpoB* (control) (Supplementary Table S1), recovered as *Bam*HI/*Eco*RI-digested fragments, and inserted between the same sites of pDG1661. In a similar manner, *litR* with its own promoter was amplified by PCR using the primers RZF/RPZR. For the substitution of the histidine residue at position 190 to alanine, the entire *litR* gene was generated by overlap-extension PCR, using the primer pairs RZF/R190ZMR and R190ZMF/RZR. The resulting plasmids carrying each promoter sequence were integrated into the *amyE* locus of *B. subtilis* 168, Δ*yvqK* (BSU33150), Δ*yvrA* (BSU33160), Δ*yvrB* (BSU33170), and Δ*yvrC* (BSU33180) by a single-crossover recombination. A *B. subtilis* strain harboring a reporter plasmid was grown in LB liquid medium under light and dark conditions at 37 °C. A β-galactosidase assay was performed using the bacterial cultures according to the standard method (15), using

o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate.

Mutational analysis of the LitR-binding site in the *litR* promoter. Base substitutions in the *litR* promoter were generated by a two-stage PCR procedure using complementary mutagenic primers. The regions upstream and downstream of a mutation site were amplified by PCR using the primers RZF/MP1R and MP1F/RZR (for pM1), RZF/MP2R and MP2F/RZR (for pM2), RZF/MP3R and MP3F/RZR (for pM3), RZF/MP4R and MP4F/RZR (for pM4), RZF/MP5R and MP5F/RZR (for pM5), RZF/MP6R and MP6F/RZR (for pM6), RZF/MP7R and MP7F/RZR (for pM7), RZF/MP8R and MP8F/RZR (for pM8), RZF/MP9R and MP9F/RZR (for pM9), RZF/MP10R and MP10F/RZR (for pM10), RZF/MP11R and MP11F/RZR (for pM11), and RZF/MP12R and MP12F/RZR (for pM12) (Supplementary Table S1). The two DNA fragments prepared in the first-round PCRs were used as the template in a second-round PCR with the primers RZF/RZR. The fragments amplified by the second-round PCR were phosphorylated by T4-polynucleotide kinase, and then cloned into the *Hinc*II site of pUC118. After the mutations were confirmed by nucleotide sequencing, the *litR* promoter sequences containing various mutations were excised with *Eco*RI and *Bam*HI, and ligated between the same sites of pDG1661, resulting in plasmids pM1 through pM12. The plasmids were integrated into the *amyE* locus of the *B. subtilis* 168 chromosome to measure the β-galactosidase activity.

5′-RACE for determination of transcriptional start points. To determine the transcriptional start site of the *litR*, *crtI1*, and *polA* promoters, the assignment of the mRNA 5′-end was performed by 5′-RACE using a 5′-Full RACE Core Set (Takara Bio) or

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Directed Amplification of Transcription Start Sites (DMTSS) (20). The 5′-Full RACE Core Set was used according to the manufacturer's instructions. DMTSS is a method for determining TSSs, in which cDNA is labeled with a homopolynucleotide tail. The cDNAs were labeled at the 3′-terminal ends with a homo adenine sequence (poly(A)) by incubation with Terminal Deoxynucleotidyl Transferase (Takara Bio) at 37 °C for 30 min. The TdT enzyme was inactivated by heating to 70 °C for 10 min. The first-round PCR was performed with A1, a specific primer for the target gene, and the DMTSS-1 primer, which contains homo thymidine (T). The following reaction program was used: denaturation and amplification for 30 cycles (95 °C for 30 s, 55 °C for 30 s, and 72 °C 45 sec), and 72 °C for 3 min. The second-round PCR was performed using the A2 and DMTSS-4 primers. The amplified DNA fragment was cloned into the T-vector pMD19. The oligonucleotide primers used for this analysis are summarized in Supplementary Table S1. The inserted DNA sequences were sequenced using an ABI3100 Genetic Analyzer or the sequencing was performed by Eurofins Genomics.

RNA preparation and semi-quantitative RT-PCR analysis. *B. megaterium* strains were grown in liquid LB medium at 37 °C. The total RNA of the *B. megaterium* strains was extracted using a Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed by using a SuperScript III First-strand Synthesis System (Thermo Fisher Scientific) using 2 µg purified total RNA, according to the manufacturer's protocol. The dsDNA was amplified with GO Taq Master Mix (Promega Corp., Madison, WI), and analyzed by agarose gel electrophoresis with ethidium bromide staining. The following PCR program was used: initial denaturation at 95 °C for 180 s and amplification for 25 cycles of 95 °C for 30 s,

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55 °C for 30 s, and 72 °C 45 s, followed by 72 °C for 3 min. The oligonucleotide primers used are listed in Supplementary Table S1.

Preparation of AdoB$_{12}$-bound LitR and SigA proteins. For the preparation of soluble LitR and LitR$_{H190A}$ recombinant proteins, each protein was expressed as a protein fused with glutathione-S-transferase (GST). The N-terminal GST fusion to LitR was constructed using the PCR cloning primers RexF and RexR (see Supplementary Table S1). These primers contain restriction sites for *Bam*HI and *Eco*RI, respectively. The amplicon was cloned into the same site of the expression plasmid pGEX-6P-2 (GE Healthcare), giving rise to the plasmid pGEXLitR. To generate LitR$_{H190A}$, a LitR protein in which histidine is substituted with alanine at amino acid position 190, the entire *litR* gene was generated by overlap-extension PCR, using the primer pairs RexF/R190ZMR and R190ZMF/RexR. The two DNA fragments amplified in a first-round PCR were used as the template in a second-round PCR with the primer set RexF/RexR. The amplified fragment was digested with *Eco*RI and *Bam*HI, and ligated between the same sites of the pGEX-6P-2 plasmid, resulting in pGEXLitR$_{H190A}$. pETSigA, an expression vector for SigA, was constructed based on pET-51b(+). *sigA* was amplified with the primer set AexF/AexR, and cloned into the *Nco*I and *Sac*I sites of pET-51b(+). SigA contained His 10 at the C-terminus. All genes cloned above were under the regulation of an isopropyl β-D-thiogalactoside (IPTG)-inducible promoter.

E. coli Rosetta2(DE3)pLysS cells harboring pGEXLitR, pGEXLitR$_{H190A}$, or pETSigA were first cultured overnight at 28 °C in LB liquid medium. Subsequently, the seed culture was inoculated at 1% into 100 mL of LB medium prepared in a 500-mL baffled Erlenmeyer flask. After 3 h of cultivation at 28 °C with rotary shaking (135 rpm), IPTG

was added at a final concentration of 0.1 mM, with continued incubation at 28 °C for 4 h. The *E. coli* cells thus obtained were harvested by centrifugation, suspended in PBS buffer (containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 1.8 mM KH_2PO_4), and disrupted with a sonicator (Astrason XL2020, Misonix, Farmingdale, NY) or a mechanical cell presser, Model APV GAULIN 1000 (APV HOMOGENISERS As, Denmark). The cell-free extract was centrifuged at 70 000 × *g* for 30 min, and the resultant supernatant was used for the affinity chromatography. The GST tag was removed by treatment with PreScission protease (GE Healthcare). The following step was performed under conditions of dim light or under dark conditions. To prepare LitR protein associated with $AdoB_{12}$, purified LitR was incubated in the presence of a 10-fold molar excess of adenosylcobalamin ($AdoB_{12}$) (SIGMA-ALDRICH Corp., St.Louis, MO) for 1 h at 37 °C. Dialysis against PBS buffer was performed to remove unbound $AdoB_{12}$ from the mixture. A U-2800A UV spectrometer or a NanoDrop 2000 was used to record the absorption spectra of the resultant $AdoB_{12}$-LitR complexes. The protein concentration was measured with a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Gel-filtration column chromatography. The molecular size of LitR was estimated by gel-filtration of 1.1 mg of the purified recombinant protein. Gel filtration was performed using a Superdex 200 HR 10/30 column on an ÄKTA FPLC system (GE Healthcare), according to the manufacturer's recommendations. The column was developed with 1X 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^{-1}. Molecular size standards (Feritin, Conalbumin, Carbonic anhydrase, Ribonuclease indicating 440, 75, 29, and 13.7 kDa, respectively) included in

a gel-filtration calibration kit (GE Healthcare) were used to determine the void volume (*Vo*) and vitamin B12 (1.35 kDa) was used to estimate total bed volume, *Vt*.

Analytical Ultracentrifugation.

AUC-SV analytical ultracentrifugation-sedimentation velocity experiments was carried out with an Optima XL-1 analytical ultracentrifuge (Beckman Coulter, Inc., Pasadena, CA) with a four hole An60Ti rotor. The non-illuminated and illuminated $AdoB_{12}$-LitR proteins (A280 = 0.6) were centrifuged at 40,000 rpm, 20 °C. Protein concentrations were monitored at 280 nm. Data were analyzed with a continuous *c*(*s*) distribution model by using a software program SEDFIT (version 11.8).

Gel-shift assay. DNA binding was determined using a gel-shift assay, as described previously (4). Probe DNA fragments were generated by PCR using the primer sets RFPF/RFPR (P*litR*), CFPF/CFPR (P*crtI1*), and PAF/PAR (P*sigA*) with the chromosome of a QM B1551 WT strain (see Supplementary Table S1). The *litR* promoter with the mutated LitR-binding site was amplified using the primer set RFPF/RFPR with pM1 to pM12, constructed above, as templates. These probe DNA fragments were labeled at the 5′ end with [γ-^{32}P] ATP (PerkinElmer, Inc., Boston, MA), using T4-polynucleotide kinase. A total of 0.5–5.0 ng of ^{32}P-labeled probe (10,000–20,000 cpm) was mixed with 0-32 pmol of recombinant LitR, prepared as described above; it was then incubated at 37 °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 µg mL^{-1} of bovine serum albumin]. Under light conditions, the sample was illuminated with white light at approximately 42 µmol s^{-1} m^{-2}. Following 10 min of exposure to white

light, reaction mixes were incubated at 37 °C for 30 min, and specific DNA-protein complexes were separated from free probe on a non-denaturing polyacrylamide gel containing 6% acrylamide. The gels were dried, and radioactive signals were detected by exposing dried gels to a Fuji imaging plate (Fuji Film, Tokyo, Japan), and images were scanned with a Typhoon 9410 or a Typhoon FLA 9500 Image Analyzer (GE Healthcare).

DNase I footprint. The LitR-binding site in the promoter region of P*litR* and P*crt* was determined using a DNase I footprint analysis, as described previously (4). The ^{32}P-labelled DNA fragments were prepared by PCR using the primers RFPF/RFPR for P*litR*, and CFPF/CFPR for P*crt* (Supplementary Table S1). The reaction mixture (50 μL) contained 10 kcpm ^{32}P-labeled DNA probe; 0-8 pmol LitR; 20 mM Tris-HCl, pH 7.2; 1.0 mM EDTA-NaOH, pH 8.0; 50 mM NaCl; and 10% glycerol. After incubation at 25 °C for 30 min, DNase I was added at a final concentration of 20 μg mL^{-1}, and the mixture was further incubated for 1 min. 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^{-1} 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 (A+G reactions) generated from the ^{32}P-labelled probe DNA fragment were used as a reference.

***In vitro* run-off transcription.** The *in vitro* run-off transcriptional assay was performed

379 as described previously (4, 21, 22). DNA templates containing the transcriptional start
380 sites of P*litR* and P*crt* were generated by PCR, using the primers PLF/PLR for Template
381 P*litR*, M7, and M12 (233 bp), PCF/PCR for Template P*crt* (306 bp), and PPF/PPR/ for
382 Template P*polA* (302 bp) (see Supplementary Table S1). A total of 0.5 pmol of template
383 DNA was mixed with 2 pmol commercial RNA polymerase core enzyme of *E. coli* (AR
384 Brown, Tokyo, Japan), 100 nmol of ribonucleotides, including [α-^{32}P] CTP (PerkinElmer),
385 and 0–8 pmol of LitR. The RNAP holoenzyme was reconstituted by combining the
386 commercial RNAP core enzyme and the *B. megaterium* σ^A recombinant protein purified
387 from *E. coli*. Transcripts were analyzed by polyacrylamide gel electrophoresis. Marker
388 10 (pBR322/*Msp*I digest) (NIPPON GENE CO., LTD., Tokyo, Japan) labeled with [γ-^{32}P]
389 ATP was used as a standard. The LitR recombinant proteins were irradiated at
390 approximately 42 µmol s^{-1} m^{-2} with blue light (λ_{max} = 450 nm), green light (λ_{max} = 530 nm),
391 and red light (λ_{max} = 660 nm). The irradiance provided by the various light treatments
392 was measured using a Model LI-250 Light Meter.
393

RESULTS

Light-induced carotenoid production and the distribution of the LitR/CarH family in *Bacillus* spp. The ability of *Bacillus* spp. to produce carotenoid has been reported (23-27); however, the correlation between carotenoid production and light has not been investigated in this genus. In our screen of light-responsive bacteria (see MATERIALS AND METHODS), the strains isolated from soil were identified to be *B. megaterium*, *B. sphaericus*, and *B. pumilus*, using the 16S rRNA gene-based phylogenetic analysis. These bacteria produced a pale or strong yellow pigment under light conditions; in contrast, the non-illuminated colonies of this strain appeared white or pale yellow (Fig. 1). This suggests that a light-responsive regulatory mechanism also exists in this group of bacteria.

To examine whether the yellow pigment was composed of carotenoids, we selected *B. megaterium* QM B1551, a strain whose genome has been completely sequenced (28), as a model. This bacterium exhibited photo-dependent carotenoid production (Fig. 1). Carotenoid production was induced by blue and green light, but not by red light (Fig. 2 and Supplementary Figure S1). The UV-visible absorption spectrum of a methanol extract of the illuminated cells showed a typical carotenoid profile, exhibiting multiple absorption peaks near 450-nm (Fig. 2). The absorption spectrum was not observed in the extract from a knockout mutant for the carotenoid biosynthesis gene (see below). This confirms that the spectrum observed here in that of carotenoids. We tried to purify the carotenoids produced by *B. megaterium* QM B1551, but their high photo-sensitivity created difficulties in further purification and identification. We also confirmed that *B. megaterium* DSM319 (28), another strain whose genome has been sequenced, also exhibited photo-dependent carotenoid production (Fig. 1 and Fig. 2).

In accordance with the observation that *B. megaterium* QM B1551 undergoes light-dependent carotenoid production, this bacterium was found to possess two putative *crt* biosynthesis operons (Supplementary Figure S2A). *BMQ_0654-BMQ_0656* encodes a putative phytoene desaturase (0654; designated as CrtI1), phytoene desaturase (0655; CrtI2), and a phytoene synthase (0656; CrtB), whereas *BMQ_2952-2949* encodes a conserved hypothetical protein (2952), a putative phospholipid/glycerol acyltransferase (2951), putative glycosyl transferase (2950), and putative phytoene desaturase (2949; CrtI3). Probably, CrtB and CrtI are involved in lycopen biosyntheis. BMQ_2950 may be involved in the glycosylation of carotenoids, because it contains a glycosyl transferase domain. *B. megaterium* DSM319 also harbors the *crt* operon in the same gene organization (data not shown). In contrast, the genomes of closely related *Bacillales* species (including *B. clausii*, *B. cellulosilyticus*, *B. thuringiensis*, *B. anthracis*, *B. cereus*, and *B. pseudofirmus*) retain *crt* operons as a single-locus operon (Supplementary Figure S2A).

B. megaterium retains a *litR* homolog [*BMQ_4356* for QM B1551 (Supplementary Figure S2B); *BMD_4342* for DSM319]. Unlike *T. thermophilus* and *M. xanthus*, the *litR* homolog of *B. megaterium* located at a locus different from that of the putative carotenoid biosynthesis genes. The LitR of *B. megaterium* QM B1551 consists of 303 amino acid residues, exhibiting 28% amino acid sequence identity to that of *T. thermophilus* (Supplementary Figure S3). The N- (corresponding to N8-Y45) and C-terminal region (K94-I167 and K179-F268) exhibited distinct similarity to the helix-turn-helix domain of a MerR family regulatory protein (*e*-value=2e-08) and the B_{12} binding domain (*e*-value=1.3e-15, *e*-value=6e-14), respectively (Supplementary Figure S3). The gene organization at the *litR* locus is conserved in the genomes of a part of

Bacillus spp., including *B. clausii*, *B. cellulosilyticus*, *B. thuringiensis*, *B. anthracis*, *B. cereus*, and *B. pseudofirmus* (Supplementary Figure S2B).

Knockout of the *crt*, *litR*, and *cbl* genes in *B. megaterium* QM B1551. To verify whether the *crtI1-crtI2-crtB* (*BMQ_0654-0656*) genes are involved in *crt* biosynthesis in *B. megaterium* QM B1551, a markerless mutant lacking these three coding sequences (Δ*crt*) was generated. As shown in Fig. 2, the Δ*crt* mutant was defective in carotenoid production under both dark and light conditions. This indicates that the gene products of *crtI1-crtI2-crtB* (hereafter, designated as *crt* operon) are responsible for carotenoid biosynthesis.

To investigate the role of *litR*, a markerless mutant for *litR* (designated Δ*litR*) was constructed. The resultant Δ*litR* produced a large amount of carotenoid under both dark and light conditions (Fig. 2). To generate a genetically complemented strain, we originally constructed a chromosome integration vector, pUCTV2thr (see MATERIALS AND METHODS). The genetically complemented strain partially restored the light-inducible carotenoid production**.** This is probably due to the introduction of the *litR* into the genomic region different from the original coding region. These results indicate that LitR is involved in the light-dependent genetic control of carotenoid production based on its function as a negative regulator.

Concordant with the fact that *B. megaterium* was previously used for the commercial production of cobalamin, *B. megaterium* has a complete anaerobic pathway for cobalamin biosynthesis (29), including a large (*cbiWHXJCDETLFGA-cysG-cbiY-btuR*) and a small (*cbiB-cobDUSC*) operon (30, 31). Although Cbl production by *B. megaterium* has been well documented, the correlation between vitamin B_{12} and

light-dependent carotenoid production has not yet been studied in this bacterium. *B. megaterium* QM B1551 encodes two proteins involved in the final step of the oxygen-independent pathway of $AdoB_{12}$ biosynthesis: (i) adenosylcobinamide-GDP ribazoletransferase (BMQ_1998) and (ii) ATP:cob(I)alamin adenosyltransferase (BMQ_2001). We constructed a markerless null mutant for *BMQ_1998*. As shown in Fig. 2, Δ*BMQ_1998* performed weak carotenoid production, irrespective of illumination. The result indicates that BMQ_1998 serves as a main biosynthesis enzyme to produce $AdoB_{12}$, and suggests that $AdoB_{12}$ is involved in the light-inducible carotenoid production.

Transcriptional analysis. To examine whether photo-dependent carotenoid production is regulated at the transcriptional level, we performed transcriptional analysis. Prior to the analysis, transcriptional start site was determined by 5′-RACE with respect to the promoter region preceding *litR* and *crtI1* (see MATERIALS AND METHODS). We also determined the transcriptional start site of *polA* (*BMQ_4764*; gene encoding DNA polymerase I), which was used as a control promoter in the *in vitro* runoff transcriptional assay (see below). Using total RNA isolated from illuminated cells, 5′-RACE assigned a single transcriptional start site 40-bp upstream of the translational initiation codon (ATG) of *litR* (Fig. 3A), 32-bp upstream for *crtI1* (Fig. 3A), and 34-bp upstream for *polA*. The potential -35 and -10 sequences of *litR* ($TTTACA_{N17}TATAAT$), *crtI* ($TTGTAT_{N17}TATAAT$), and *polA* ($TTGTCC_{N18}TAAAAT$) were similar to promoter sequences recognized by *B. subtilis* σ^A-RNA polymerase (Fig. 3A), for which the consensus sequence is $TTGACA_{N16–18}TATAAT$ (32). Hereafter, these promoters will be designated as P*litR*, P*crtI1*, and P*polA*, respectively.

We examined the mRNA levels of *litR* and *crtI1* under dark and light conditions by semi-quantitative RT-PCR analyses. An essential sigma factor gene, *sigA,* was used as the control, because illumination did not affect the growth of *B. megaterium* (data not shown), which suggests that transcription of *sigA* is not affected by illumination.

As shown in Fig. 4A, the transcription of *litR* and *crtI1* in the WT strain increased in response to illumination. In contrast, *crtI1* transcription occurred in Δ*litR* under both conditions. We were unable to evaluate the transcriptional activity of *litR* in this mutational background because the coding sequence had been deleted, from initiation codon to stop codon. The transcription of *litR* and *crtI1* in the complemented strains was partially induced by light. This complementation analysis suggested mutation effects were due to gene disruption.

BMQ_2952-2949 (Supplementary Figure S2A) encodes the putative enzymes involved in the modification of carotenoids such as glycosylation and acylation. The transcription level of *BMQ_2949* (*crtI3*) was constitutive (data not shown), indicating that LitR only regulates *BMQ_0654-BMQ_0656*, and thus is involved in the early step of the carotenoid biosynthesis.

Functional analysis of LitR in a heterologous host, *B. subtilis*. In order to investigate whether LitR serves as a photosensitive regulator and whether an additional protein is required for light-inducible transcription, we examined the *in vivo* function of *litR* in *B. subtilis* 168, as a heterologous host. Since *B. subtilis* 168 does not retain a homolog of the LitR/CarH family (Supplementary Figure S2B), it is suitable for the analysis. A chromosomal integration plasmid, pDG1661, carrying each genes or promoters fused to the *lacZ* gene was used to monitor light-dependent transcription via

β-galactosidase activity (see MATERIALS AND METHODS).

The P*litR-litR-lacZ*-containing *B. subtilis* transformant showed a higher transcriptional activity in the light condition than in the dark condition (Fig. 4B). In contrast, *B. subtilis* 168 carrying P*litR-lacZ* (the construction lacking LitR coding sequence) and the P*rpoB-lacZ* promoter did not exhibit significant differences in transcriptional activity between light and dark conditions. This result suggests that LitR expressed in the heterologous host serves as a photo-dependent transcriptional regulator similarly as in its original host, *B. megaterium* QM B1551.

We also constructed a 168 strain harboring $litR_{H190A}$, in which the histidine at residue 190 (^{190}His) was substituted with alanine (Supplementary Figure S3). The ^{190}His of LitR is a conserved amino acid residue in the Cbl-binding domain predicted for the involvement in the association of LitR with $AdoB_{12}$. As shown in Fig. 4B, the transformant harboring the $litR_{H190A}$-*lacZ* fusion showed constitutive transcriptional activity under both dark and light conditions. The results indicate that *litR* is exclusive for the light-responsive transcription, and that ^{190}His is essential for the function of LitR.

The addition of Cbl derivatives to the LB medium was not required for the light-inducible transcription in *B. subtilis* described above. Since *B. subtilis* retains *yvrA*, *yvrB*, and *yvrC* encoding a putative B_{12} uptake transporter, and *yvqK* encoding a homolog of ATP:cob(I)alamin adenosyltransferase, which is involved in the final step of $AdoB_{12}$ biosynthesis (33), we assumed that an intermediate of Cbl may be taken up from LB medium and used for the synthesis of $AdoB_{12}$. Actually our bioassay using *L. leichmannii* as an indicator strain demonstrated that LB medium contains 0.2 ng/ml. On the other hand, fresh LB medium in which *B. subtilis* was cultured overnight contained 0.1 ng/ml Cbl. This indicates that about a half of Cbl contained in LB was utilized by this

bacterium. We also studied the light-inducible transcription in the mutants for the *yvr* and *yvq* genes and confirmed that it does not occur in those mutants (Supplementary Figure S4). This leads us to the assumption that an inactive cobalamin such as CNB_{12} in LB medium is taken up by YvrABC transporter and converted into $AdoB_{12}$ by YvqK.

Association of LitR recombinant protein with $AdoB_{12}$. To examine the biochemical properties of LitR and $LitR_{H190A}$, these recombinant proteins were prepared in an *E. coli* expression system. Both proteins were successfully obtained as soluble proteins in the *E. coli* expression system (see MATERIALS AND METHODS).

The interaction of $AdoB_{12}$ with the prepared LitR proteins was analyzed by dialysis equilibration using a spectrophotometer, based on their absorption spectra (Fig. 5). Unexpectedly, the UV-visible absorption spectrum of the $AdoB_{12}$-treated LitR protein showed a typical OHB_{12} profile, exhibiting multiple absorption peaks near 350-nm and 550-nm. Illumination did not cause any spectrum change in the $AdoB_{12}$-LitR complex. In contrast, $LitR_{H190A}$ treated with $AdoB_{12}$ did not show the absorption spectrum of either $AdoB_{12}$ or OHB_{12}. Hereafter, we refer to the complex between LitR and $AdoB_{12}$ as $AdoB_{12}$-LitR.

Light-induced subunit dissociation of the $AdoB_{12}$-LitR protein complex. To determine their subunit structures based on estimated relative molecular masses, the illuminated and dark-incubated $AdoB_{12}$-LitR proteins were subjected to analytical gel-filtration chromatography (see MATERIALS AND METHODS). LitR protein, purified to near-homogeneity, migrated as a ca. M_r 36,500 on an SDS-polyacrylamide gel, which was almost identical to the calculated molecular masses (35.88 kDa) (data not shown).

The dark-incubated and illuminated $AdoB_{12}$-LitR proteins were eluted from the gel-filtration column at positions corresponding to M_r 139,000 and 89,000 (Fig. 6A). The light-dependent change of the molecular weight was also observed by the analytical ultracentrifugation (Fig. 6B); the molecular weight of the dark-incubated and illuminate $AdoB_{12}$-LitR proteins were estimated to M_r 109,000 and 60,700, and 64,200, respectively. We speculate that the dark- and light-state of $AdoB_{12}$-LitR forms a tetramer and a dimer, respectively, in a solution. We also estimated the relative molecular mass of the B_{12}-free Apo-LitR proteins using gel-filtration chromatography. Both of the dark-incubated and illuminated Apo-LitR proteins were eluted at positions corresponding to M_r 123,000 (Supplementary Figure S5). This result indicates that $AdoB_{12}$-LitR of *B. megaterium* undergoes subunit dissociation in response to illumination, and $AdoB_{12}$ is essential for the light-induced dissociation.

Light-sensitive DNA-binding activity of $AdoB_{12}$-LitR. We next performed a gel-shift assay to examine the interaction of $AdoB_{12}$-LitR with the light-inducible promoters P*litR* and P*crtI1*. The result showed that $AdoB_{12}$-LitR prepared under dark conditions caused a specific gel-shift of a DNA probe containing P*litR* and P*crtI1* (Fig. 7A). In contrast, irradiation of $AdoB_{12}$-LitR prior to its addition to the reaction did not retard the DNA probe, or produced a smeared signal. Similarly, the $AdoB_{12}$-unbound LitR did not cause a band shift (data not shown). The gel-shift induced by the $AdoB_{12}$-LitR complex was not detected with a DNA probe containing the *sigA* promoter region. We also examined the loss of the DNA-binding activity of $AdoB_{12}$-LitR along with illumination time (Fig. 7B). At least 3 min of exposure to light at 100 μmol s^{-1} m^{-2} was required for the inactivation of $AdoB_{12}$-LitR.

We also examined the DNA-binding activity of the $LitR_{H190A}$ mutant using this assay (Fig. 7A). No marked gel shift was detected when using the $LitR_{H190A}$ protein, indicating that the ^{190}His residue is essential for the $AdoB_{12}$-interaction and involved in the DNA-binding activity. Collectively, these results showed that $AdoB_{12}$-LitR is a photo-sensitive DNA-binding protein, and that $AdoB_{12}$ is required for the light-sensitive DNA-binding activity of LitR.

Determination of LitR-binding sites. DNase I footprint analysis was performed to identify each of the $AdoB_{12}$-LitR binding sites in the P*litR* and P*crtI1*. Figure 8 shows the region protected by binding of $AdoB_{12}$-LitR prepared under dark conditions. For P*litR*, nucleotide positions –36 to –67 of the sense strand and positions –27 to –69 of the antisense strand, with respect to the transcriptional start site, were protected from DNase I digestion (see also Fig. 3A). In the P*crtI1*, nucleotide positions –13 to +22 of the sense strand and positions –15 to +18 of the antisense strand were protected (Fig. 8 and 3A).

Alignment of two LitR-binding sequences yields a definitive consensus sequence, 5′-TG(T/A)A(C/T)A-N_{16}-T(G/A)TA(C/T)A-3′ shown in Fig. 3B. To confirm whether the predicted consensus sequence is recognized by LitR, we performed the reporter assay using *B. subtilis* 168 harboring *litR-lacZ* with respect to the various types of mutated promoter. The mutated nucleotide sequences are summarized in Table 1. The reporter assay with M7 (5′-TGA<u>CAC</u>-N_{16}-TATACA-3′), M9 (5′-TGAACA-N_{14}-TATACA-3′), M11 (5′-TGAACA-N_{16}-TATA<u>C</u>-3′) and M12 (5′-TGAACA-N_{16}-TAT<u>CAC</u>-3′) showed an identical transcription level under both dark and light conditions (Supplementary Figure S6). Consistent with the results of the reporter assay, recombinant protein $AdoB_{12}$-LitR

610 was unable to bind to the promoter region of M7, M9 or M12 in the gel-shift assay
611 (Supplementary Figure S7). These results indicate that the consensus sequence as well
612 as the length of the spacer is important for the recognition of LitR. On the other hand,
613 AdoB12-LitR was able to bind M11. The reason for the inconsistency is not known, but
614 the mutated sequence may allow weak and constitutive binding of LitR, hence result in
615 the low but light-independent transcription.
616
617 ***In vitro* runoff transcription analysis.** To reproduce the light-dependent transcription
618 observed *in vivo*, we performed an *in vitro* transcriptional analysis with components: the
619 $AdoB_{12}$-LitR complex, the C-terminally histidine-tagged recombinant σ^A protein of *B.*
620 *megaterium* (see MATERIALS AND METHODS), and commercially available *E. coli*
621 RNA polymerase core enzyme. Concordant with the fact that the −10 and −35 regions of
622 P*litR,* P*crtI1*, and P*polA* showed high similarity to a consensus sequence recognized by
623 *B. subtilis* 168 σ^A, the RNA polymerase core enzyme containing σ^A ($E\sigma^A$) generated
624 transcripts of expected molecular sizes according to the relative position of the
625 transcriptional start site (87 bases for template P*litR*, 87 bases for template P*crtI1*, and
626 91 bases for template P*polA*) (Fig. 9A). $E\sigma^A$ also generated a specific transcript for
627 P*polA*, irrespective of the presence of the $AdoB_{12}$-LitR complex (Fig. 9A), indicating that
628 $AdoB_{12}$-LitR does not act on P*polA*.
629 We then examined the light-dependent repressor activity of $AdoB_{12}$-LitR. As shown in
630 Fig. 9A, the amount of *litR* and *crtI1* transcript generated by $E\sigma^A$ was markedly reduced,
631 along with the increase of $AdoB_{12}$-LitR protein (0–8 pmol) added to the reaction, under
632 dark conditions. Meanwhile, the addition of illuminated $AdoB_{12}$-LitR did not inhibit their
633 transcription. The $AdoB_{12}$-LitR-dependent repression in the dark was not observed

when the mutated M7 (5′-TGACAC-N_{16}-TATACA-3′) and M12 (5′-TGAACA-N_{16}-TATCAC-3′) promoters were used as templates. These results indicate that the $AdoB_{12}$-LitR complex serves as an active repressor for the transcriptional initiation at P*litR* and P*crtI1*, and the repression is abolished by illumination. The light-dependent transcriptional repression was not observed with respect to the $AdoB_{12}$-treated $LitR_{H190A}$ mutant protein (Fig. 9B), while the weak transcriptional repression of P*crtI1* by $AdoB_{12}$ + $LitR_{H190A}$ was still observed. The reason for this phenomenon is not clear. The mutation may confer a conformation change that causes light-independent repression specific to *crtI* promoter.

We examined the type of Cbl that confers LitR repressor activity (Fig. 9C). $AdoB_{12}$ was the only Cbl that decreased the P*litR* transcription level under dark condition. We also determined which light wavelengths affect LitR activity (Fig. 9D). Illumination with white light, and light wavelengths of 365-nm, 450-nm, and 530-nm induced transcription, whereas illumination of 633-nm light and dark condition did not affect the transcription level. The light wavelengths that inhibited $AdoB_{12}$-LitR activity were consistent with the absorption spectrum of $AdoB_{12}$. Collectively, these results demonstrate that the $AdoB_{12}$-LitR complex serves as a light-sensitive repressor for P*litR* and P*crtI1*.

DISCUSSION

LitR/CarH homologs are found in phylogenetically diverse bacterial genera; however, the biochemical properties of Gram-positive bacteria in this family remain poorly understood. In this study, we showed that the LitR protein derived from *B. megaterium*, a Gram-positive bacterium, serves as a light-sensitive repressor protein that stimulates the light-inducible transcription of *crt* genes. We successfully reproduced light-inducible transcription of *crt* genes *in vitro*. The evidence obtained in the present and previous studies suggests that the $AdoB_{12}$-LitR-mediated regulatory mechanism is common to light-induced carotenoid production in both Gram-positive and Gram-negative bacteria.

Fig. 10 illustrates the molecular mechanism underlying light-inducible carotenoid production in *B. megaterium* QM B1551 based on the genetic and biochemical data obtained in this study. The $AdoB_{12}$-LitR recombinant protein specifically binds to the promoter region of *litR* itself and the *crt* operon, and represses their expression. The binding site is located upstream (–36 to –67) and near (–13 to +22) the transcriptional start site of the promoters preceding *litR* and *crt*, respectively. Illumination caused the subunit dissociation of $AdoB_{12}$-LitR and weakened the DNA-binding activity, which in turn allowed the σ^A-containing RNA polymerase holoenzyme to bind to the promoters to generate the transcripts for both the genes. The LitR protein was also expressed under illumination (Fig. 5A) and displayed a weak DNA-binding activity (Fig. 7A), which may contribute to prevent the carotenoid overproduction. Consequently, the carotenoid production by the wild-type *B. megaterium* under illumination was lower than that of the *litR* null mutant under the same condition (Fig. 2). The Δ*BMQ_1998* exhibited a moderate level of carotenoid production when compared to the wild-type strain under dark and illumination conditions (Fig. 2). The underlying reason for this result is unclear

676 at present. Apo-LitR may have a regulatory activity different from that of AdoB$_{12}$-LitR.

677 The molecular mechanism of light-inducible transcription in *B. megaterium* QM B1551
678 appears to be simple compared to that in *M. xanthus* and *T. thermophilus*. This is based
679 on two pieces evidence. First, disruption of *litR* caused overproduction of carotenoid,
680 indicating that LitR is the only or the major negative regulator for the photoresponse.
681 Second, the light-induced transcription occurred *in vitro* by adding only two proteins,
682 AdoB$_{12}$-LitR and RNAP, indicating that any other regulator is not required. In contrast,
683 the mechanism known in *M. xanthus* is highly complex. The two photo-dependent
684 signaling pathways, the singlet oxygen ($^1O_2$)-dependent and the B$_{12}$-dependent pathway,
685 involve multiple regulators including two MerR family regulators (CarA and CarH), sigma
686 factor, anti-sigma factor, anti-repressor, etc. (1, 34). Although *T. thermophilus* appears
687 to have only the B$_{12}$-dependent pathway as does *B. megaterium*, light-inducible
688 transcription in this thermophile requires two transcriptional regulators, LitR and LdrP
689 (4).

690 YtvA, originally found in *B. subtilis*, is a LOV domain-containing blue light responsive
691 photoreceptor. This protein functions as a blue light-inducible molecular switch (35, 36,
692 37). By absorbing blue light, YtvA activates σ^B, a stress response sigma factor, hence
693 initiates the phosphorylation cascade downstream of the σ^B-dependent general stress
694 response. We suppose a similar photo-dependent mechanism exists in *B. megaterium*
695 because it retains a *ytvA* homolog (*BMQ_3060*). The induction of σ^B-dependent general
696 stress response by light in *B. subtilis* requires the additional salt stress (37). This means
697 the YtvA-mediated response is not specific to light. In contrast, LitR system enables the
698 quick response to the illumination.

699 The *in vitro* transcription experiment clearly demonstrated that AdoB$_{12}$-LitR and

700 σ^A-containing RNA polymerase activate the transcription in a light-dependent manner
701 (Fig. 9). This assay also demonstrated that $AdoB_{12}$ confers the repressor activity on LitR
702 sensitive to the specific light wavelength. In our study, we performed the same assay
703 with LitR protein from *T. thermophilus* under various conditions, but failed to reproduce
704 the light-dependent transcription. Similarly, *in vitro* reconstruction has not yet been
705 reported in *M. xanthus*, probably due to the difficulty in the preparation of the native
706 recombinant protein. The data presented in this study provide the first example of *in*
707 *vitro* reproduction of light-inducible transcription by a member of the LitR/CarH protein
708 family.

709 The involvement of $AdoB_{12}$ in the proper activity of LitR was verified by the gel-shift
710 assay (Fig. 7A) and the *in vitro* runoff transcription assay (Fig. 9C). However,
711 $AdoB_{12}$-treated LitR from *B. megaterium* showed a similar spectrum to OHB_{12} under
712 both dark and light conditions (Fig. 5). In contrast, the *T. thermophilus* LitR showed a
713 similar spectrum to $AdoB_{12}$ under dark conditions and to OHB_{12} under light conditions (1,
714 11) . At present, the reason for this inconsistency is unclear. However, one possibility is
715 that the adenosyl moiety of $AdoB_{12}$ is located in the internal region of the protein, which
716 could cause LitR to exhibit a similar spectrum to OHB_{12} under both conditions.

717 Gel-filtration chromatography and analytical ultracentrifugation demonstrated that *B.*
718 *megaterium* $AdoB_{12}$-LitR shows the subunit dissociation in response to illumination (Fig.
719 6). *T. thermophilus* $AdoB_{12}$-LitR comprises a tetramer, which undergoes subunit
720 dissociation in response to illumination (1, 11). Thus, our result indicates that the
721 biochemical property of *B. megaterium* LitR slightly differ from that of *T. thermophilus*
722 LitR in terms of the subunit structure. We expect that detailed structural characterization
723 of $AdoB_{12}$-LitR will provide critical information regarding the light-induced conformational

724 changes in LitR and the significance in the transcriptional control in *B. megaterium*.

725

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735 **FIGURE LEGENDS**

736 **Figure 1. Light-inducible carotenoid production of *Bacillus* spp.** Colonies of *B.*
737 *megaterium*, *B. pumilus*, and *B. sphaericus* originally isolated from soil, and of *B.*
738 *megaterium* NBRC 15308, QM B1551, and DSM319 were grown at 37 °C on LB solid
739 medium under dark and light conditions are shown. Colonies producing carotenoids
740 appear yellow, whereas non-producing colonies appear white or cream.

741

742 **Figure 2. Light-induced carotenoid production in *Bacillus megaterium*.** UV-visible
743 spectra are shown with regard to the crude carotenoid fraction extracted from cells of *B.*
744 *megaterium* QM B1551 WT, Δ*litR*, Δ*litR*/*litR,* Δ*BMQ_1998*, Δ*crt*, and *B. megaterium*
745 DSM319 grown at 37 °C for 15 h under dark (solid line) and light (dashed line)
746 conditions.

747

748 **Figure 3. Promoter region of *litR* and *crtI1*.** (A) Nucleotide sequence of the promoter
749 region preceding *litR* and *crtI1*. The C-terminal and N-terminal amino acid sequences of
750 BMQ_4357, and LitR and CrtI1, respectively, are also shown. Possible −10 and −35
751 hexamer sequences of the *litR* and *crtI1* promoters are indicated by small letters. The
752 transcriptional start sites (designated as +1), determined by 5′-RACE, are indicated by
753 the bent arrow. (B) Alignment of the LitR-binding sequences. The consensus sequence
754 is also shown.

755

756 **Figure 4. Transcriptional analysis of *litR* and *crt* promoters in *B. megaterium* QM**
757 **B1551 (A) and in a heterologous host, *B. subtilis* 168 (B).** (A) Quantification of
758 transcripts using semi-quantitative RT-PCR. The amounts of *litR*, *crtI1,* and *sigA*

(control) transcripts produced in the wild-type strain (WT), the *litR* mutant (Δ*litR*), and the genetically complemented *litR* mutant (Δ*litR*/*litR*) were estimated. The cDNA synthesis was performed in the presence (+) or absence (−) of Reverse Transcriptase (RT). ND, Not detected due to the elimination of primer annealing site. (B) A chromosome integration plasmid, pDG1661, was used to monitor the transcriptional activities of promoters preceding *litR* via β-galactosidase activity in *B. subtilis* 168. The promoter preceding *rpoB* (encoding a β-subunit of RNA polymerase) (P*rpoB*) was used as a control. The transformants were grown in liquid LB medium under dark conditions (closed circle) or under light conditions (opened square).

Figure 5. Absorption spectra of LitR recombinant proteins. Spectra of free $AdoB_{12}$ (panel A), free OHB_{12} (panel B), $AdoB_{12}$-treated $LitR_{H190A}$ (panel C), and $AdoB_{12}$-LitR (panel D). In each panel, B_{12} or LitR under dark conditions are shown by a solid line, whereas the illuminated B_{12} or LitR proteins are shown by a dashed line.

Figure 6. Light-inducible subunit dissociation of $AdoB_{12}$-LitR protein analyzed by Gel-filtration (A) and Analytical ultracentrifugation (B). (A) To estimate the relative molecular mass of the $AdoB_{12}$-LitR protein, the purified recombinant proteins were analyzed by gel-filtration. The elution volume of $AdoB_{12}$-LitR on a Superdex 200 HR 10/30 column are indicated. The panel shows the elution profiles for $AdoB_{12}$-LitR under dark (solid line) and illuminated (dashed line) conditions. Each protein examined eluted at a position corresponding to the tetrameric (M_r 13,900) and dimeric (M_r 8,900) structure, respectively. (B) Sedimentation velocity experiments were performed with the non-illuminated (upper panel) and illuminated (lower panel) $AdoB_{12}$-LitR. The

783 sedimentation coefficients, *c*(s) 4.35 and 6.45 S for the non-illuminated $AdoB_{12}$-LitR and
784 4.26 S for the illuminated $AdoB_{12}$-LitR are shown. The molecular weight of each protein
785 was estimated to be M_r 60,700 and 109,000 and 64,200 on the basis of the conversion
786 of *c*(*s*) to *c*(*M*).
787
788 **Figure 7. Gel-shift assay of $AdoB_{12}$-LitR.** (A) The amounts of $AdoB_{12}$-LitR and
789 $AdoB_{12}$-$LitR_{H190A}$ used in lanes 1, 2, 3, 4, and 5 were 0, 4, 8, 16, and 32 pmol,
790 respectively. Purified $AdoB_{12}$-LitR or $LitR_{H190A}$ was mixed with the probes for P*litR* (135
791 bp), P*crtI1* (146 bp), and P*sigA* (230 bp) and applied to a non-denaturing
792 polyacrylamide gel. Open and closed triangles indicate the probe and protein-DNA
793 complex, respectively. (B) $AdoB_{12}$-LitR (4 pmol) was incubated in the dark (9 min) or
794 under white light conditions (0–9 min) prior to the addition of ^{32}P-labeled probes of P*litR*.
795 Only the shifted band (the LitR-$AdoB_{12}$ + DNA complex) is shown.
796
797 **Figure 8. DNase I footprint for determining the LitR-binding site in the *litR* and**
798 ***crtI1* promoter.** The assay was performed on the sense (+) and antisense (-) strands.
799 The amounts of recombinant $AdoB_{12}$-LitR used were 0 pmol (lane 1 and 7), 0.5 pmol
800 (lane 2), 1 pmol (lane 3), 2 pmol (lane 4), 4 pmol (lane 5), and 8 pmol (lane 6) for the *litR*
801 promoter, and 0 pmol (lane 1 and 8), 0.25 pmol (lane 2), 0.5 pmol (lane 3), 1 pmol (lane
802 4), 2 pmol (lane 5), 4 pmol (lane 6), and 8 pmol (lane 7) for the *crtI1* promoter. Positional
803 numbering is based on the transcriptional start point of each promoter, numbered as +1.
804 The DNase I digests were run with the chemically cleaved probes (G+A lanes for the
805 both strands).
806

Figure 9. *In vitro* run-off transcription assay. (A) The indicated amounts of the *E. coli* RNA polymerase core enzyme containing *B. megaterium* σ^A ($E\sigma^A$) and the $AdoB_{12}$-LitR recombinant protein were added to the reaction mixture with the promoter DNA fragments. Transcripts of the predicted lengths were detected: 87 bp for P*litR*, M7, and M12, 91 bp for P*crtI1*, and 87 bp for P*polA*. (B) The $AdoB_{12}$-$LitR_{H190A}$ recombinant protein was added to the reaction mixture. (C) The effects of $AdoB_{12}$, OHB_{12} (hydroxocobalamin), CNB_{12} (cyanocobalamin) and MeB_{12} (methylcobalamin) were examined using $AdoB_{12}$-LitR and the DNA fragments containing P*litR*. (D) The effects of different light wavelengths, white light, 365-nm, 450-nm, and 530-nm, and 633-nm, were examined using the $AdoB_{12}$-LitR protein and P*litR*. D and L denote dark and white light, respectively.

Figure 10. Working model for light-inducible transcriptional control of the *litR* and *crt* genes by $AdoB_{12}$-LitR. LitR is bound by $AdoB_{12}$ and associates with the promoter region of *litR* and *crtI1*, repressing the transcription of both genes under dark conditions. Under light conditions, the absorption of light by the tetrameric $AdoB_{12}$-LitR complex causes its dissociation into dimeric form probably due to the photolysis of $AdoB_{12}$. The dissociation inactivates LitR and allows RNA polymerase (RNAP) holoenzyme to initiate mRNA synthesis of the *litR* and *crt* operon consisting of *crtI1-crtI2-crtB*.

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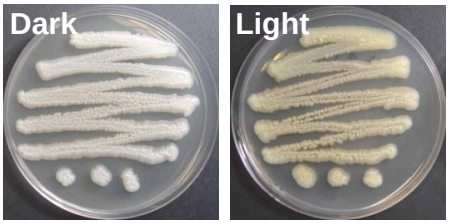

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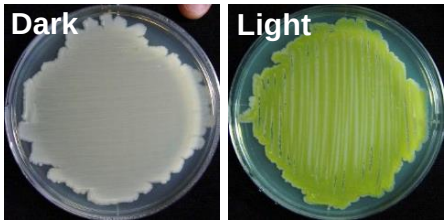

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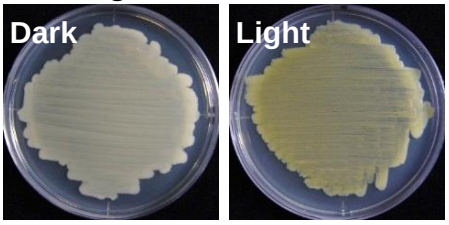

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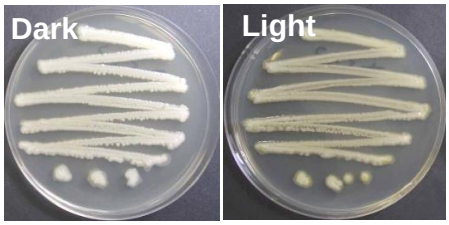

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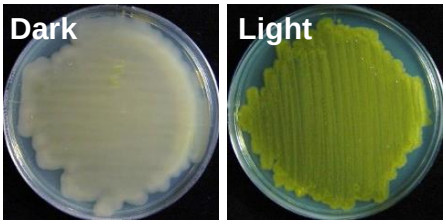

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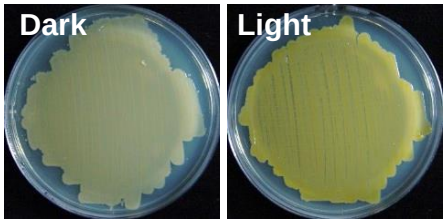

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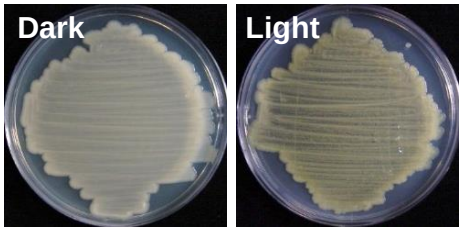

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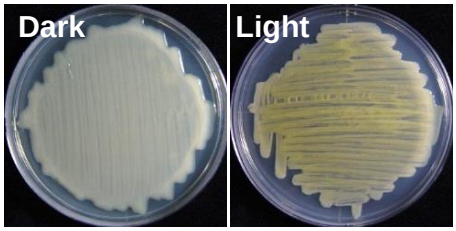
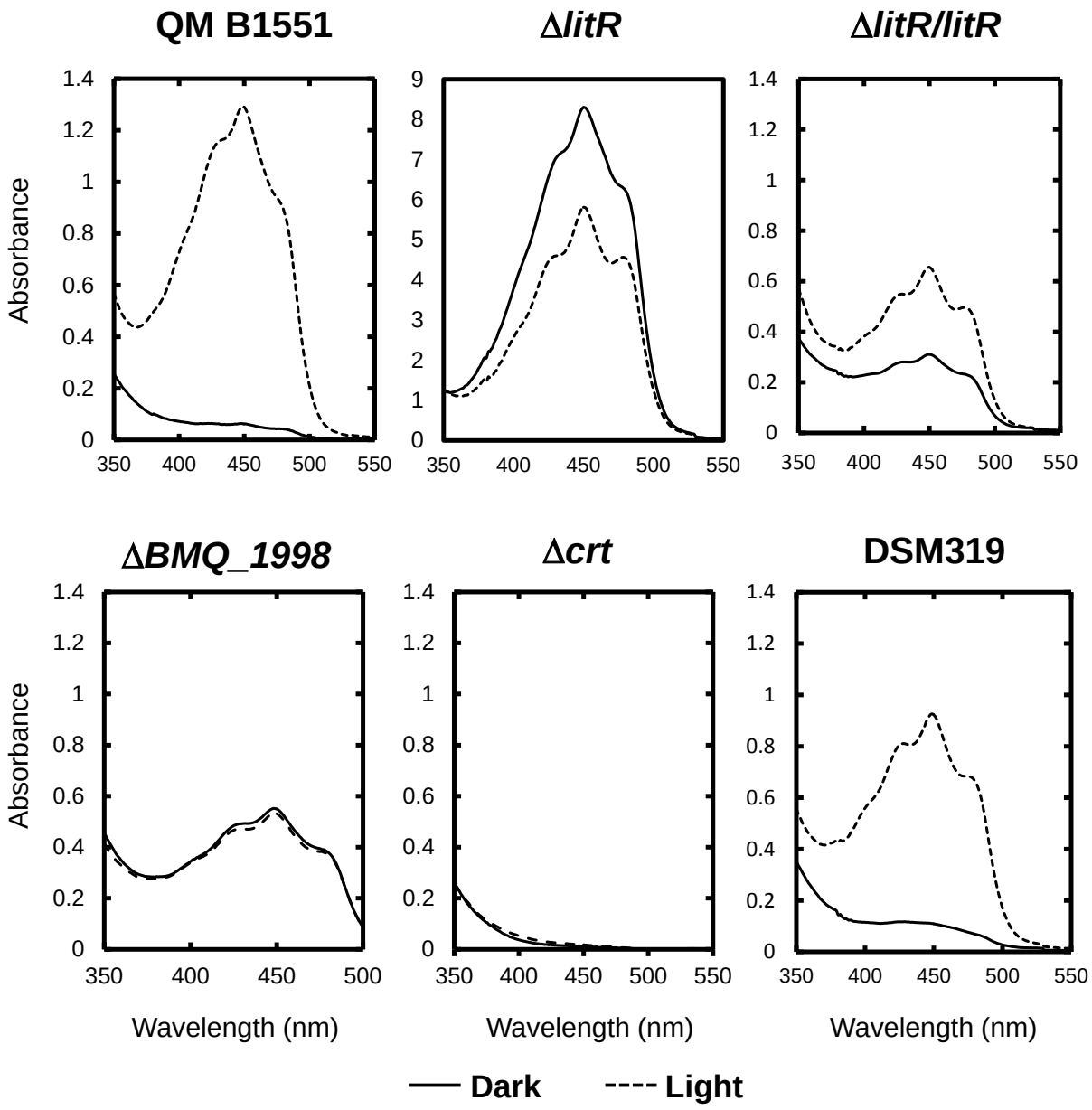


Takano *et al.* Fig. 1



Takano *et al.* Fig. 2

A

litR

```
                                                                    -35
(+) 5′CTTTAAAAAGGAAAGAATAGCCTTGAACAAAATCTTTACATAGCATATACAAATtttaca 3′
(-) 3′GAAATTTTTCCTTTCTTATCGGAACTTGTTTTAGAAATGTATCGTATATGTTTAAAATGT 5′
        L  K  R  K  E  *
                          -10      +1
   5′CACAGGATAACTTATCCtataatTCAATTAATAGATACAGTTCACGAAGAGTAGTTGGAG 3′
   3′GTGTCCTATTGAATAGGATATTAAGTTAATTATCTATGTCAAGTGCTTCTCATCAACCTC 5′

   5′GTTACGTATGGCTCACGAAGGGAAGTATAATATTAAAGCCATCTCAAATATGGTCGGAAT 3′
   3′CAATGCATACCGAGTGCTTCCCTTCATATTATAATTTCGGTAGAGTTTATACCAGCCTTA 5′
      litR > fM  A  H  E  G  K  Y  N  I  K  A  I  S  N  M  V  G  I
```

crtI1

```
                                                          -35
(+) 5′AATGGTTTAGTTATTGGTAATTTTATGATTCGAATAaagacaAGCAGTTTATCGttgtat 3′
(-) 3′TTACCAAATCAATAACCATTAAAATACTAAGCTTATTTCTGTTCGTCAAATAGCAACATA 5′
                      -10       +1
   5′TTAATAATAAAGATTTGtataatGTTTGTATAACTATTGTATAGGATTAGGAGTGTATGT 3′
   3′AATTATTATTTCTAAACATATTACAAACATATTGATAACATATCCTAATCCTCACATACA 5′

   5′ATGCAAAAAAACGTAATCGTGATTGGAGCAGGTCCAGGAGGACTAGCGGCAGCGATGCTG 3′
   3′TACGTTTTTTTGCATTAGCACTAACCTCGTCCAGGTCCTCCTGATCGCCGTCGCTACGAC 5′
     fM  Q  K  N  V  I  V  I  G  A  G  P  G  G  L  A  A  A  M  L
    crtI1 >
```

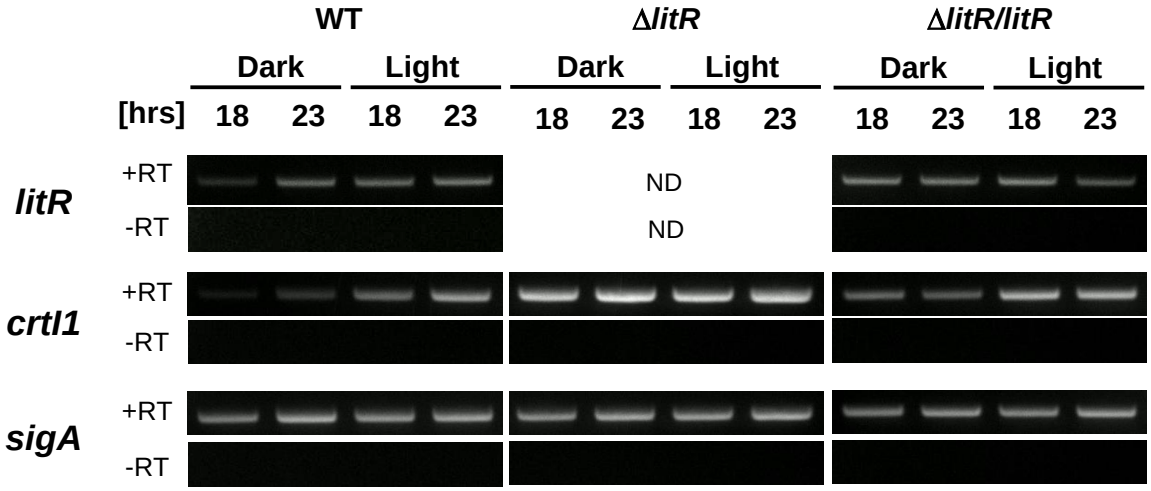
B

```
PlitR     5′-CCTTGAACAAAATCTTTACATAGCATATACA-3′
PcrtI1       5′-TGTATAATGTTTGTATAACTATTGTATAGGA-3′

Consensus  TG(T/A)A(C/T)A-N16-T(G/A)TA(C/T)A
```

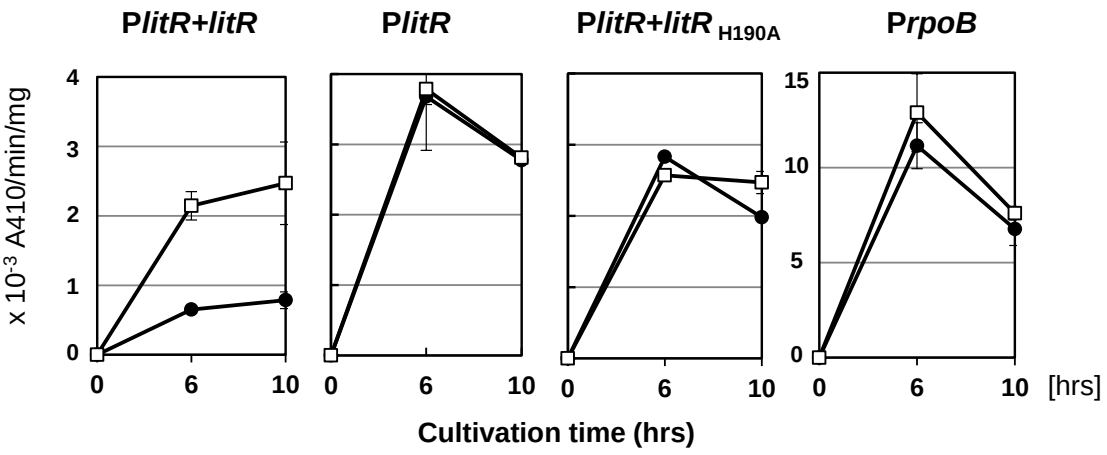
Takano *et al.* Fig. 3

A

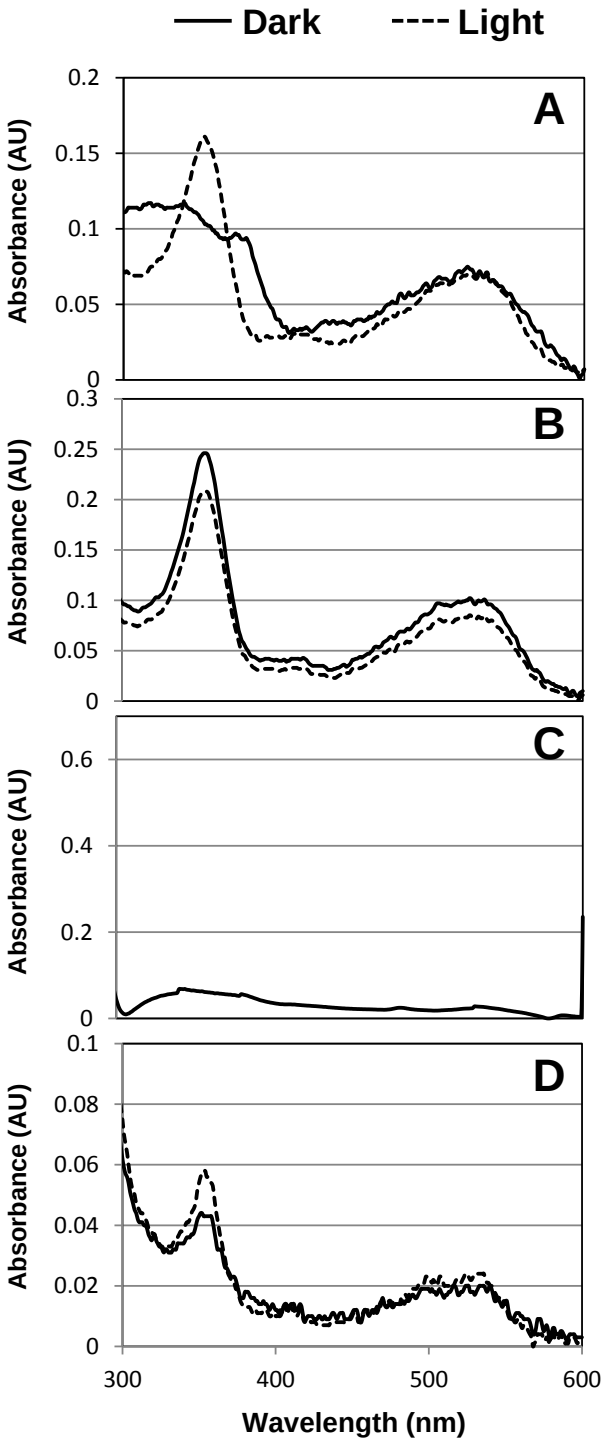


Takano *et al.* Fig. 4A

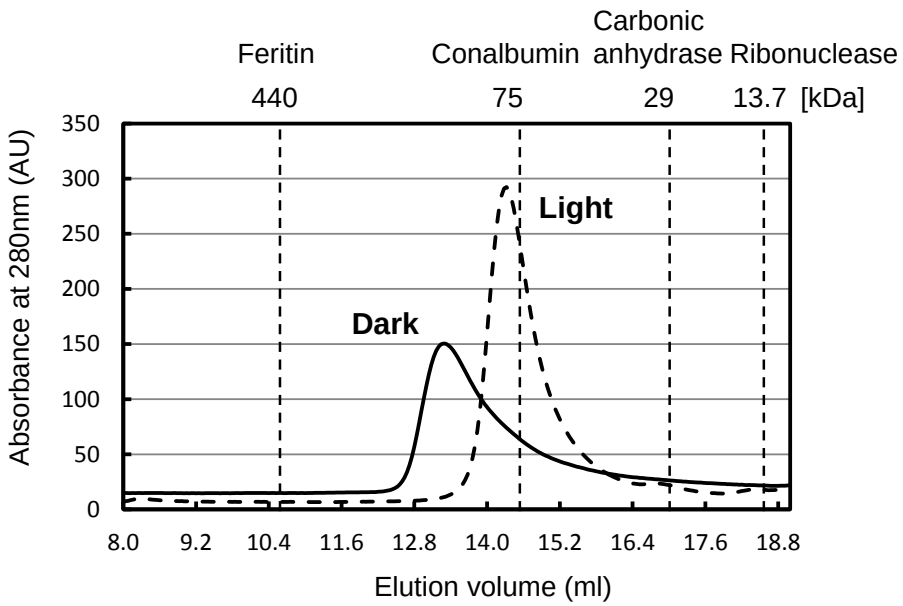
B



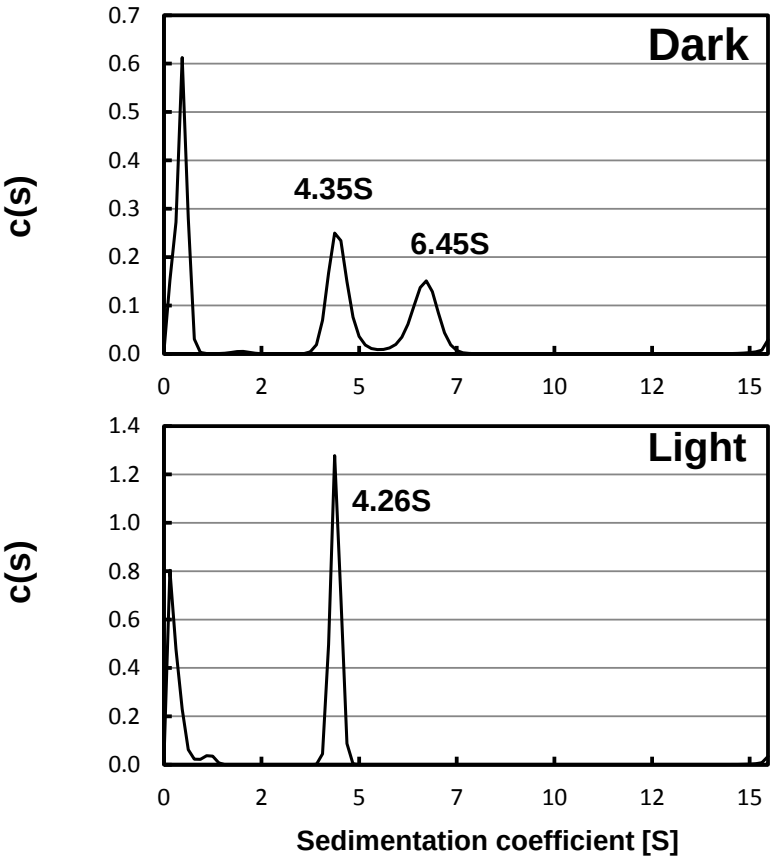
Takano *et al.* Fig. 4B



Takano *et al.* Fig. 5

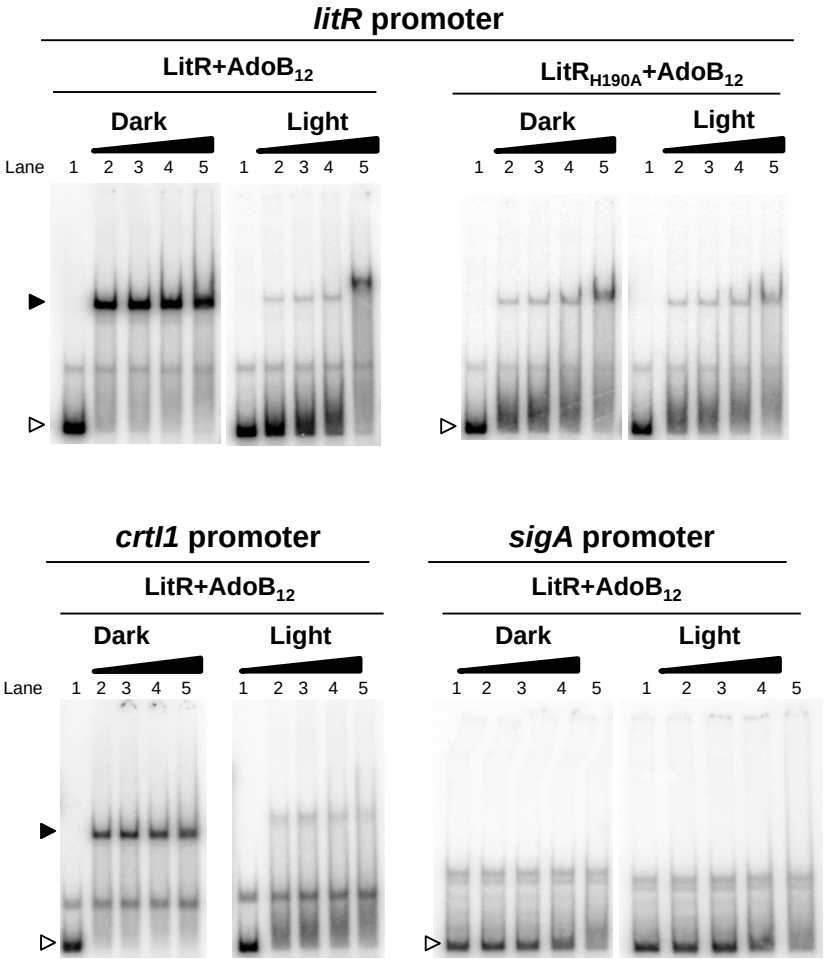


Takano *et al.* Fig. 6A

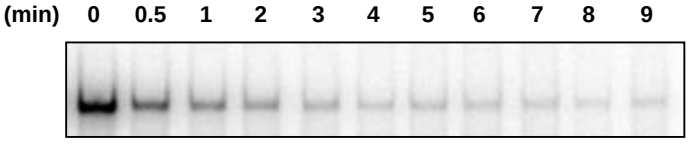


Takano *et al.* Fig. 6B

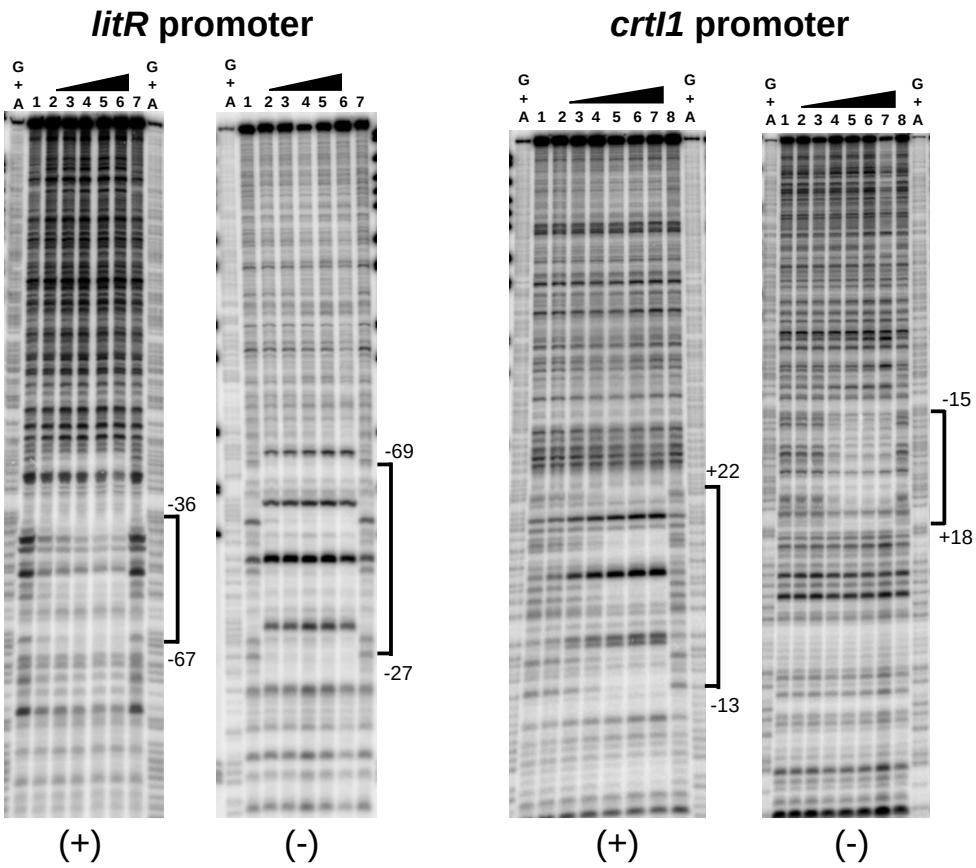
A



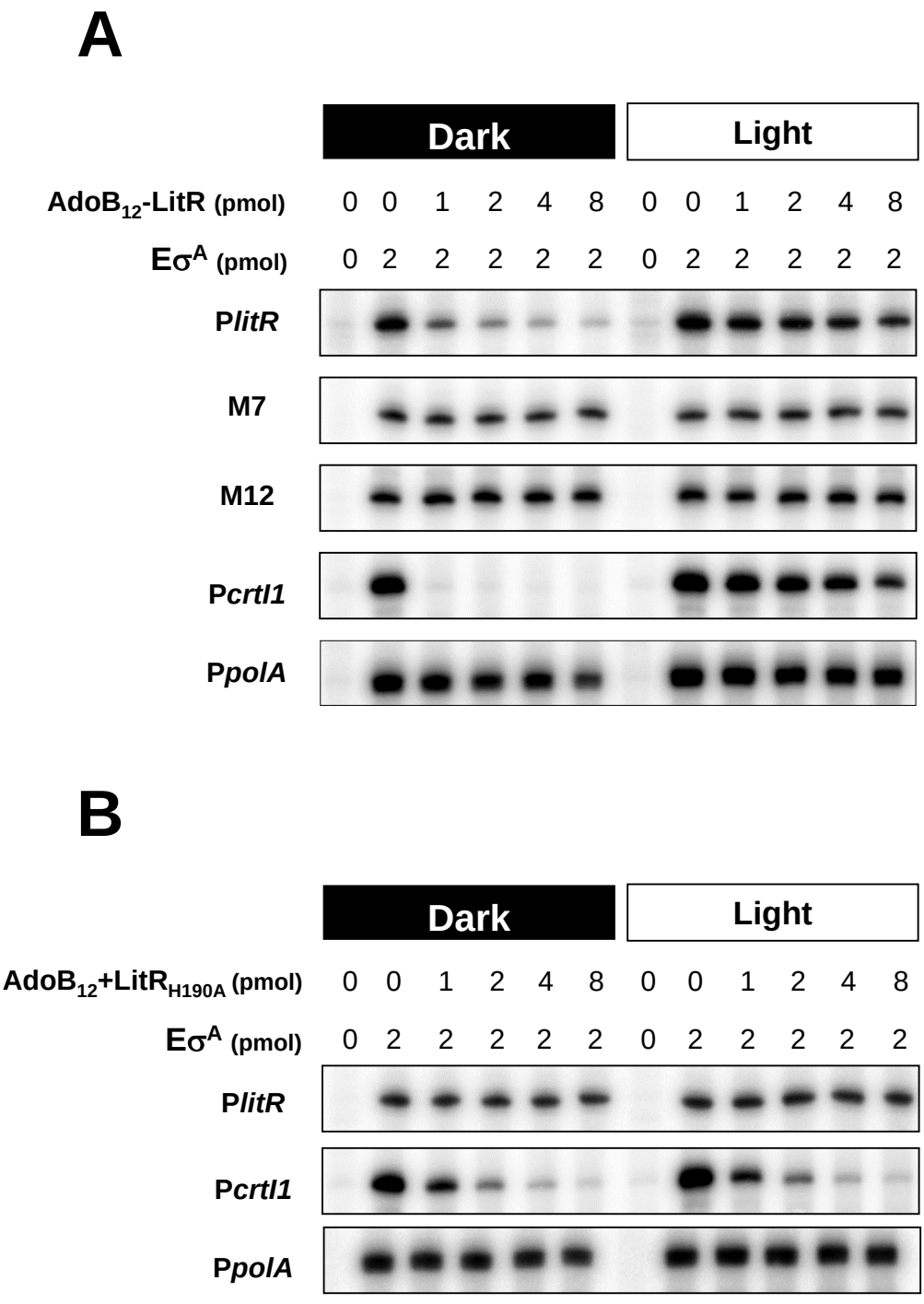
B



Takano *et al.* Fig. 7

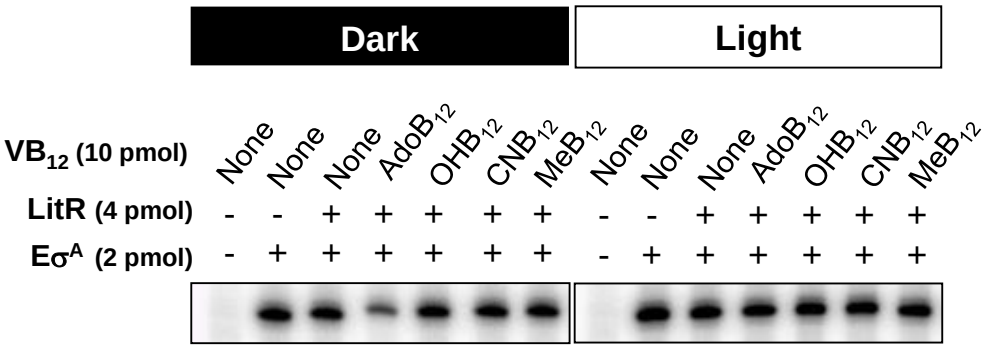


Takano *et al.* Fig. 8

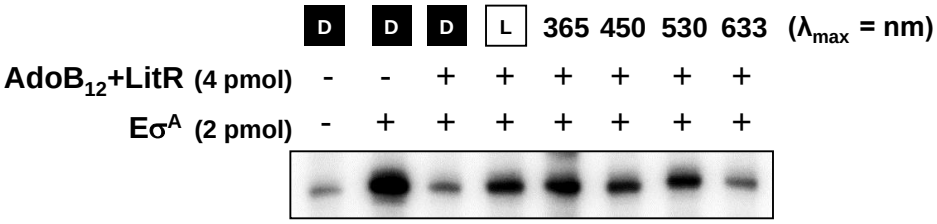


Takano *et al.* Fig. 9AB

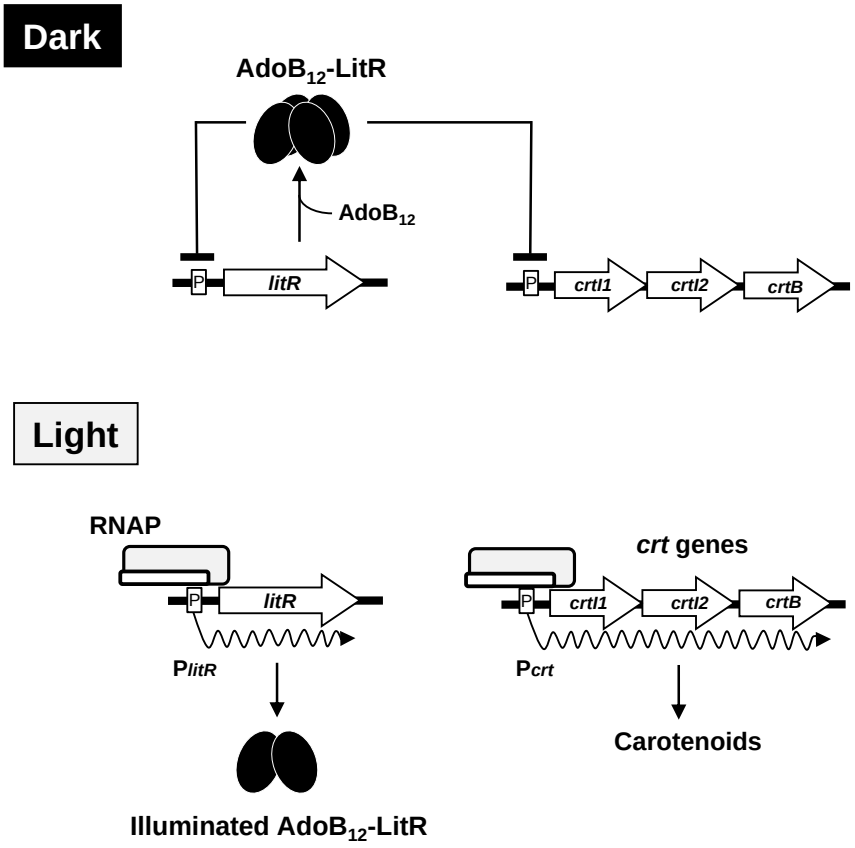
C



D



Takano *et al.* Fig. 9CD



Takano *et al.* Fig. 10

Table 1. The nucleotide sequence of the mutated LitR-binding site and DNA-binding activity of AdoB$_{12}$-LitR to the mutated LitR-binding site. The predicted consensus sequence for LitR binding is shown in bold letters. The mutated and deleted residue is indicated by an underline and a dash, respectively.

Name	LitR-Binding	Mutation site in *litR* promoter
WT	++	AGCCT**TGAACA**AAATCTTTACATAGCA**TATACA**AATTTT
M1	++	AGCCT**C**GAACAAAATCTTTACATAGCA**TATACA**AATTTT
M2	++	AGCCT**T**TAACAAAATCTTTACATAGCA**TATACA**AATTTT
M3	++	AGCCT**TG**CACAAAATCTTTACATAGCA**TATACA**AATTTT
M4	+	AGCCT**TGA**CCAAAATCTTTACATAGCA**TATACA**AATTTT
M5	+	AGCCT**TGAA**AAAATCTTTACATAGCA**TATACA**AATTTT
M6	++	AGCCT**TGAAC**CAAATCTTTACATAGCA**TATACA**AATTTT
M7	-	AGCCT**TGA**CAC AAATCTTTACATAGCA**TATACA**AATTTT
M8	++	AGCCT**GT**AACAAAATCTTTACATAGCA**TATACA**AATTTT
M9	-	AGCCT**TGAACA**AAATCTT- -CATAGCA**TATACA**AATTTT
M10	++	AGCCT**TGAACA**AAATCTTTACATAGCA**G**ATACAAATTTT
M11	++	AGCCT**TGAACA**AAATCTTTACATAGCA**TATAC**CAATTTT
M12	-	AGCCT**TGAACA**AAATCTTTACATAGCA**TATCAC**AATTTT