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Control of Peripheral Light-Harvesting Complex Synthesis by a Bacteriophytochrome in the Aerobic Photosynthetic Bacterium *Bradyrhizobium* Strain BTAi1▽

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The recent sequence analysis of the photosynthetic and plant-symbiotic *Bradyrhizobium* sp. strain BTAi1 revealed the unexpected presence of a *pucBA* operon encoding the apoproteins of peripheral light-harvesting (LH) complexes. This *pucBA* operon is found close to a bacteriophytochrome gene ($BphP3_{B\ BTAi1}$) and a two-component transcriptional regulator gene (TF_{BTAi1} gene). In this study, we show that $BphP3_{B\ BTAi1}$ acts as a bona fide bacteriophytochrome and controls, according to light conditions, the expression of the *pucBA* operon found in its vicinity. This light regulatory pathway is very similar to the one previously described for chromo-$BphP4_{Rp}$ in *Rhodopseudomonas palustris* and conducts the synthesis of a peripheral LH complex. This LH complex presents a single absorption band at low temperature, centered at 803 nm. Fluorescence emission analysis of intact cells indicates that this peripheral LH complex does not act as an efficient light antenna. One putative function of this LH complex could be to evacuate excess light energy in order to protect *Bradyrhizobium* strain BTAi1, an aerobic anoxygenic photosynthetic bacterium, against photooxidative damage during photosynthesis.

Anoxygenic phototrophic bacteria have the ability to transform light energy into biochemical amenable energy for their growth and motion. The collection of light and its transformation into chemical energy are mediated by the so-called photosynthetic apparatus. This complex system is composed of three multimeric transmembrane protein complexes: the light-harvesting (LH) complexes, the photochemical reaction center (RC), and the cytochrome bc_1 complex located in the intracytoplasmic membrane. Light collected by the peripheral LH complexes is transferred first to the LH1 complex, which absorbs at around 870 nm, and then to the RC, where a charge separation occurs. This initiates a cyclic electron transfer between the RC and cytochrome bc_1 via electron carrier proteins in the periplasmic space and quinone molecules in the membrane. This cyclic electron transfer is coupled to the translocation of protons and to the formation of a proton motive force across the inner membrane, ultimately used for ATP synthesis. To optimize light collection, various peripheral LH complexes, coded by different *pucBA* genes, are expressed according to environmental conditions. In most cases, peripheral LH complexes absorb at 800 and 850 nm and are designated LH2. However, other peripheral LH complexes, which differ by their absorption properties and carotenoid content, have been described. At low light intensities and/or low temperatures, *Rhodopseudomonas acidophila* synthesizes an LH complex, designated LH3, which absorbs at 800 and 820 nm (4, 8). An atypical LH complex (LH4), presenting a single band around 805 nm, is synthesized by *Rhodopseudomonas palustris* cells when grown at a low light intensity (16).

In general, the photosynthetic activity of anoxygenic phototrophic bacteria takes place under anaerobic or semiaerobic conditions, since the synthesis of the photosynthetic apparatus is switched on at a low oxygen tension level (5). However, one exception is found for the aerobic anoxygenic phototrophs (AAPs). These bacteria, which are unable to grow in strict anaerobiosis, require oxygen to grow and synthesize their photosynthetic apparatus (32). Shiba et al. (23) were the first to report on the presence of AAPs isolated from seawater, sand, and bottom sediments of Tokyo Bay. Since then, AAPs have been isolated from very diverse ecological niches, including deep-sea hydrothermal vent plume waters and nitrogen-fixing stem nodules (10, 32). The inability to perform an efficient photoinduced cyclic electron transfer under anaerobic conditions is not related to differences in the composition of the photosynthetic apparatus or significant changes in amino acid sequences of LH or RC polypeptides (32). Several explanations have been put forward to explain the growth particularity of AAPs, but the molecular bases underlying this phenomenon are still a matter of debate. Most of the AAPs studied so far possess only the LH1 complex as an antenna. The peripheral LH complexes found in some of the AAPs are distinct from the "classical" LH2 complex of anoxygenic purple bacteria. For example, the absorption maxima of the LH2 complex of *Erythromicrobium hydrolyticum*, *Erythromicrobium ezovicum*, and *Erythromicrobium ramosum* peak at 798 and 832 nm, reminiscent of the LH3 complex (33, 34). The only peripheral LH

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complex present in *Roseobacter denitrificans* OCh114, previously named *Erythrobacter*, exhibits one peak at 806 nm, similar to the LH4 complex of the anaerobic photosynthetic bacterium *R. palustris* (24).

The recent sequencing of the genomes of the symbiotic AAP *Bradyrhizobium* strains (ORS278 and BTAi1) revealed the presence of three bacteriophytochrome (BphP) genes in each strain. Two of them ($BphP1_B$ and $BphP2_B$) are perfectly conserved and are found in synteny in both strains. The third BphP gene is specific to each strain and is denominated $BphP3_{B\ BTAi1}$ or $BphP3_{B\ ORS278}$. The product of $BphP1_B$, found in the photosynthesis gene cluster, was previously shown to play a key role in the control of photosystem synthesis in both strains (9, 19). This BphP protein activates the expression of the main photosynthesis genes by antagonizing the action of the repressor PpsR2 (9, 14). The role of the second BphP protein ($BphP2_B$), whose homologs are found in various bacteria (11), remains unknown. No obvious phenotype is observed for the corresponding deletion mutants in *Bradyrhizobium* ORS278 or *R. palustris* (unpublished data). A recent study of the specific BphP found in strain ORS278 ($BphP3_{B\ ORS278}$) clearly indicates an acquisition by lateral gene transfer (18). $BphP3_{B\ ORS278}$ is supposed to control the synthesis of gas vesicles whose genes are found at the vicinity of its gene, but this has not been demonstrated experimentally (18).

The gene of a specific BphP protein of BTAi1 (BBta_3079 or $BphP3_{B\ BTAi1}$) is found at the vicinity of a two-component transcriptional regulator gene (BBta_3078) and a putative *pucBAC* operon (*BBta_3081-BBta_3082-BBta_3083*) (https://www.genoscope.cns.fr/agc/mage or http://genome.jgi-psf.org/finished_microbes/bra_b/bra_b.home.html). The presence of *pucBA* genes is surprising, since the synthesis of peripheral LH complexes has not been evidenced so far in this bacterium regardless of the growth mode (i.e., isolation from stem nodules or growth in the laboratory). This genomic organization is also encountered in several strains of *R. palustris* for which the *pucBA.e* operon is found close to a *BphP* gene ($BphP4_{Rp}$) homolog to $BphP3_{B\ BTAi1}$ (31). Interestingly, it has been shown that $BphP4_{Rp}$ acts, depending on the considered strains of *R. palustris*, either as a light-sensitive (chromo-$BphP4_{Rp}$) or a redox-sensitive (achromo-$BphP4_{Rp}$) kinase to control the synthesis of an LH2 complex (31).

By combining biophysical, biochemical, and genetic approaches, we demonstrate in the present study that $BphP3_{B\ BTAi1}$ acts as a bona fide BphP protein to control the synthesis of a peripheral LH complex. The ecological bases of this light regulation are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bradyrhizobium* BTAi1 was grown in solid medium on petri dishes under different light conditions as previously described (19). Coillumination of cultures under 700- and 770-nm wavelengths was provided by light-emitting diodes (ELD770-524 and ELD700-524 from Roithner) with an irradiance of 200 μmol and 100 μmol of photons/m^2/s, respectively.

Expression and protein procedures. The entire $BphP3_{B\ BTAi1}$ gene was amplified by PCR. Primers designed to add appropriate restriction sites for expression as a His_6-tagged version in the pBAD/HisB expression vector (Invitrogen) were used. The 38-amino-acid tag is located at the N terminus of the recombinant protein. This tag contains, in addition to the polyhistidine region, an Xpress epitope and an enterokinase recognition cleavage site that could be used to immunodetect the protein or to remove the tag after purification, respectively. In order to reconstitute $BphP3_{B\ BTAi1}$ holo-BphPs in vivo, the *hmuO* gene from *Bradyrhizobium* ORS278 was inserted in the above pBAD:*BrBphP3.BTAi1* constructs (described in reference 15). The recombinant proteins were overexpressed in *Escherichia coli* LMG194 and purified as previously described (15). To test the reduction/oxidation of the $BphP3_{B\ BTAi1}$ protein, reducing conditions were created with 1 mM dithiothreitol (DTT) and oxidizing conditions with 1 mM $K_3Fe(CN)_6$. After addition of the oxidizing or reducing reagent, the protein samples were incubated for at least 30 min on ice and then analyzed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein kinase assays. Protein kinase reactions were carried out in triplicate as previously described (15). For the phosphotransfer experiments, $BphP3_{B\ BTAi1}$ was phosphorylated for 15 min in the presence of [γ-^{32}P]ATP following a 705-nm illumination. This phosphorylation step was followed by a 25-min incubation in the presence of a stoichiometric amount of the Rpa1489 protein (31). ^{32}P-labeled products were quantified by using a Typhoon phosphorimager (Amersham Biosciences).

Construction of $BphP3_{B\ BTAi1}$ and $pucBA_{B\ BTAi1}$ mutants. For the construction of the $BphP3_{B\ BTAi1}$ null mutant, the gene was introduced into the pJQ200-SK suicide vector (22). The *lacZ*-Km^r cassette of pKOK5 (20) was then inserted directly into the unique XhoI site of the $BphP3_{B\ BTAi1}$ gene. To study the effect of light on *pucBA* gene expression, the *pucBA* genes were replaced by the *lacZ*-Km^r cassette, which contains the *lacZ* reporter gene. The region flanking *pucBA* was ligated thanks to the following pairs of primers: up.pucBA.F (5′-CCAT**GGATCC**CATCAGTGTCACGATTTCGGTGAAT-3′) and up-.pucBA.R (5′-CAAACTCTGTT*GTCGAC*GTCCGGCATCACGTCACCTC CTGTATTG-3′) and dw.pucBA.F (5′-ATGCCGGAC*GTCGAC*AACAGAG TTTGTAGGCGGCGCCGTTCGCGGACAATG-3′) and dw.pucBA.R (5′-C AG**GGGCCC**CGACAGCATGATCCCGACCAGCGATG-3′) (the primers up.pucBA.R and dw.pucBA.F contain an overlapping sequence of 26 bp [underlined] to facilitate overlap extension PCR). The ligated product was amplified and introduced into the pJQ200-SK plasmid thanks to restriction sites designed in each primer (in bold). The 4.7-kb SalI *lacZ*-Km^r cassette of pKOK5 was then inserted into the SalI site designed in the overlapping sequences of the up.pucBA.R and dw.pucBA.F primers (in italics). These two constructions were introduced and delivered by conjugation into the BTAi1 strain as previously described (13). Double recombinants were selected on sucrose and confirmed by PCR.

Light action spectrum of *pucBA* gene expression. The mutant harboring the *lacZ-pucBA* fusion was grown under continuous illumination with low irradiance (6.6 μmol · m^{-1} · s^{-1}) of different wavelengths as described previously (19). After growth, the cells under the illuminated area were resuspended in 3 ml of water and β-galactosidase activity was measured as previously described (13).

Absorbance and fluorescence measurements. Absorption and fluorescence spectra were measured with a Cary 50 spectrophotometer and a Cary Eclipse spectrofluorometer as previously described (15). For both spectrometers, the intensity of the measuring beam (provided by short flashes) is low enough to induce no significant photochemistry. Excitation of purified His-tagged $BphP3_{B\ BTAi1}$ was provided by light-emitting diodes emitting at 770 nm or 705 nm (ELD770-524 and ELD 700-524; Roithner) with an irradiance of 15 μmol of photon/m^2/s.

Analysis of *pucBA* and $BphP3_{B\ BTAi1}$ genes. The presence of *pucBA* and $BphP3_{B\ BTAi1}$ genes in different photosynthetic *Bradyrhizobium* strains was examined by PCR using the following pairs of primers: pucBA.BTAi1.f (5′-GGCAACGCGCTCGCG AGAGTGAAG-3′) and pucBA.BTAi1.r (5′-CTGACCCGATTCATCATCTCTG AAAC-3′) and Br.BphP.BTAi1.f (5′-CCTGGTCCGGTTTCGGCCCAATC-3′) and Br.BphP.BTAi1.r (5′-CTCATGGCCGATCGCCTGCAATC-3′). The localization of the primers is indicated in Fig. 1. A touchdown PCR was done as follows: initial denaturation at 94°C for 5 min, followed by 20 cycles consisting of a 30-s denaturation at 94°C, 30-s annealing temperature from 60 to 50°C, and 1-min primer extension at 72°C, followed by 15 cycles consisting of a 30-s denaturation at 94°C, 30-s annealing temperature at 50°C, and 1-min primer extension at 72°C.

RESULTS

Identification of *pucBAC* operon close to putative *BphP* gene in BTAi1 genome. Comparison of the genomes of two photosynthetic *Bradyrhizobium* strains (ORS278 and BTAi1), recently annotated, reveals the specific presence of a putative *pucBAC* operon (*BBta_3081-BBta_3082-BBta_3083*) in the BTAi1 strain (Fig. 1A). The *pucBA* genes encode the apopro-

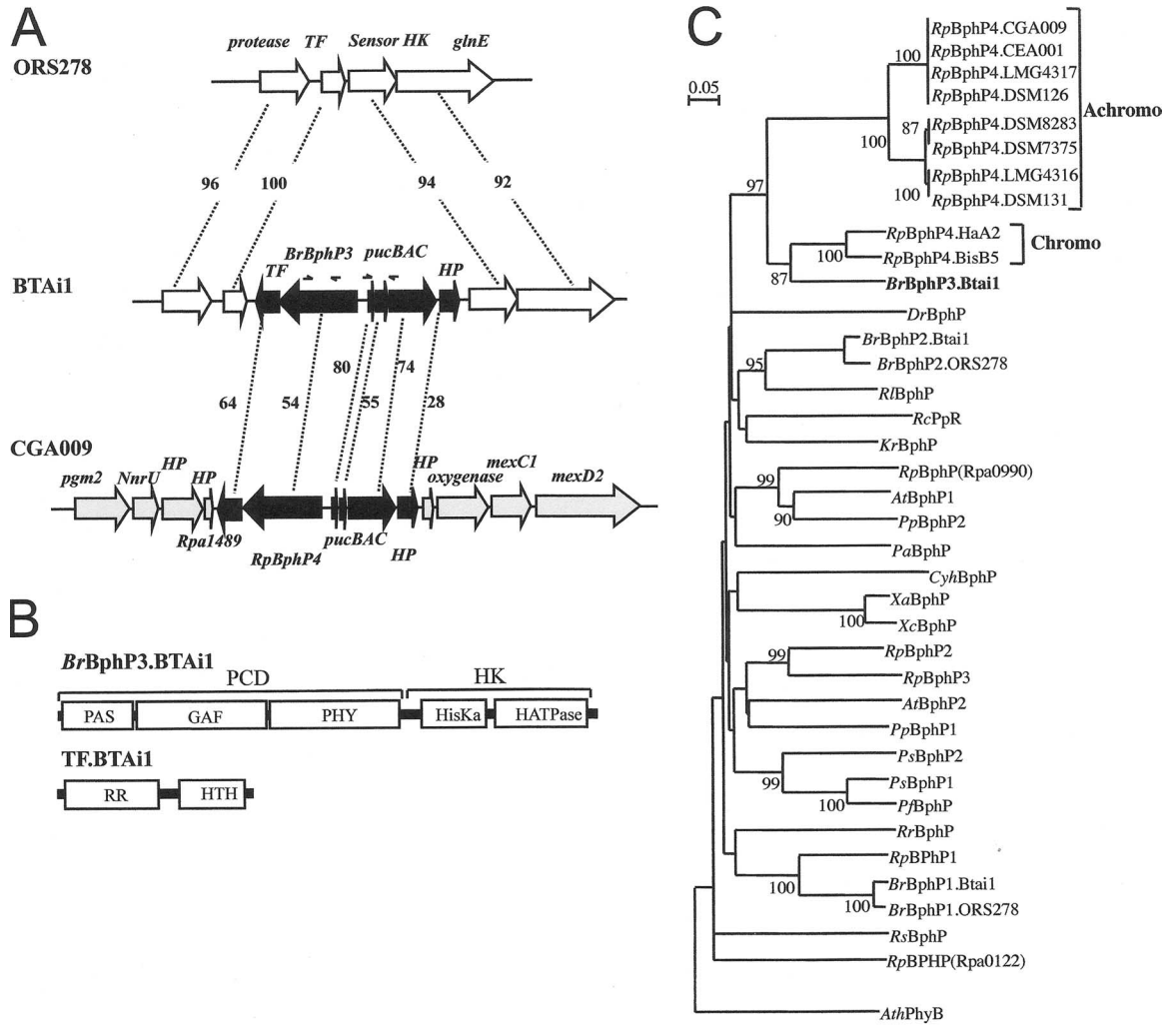

FIG. 1. Molecular characterization of $BphP3_{B\ BTAi1}$. (A) Comparative genomic analysis of the region surrounding the *pucBA* operon and $BphP3_{B\ BTAi1}$ (*BrBphP3*) in the *Bradyrhizobium* BTAi1 and ORS278 strains and in *R. palustris* CGA009. The values given between the genes correspond to the percentages of identity of the corresponding proteins. The arrows indicate the localization of the primers used to search for *pucBA* and *BphP* genes in different photosynthetic bradyrhizobia. Abbreviations: TF, transcriptional factor; HP, hypothetical protein. (B) Predicted domain structure of $BphP3_{B\ BTAi1}$ (*Br*BphP3.BTAi1) and TF_{BTAi1} (TF.BTAi1). HK, histidine kinase domain; HisKa, phosphoacceptor domain; HATPase, ATP binding domain; RR, response regulator domain; HTH, helix-turn-helix domain. (C) Phylogenetic analysis of the BphP family based on an alignment of the GAF domain. The sequences were aligned by using the CLUSTALX software program, and the tree was generated by the neighbor-joining method and displayed using the NJPLOT software program. Bootstrap values, expressed as percentages of 1,000 replications, are given at the branching points. The end points of the GAF domain of each sequence were determined by Pfam analysis (1). Species abbreviations: *At*, *Agrobacterium tumefaciens*; *Ath*, *Arabidospsis thaliana*; *Br*, *Bradyrhizobium* sp.; *Dr*, *Deinococcus radiodurans*; *Pa*, *Pseudomonas aeruginosa*; *Pf*, *Pseudomonas fluorescens*; *Pp*, *Pseudomonas putida*; *Ps*, *Pseudomonas syringae*; *Rc*, *Rhodospirillum centenum*; *Rl*, *Rhizobium leguminosarum*; *Rp*, *R. palustris*; *Rr*, *Rhodospirillum rubrum*; *Rs*, *Rhodobacter sphaeroides*; *Xa*, *Xanthomonas axonopodis*; *Xc*, *Xanthomonas campestris*.

teins of a peripheral LH complex. The *pucC* gene is required for a high-level transcription of these genes but also for a proper assembly of LH1 complexes (17). As already mentioned, this *pucBAC* operon is found close to a putative *bphP* gene (BBta_3079) and a two-component transcriptional regulator gene (BBta_3078) (Fig. 1A). We specify the products of these two genes as $BphP3_{B\ BTAi1}$ and the transcription factor (TF) TF_{BTAi1}, respectively. A similar gene organization was previously found in several *R. palustris* strains (Fig. 1A).

Pfam analysis by comparison of the protein translation to the Pfam database (1) revealed that $BphP3_{B\ BTAi1}$ possesses the classical BphP architecture (Fig. 1B) with a photosensory core domain (PCD) at the N terminus and a C-terminal histidine kinase module involved in signal transduction. TF_{BTAi1} consists of an N-terminal response regulator domain and a C-terminal helix-turn-helix DNA binding domain (Fig. 1B).

Phylogenetic analysis using the GAF domain of $BphP3_{B\ BTAi1}$ (Fig. 1C), a subdomain of the PCD, shows that this BphP is closely related to the $BphP4_{Rp}$ clade (bootstrap value equal to 97%). A similar clustering pattern was obtained using the PHY domain (data not shown). It should be noted that $BphP3_{B\ BTAi1}$ displays a higher identity level with chromo-$BPhP4_{Rp}$ proteins (64% with $BphP4_{Rp}$ proteins from the *R. palustris* HaA2 and BisB5 strains) than with achromo-$BphP4_{Rp}$ (54% with $BphP4_{Rp}$

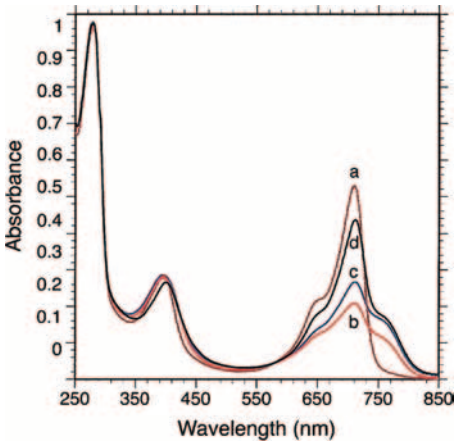

FIG. 2. Spectral characterization of the His-tagged BphP3$_{B\ BTAi1}$ recombinant protein. Absorption spectra of purified BphP3$_{B\ BTAi1}$ are as follows: spectrum a (brown line), recorded after 30 min of dark adaptation following a 770-nm preillumination; spectrum b (red line), after 705-nm illumination; spectrum c (blue line), after 2 min of dark after 705-nm illumination; spectrum d (black line), after 30 min of dark after 705-nm illumination.

from CGA009). In agreement, the PCD of BphP3$_{B\ BTAi1}$ presents the canonical Cys residue, used by BphP as a bilin attachment site, at position 10, in contrast to achromo-BphP4$_{Rp}$ proteins, which lack this residue. Altogether these data suggest that BphP3$_{B\ BTAi1}$ acts as a bona fide BphP protein involved in the control of the *pucBAC* operon found at its vicinity, as already demonstrated in the case of *R. palustris* (31).

Expression, purification, and biochemical and biophysical properties of BphP3$_{B\ BTAi1}$. The His-tagged BphP3$_{B\ BTAi1}$ protein was coexpressed with a heme oxygenase (required for chromophore synthesis) in *E. coli* and then purified by affinity chromatography. The absorption spectrum of purified recombinant BphP3$_{B\ BTAi1}$ is characteristic of a bona fide BphP (Fig. 2). Similar to the case with chromo-BphP4$_{Rp}$ proteins, BphP3$_{B\ BTAi1}$ efficiently binds a chromophore, as determined by fluorescence of the chromoprotein in the presence of zinc and UV light after SDS-PAGE separation (data not shown) (2).

BphP3$_{B\ BTAi1}$ has unusual photochemical properties which are very similar to those observed for chromo-BphP4$_{Rp}$ proteins (31). The dark-adapted state of BphP3$_{B\ BTAi1}$ (reached after 30 min of darkness) depends upon the nature of the light it has previously absorbed. When BphP3$_{B\ BTAi1}$ is illuminated with infrared light (770 nm) and allowed to adapt to darkness for 30 min, its absorption spectrum is characteristic of the red-absorbing form of a Bph (Pr state) (Fig. 2, spectrum a), with an absorption maximum centered at 710 nm. After illumination with red light (705 nm) and 30 min of dark adaptation, the dark-adapted state is a mixture of Pr and Pfr (far-red-absorbing form) states, which absorbed at 760 and 710 nm, respectively (Fig. 2, spectrum d). Starting from the Pr state (illumination by 770-nm light), red light (705 nm) induces a marked bleaching of this form but the formation of only a small amount of the Pfr state, as shown by minor absorption increase around 760 nm (Fig. 2, spectrum b). This light-induced state (large bleaching of the Pr state and minor formation of the Pfr state) appears similar to the "meta-R" state of Agp1 BphP (3) and phyA (6), a very short-lived intermediate during the dark transition from Pr to Pfr. In BphP3$_{B\ BTAi1}$, this state transforms over 2 min to the Pfr state, as shown by the absorption increase around 760 nm (Fig. 2, spectrum c). In fact, the maximum amount of Pfr state is obtained after a 705-nm illumination and a dark adaptation of 5 to 15 min. Conversely, illumination with far-red light induces a rapid and complete formation of the Pr form (not shown). Thus, the peculiar photochemical properties of BphP3$_{B\ BTAi1}$ are probably a consequence of a very slow conversion from the intermediate meta-R state to the Pfr state. Steady-state excitation and emission fluorescence measurements show that the Pr state of BphP3$_{B\ BTAi1}$ is the main fluorescent state, as observed for other (bacterio)phytochromes (data not shown).

Light signaling pathway of BphP3$_{B\ BTAi1}$. The phosphorylation state of BphP3$_{B\ BTAi1}$ was studied under different illumination conditions by in vitro incubation in the presence of [γ-^{32}P]ATP (Fig. 3A). As previously observed for chromo-BphP4$_{Rp}$ proteins (31), BphP3$_{B\ BTAi1}$ was maximally phosphorylated in its Pfr form following a 705-nm illumination (Fig. 3A, lane a) while a transition to the Pr state induced a significant decrease in the phosphorylation level (50% $\pm$ 7%) (Fig. 3A, lane b). We next addressed the question of the effect of redox conditions on kinase activity of BphP3$_{B\ BTAi1}$. No obvious difference in the phosphorylation state of BphP3$_{B\ BTAi1}$ was observed after treatment with reducing (DTT) or oxidizing (potassium ferricyanide) agents (Fig. 3B). This is in agreement with the lack of the two Cys residues previously shown to be involved in redox sensitivity of achromo-BphP4$_{Rp}$ proteins (31). Altogether these data indicate that BphP3$_{B\ BTAi1}$ acts as a light-regulated histidine kinase.

Sequence alignment of the putative transcriptional factor TF$_{BTAi1}$ from *Bradyrhizobium* BTAi1 and its homolog, Rpa1489, from *R. palustris* strain CGA009 revealed a high sequence identity of 64% (Fig. 1A). Unfortunately, the TF$_{BTAi1}$ recombinant protein was expressed only as inclusion bodies in *E. coli*. We therefore used its homolog, Rpa1489, to assess the phosphotransfer capability of purified BphP3$_{B\ BTAi1}$. As shown in Fig. 3C, we observed an efficient phosphotransfer from BphP3$_{B\ BTAi1}$ to purified Rpa1489. This phosphotransfer is maximal when BphP3$_{B\ BTAi1}$ is in its Pfr form. Taking into account the high identity between Rpa1489 and TF$_{BTAi1}$, this suggests a two-component regulatory system where BphP3$_{B\ BTAi1}$ is the first element and TF$_{BTAi1}$ the cognate response regulator.

Expression of *pucBA* and phenotype of *BphP3$_{B\ BTAi1}$* deletion mutant. To check the possibility that *pucBA* is expressed via the pair BphP3$_{B\ BTAi1}$/TF$_{BTAi1}$, we examined the absorption spectra of *Bradyrhizobium* BTAi1 cells (wild-type [WT] strain) and the expression of β-galactosidase activity in the BTAi1 Δ*pucBA* mutant grown under illumination of various wavelengths between 590 and 875 nm. Figure 4A shows the absorption spectra of intact cells illuminated at a few selected wavelengths (648, 732, and 769 nm). Whereas illumination with 648- or 769-nm light induces almost exclusively the synthesis of the RC and the LH1 complex, the synthesis of an additional LH complex, absorbing around 810 nm, is observed under 732-nm lighting. The action spectrum of LH1 synthesis (Fig. 4B) is very similar to that reported previously for the related species *Bradyrhizobium* ORS278 (9). It corresponds to the dark-adapted form of BphP1$_{B\ BTAi1}$ (not shown). The

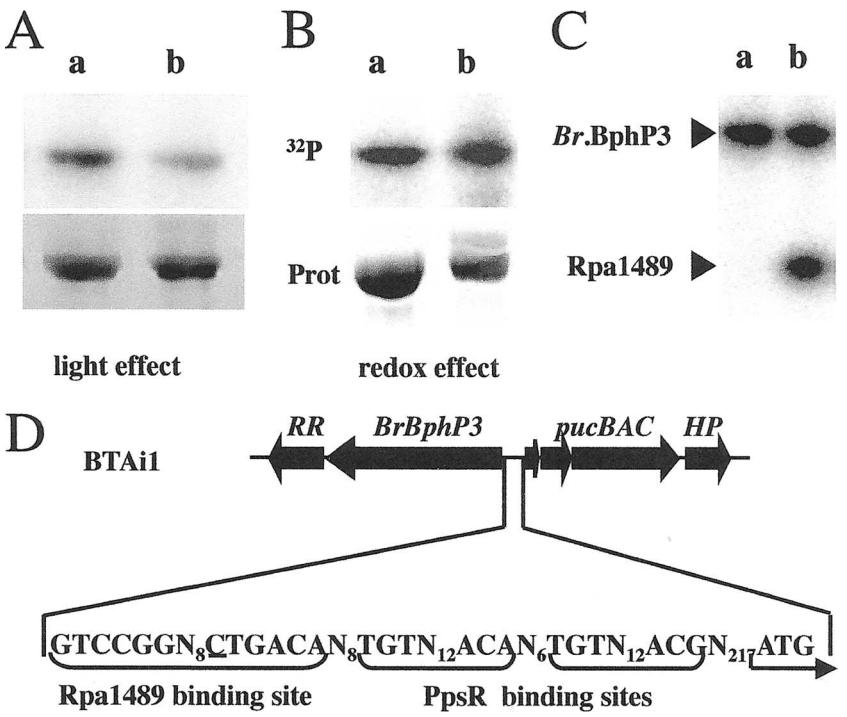

FIG. 3. BphP3$_{B\ BTAi1}$ acts as a light-regulated histidine kinase. (A) Effect of light conditions on kinase activity of BphP3$_{B\ BTAi1}$. The chromoprotein was converted preferentially to its Pfr (a) or Pr (b) forms. The maximal amount of the Pfr form was obtained by 15 min of preillumination at 705 nm, followed by 15 min of dark adaptation, and that of the Pr form by illumination at 770 nm. Proteins were incubated with [γ-^{32}P]ATP for 15 min. The reaction products were separated by SDS-PAGE, and the gel was subjected to autoradiography (top) or stained by Coomassie blue (bottom). (B) Effect of redox conditions on kinase activity of BphP3$_{B\ BTAi1}$. For the redox effect, the sample was subjected to the following conditions: a 15 min preillumination with 705-nm light, followed by a 15-min dark adaptation; DTT (1 mM) (a) or ferricyanide (1 mM) (b) was used. (C) Phosphotransfer between BphP3$_{B\ BTAi1}$ (*Br*BphP3) and the Rpa1489 recombinant protein. BphP3$_{B\ BTAi1}$ was preferentially placed in its Pfr form by a 705-nm illumination followed by 15 min of dark adaptation. Lane a, BphP3$_{B\ BTAi1}$ alone; lane b, BphP3$_{B\ BTAi1}$ plus Rpa1489. (D) Sequence analysis of the *pucBA* promoter region of BTAi1 showing the presence of Rpa1489 and PpsR binding sites.

synthesis of the additional LH complex is maximal for lights centered at 725 nm (Fig. 4B). A similar action spectrum is obtained for the expression of β-galactosidase activity measured for the BTAi1 Δ*pucBA* mutant (Fig. 4B), demonstrating that this additional LH complex is the product of the *pucBA* genes. These action spectra are very similar to the absorption spectrum of BphP3$_{B\ BTAi1}$ in its dark-adapted state but slightly shifted to longer wavelengths (725 nm instead of 710 nm). The rationale of this observation is that the synthesis of the additional LH complex requires not only the action of BphP3$_{B\ BTAi1}$ but also the activation of BphP1$_{B\ BTAi1}$. Indeed, activation of BphP1$_{B\ BTAi1}$ induces the synthesis of various enzymes and proteins involved in the synthesis of the photosynthetic apparatus (*bch*, *crt*, and *puf* genes) by antagonizing the repressive activity of PpsR2, which is a prerequisite for the formation of LH complexes. To verify this proposal, WT cells were illuminated with 770-nm light alone or with 700- plus 770-nm light. The 770-nm light, by exciting BphP1$_B$, induces all the enzymes necessary for synthesis of the photosynthetic apparatus, while the 700-nm light preferentially excites BphP3$_{B\ BTAi1}$ to induce the transcription of the *pucBA* genes. Figure 4C compares the absorption spectra of intact cells of *Bradyrhizobium* BTAi1 grown under these two conditions. While illumination with 770-nm light induces solely the formation of the RC-LH1 complex, the synthesis of an extra LH complex is observed with an additional 700-nm illumination (Fig. 4C). At room temperature, the extra LH complex exhibits an absorption band centered at 805 nm with a shoulder around 815 nm. A definite proof of the essential role played by BphP3$_{B\ BTAi1}$ in the synthesis of this additional LH complex is shown by the phenotype of a BphP3$_{B\ BTAi1}$ deletion mutant, which does not synthesize this additional LH complex regardless of the illumination conditions (Fig. 4D).

Distribution of the *pucBA* operon in photosynthetic *Bradyrhizobium* strains. A comparative genomic analysis reveals that the region of 5 kb containing this light regulatory system and the *pucBA* operon is found in a synteny group of four genes that is perfectly conserved in the ORS278 genome (Fig. 1A) and other rhizobial genomes (not shown). This suggests that this DNA region of the BTAi1 chromosome was acquired by lateral gene transfer, possibly from an *R. palustris* strain. To strengthen this hypothesis, we searched by PCR for the presence of both the *pucBA* operon and the *BphP3*$_{B\ BTAi1}$ gene in 107 photosynthetic *Bradyrhizobium* strains isolated from various countries (Senegal, Mexico, Guyana, etc.). None of them gave PCR products. However, we found the corresponding PCR products for the *Blastobacter denitrificans* type strain (LMG 8443), a bacterium isolated from a pond in Germany, recently proposed to be renamed *Bradyrhizobium denitrificans* based on its ability to nodulate *Aeschynomene* plants and on the phylogenetic analysis of 23S rRNA gene sequences, which implies its grouping with the photosynthetic *Bradyrhizobium* species, more particularly with strain BTAi1 (29, 30). Sequence

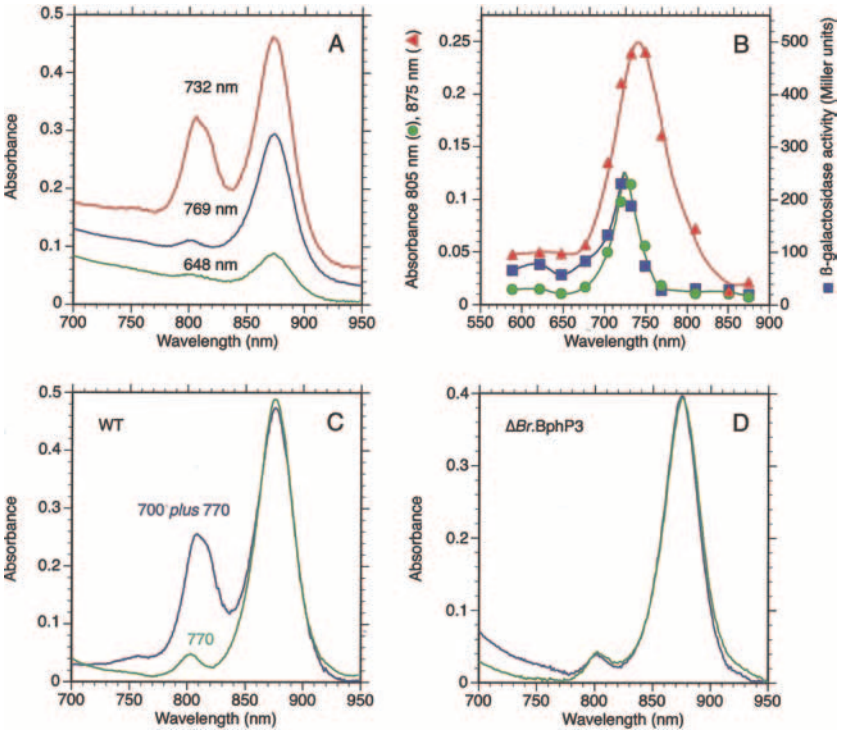

FIG. 4. BphP3$_{B\ BTAi1}$ controls the peripheral LH synthesis in *Bradyrhizobium* BTAi1. (A) Absorption spectra of *Bradyrhizobium* BTAi1 cells (WT) grown under semiaerobic conditions, subjected to illumination at various wavelengths (648 nm, green line; 732 nm, red line; 769 nm, blue line). (B) Wavelength dependence of the synthesis of RC/LH1 (red line) or of the peripheral LH complexes (green line) or of the expression of a *pucBA-lacZ* fusion (blue line). (C) Absorption spectra of *Bradyrhizobium* BTAi1 cells (WT) grown under 770-nm illumination (green line) or under 700- plus 770-nm light (blue line). (D) Absorption spectra of *Bradyrhizobium* strain BTAi1 (ΔBphP3$_B$) cells (deletion mutant) grown under 770-nm illumination (green line) or under 700- plus 770-nm lights (blue line).

analysis of the PCR products of 762 and 772 bp, respectively, confirms a very high identity between these two regions of the *Blastobacter denitrificans* and BTAi1 genomes: 100% identity for the *pucBA* operon and only one mismatch with the *BphP3*$_{B\ BTAi1}$ gene. Similar to what we observed for the BTAi1 strain, the expression of the *pucBA* genes of *Blastobacter denitrificans*, revealed by a 700-nm illumination, was required for the synthesis of the peripheral LH complex (not shown). Altogether these data indicate that the presence of the peripheral LH complex in photosynthetic bradyrhizobia is very unusual, limited to some rare strains, which acquired this property by lateral gene transfer.

DISCUSSION

Transduction pathway of *pucBA* operon of *Bradyrhizobium* BTAi1. In the present report, we put forward evidence that the third BphP (BphP3$_{B\ BTAi1}$) found specifically in the photosynthetic *Bradyrhizobium* BTAi1 strain is involved in control of the expression of a *pucBA* operon encoding the apoproteins of an LH complex. Our model is based on the high level of identity observed between BphP3$_{B\ BTAi1}$ and BphP4$_{Rp}$, the conserved arrangement surrounding their genes (Fig. 1A), and the phosphotransfer observed between BphP3$_{B\ BTAi1}$ and Rpa1489, a homolog of TF$_{BTAi1}$. The transcriptional factor Rpa1489 binds to the palindromic motif TGTCCGN$_8$CGGACA that is found on the *pucBA.b/e* promoter regions of *R. palustris* strains (31). This promoter site is located downstream from a TGTN$_{12}$ACA palindrome corresponding to a PpsR binding site, another key regulator of photosynthesis gene expression in purple bacteria (7). Both Rpa1489 and PpsR binding motifs are present in the promoter region of the *pucBA* operon from *Bradyrhizobium* BTAi1, as shown in Fig. 3D. The two photosynthetic *Bradyrhizobium* strains and *R. palustris* species display the unusual properties of having two distinct PpsRs (encoded by *ppsR1* and *ppsR2*) (19). From these observations, we propose that the *pucBA* operon from *Bradyrhizobium* BTAi1 is regulated by a complex regulatory network involving the concerted action of the BphP3$_{B\ BTAi1}$/TF$_{BTAi1}$ two-component system and the PpsR1 and PpsR2 proteins, as already demonstrated for *R. palustris* strains (31).

Properties and function of the peripheral LH complex of *Bradyrhizobium* BTAi1. The absorption property of this LH complex at room temperature (Fig. 5B) is very similar to those of the LH3 complex found in *R. palustris* or *R. acidophila*. In agreement with this assignment, examination of the amino acid sequence of the *pucA* product revealed a PhePhe sequence at positions +13 and +14 found in LH3 and LH4 complexes of *R. palustris* (28). A Thr found at position −5 from the His binding the chromophore is also indicative of an LH3 complex. In addition, the low-resolution structure obtained by Hartigan et al. (16) shows that the Met at the same position in the LH4 complex of *R. palustris* is a putative ligand of the extra bacteriochlorophyll of this complex, which is absent in the LH3

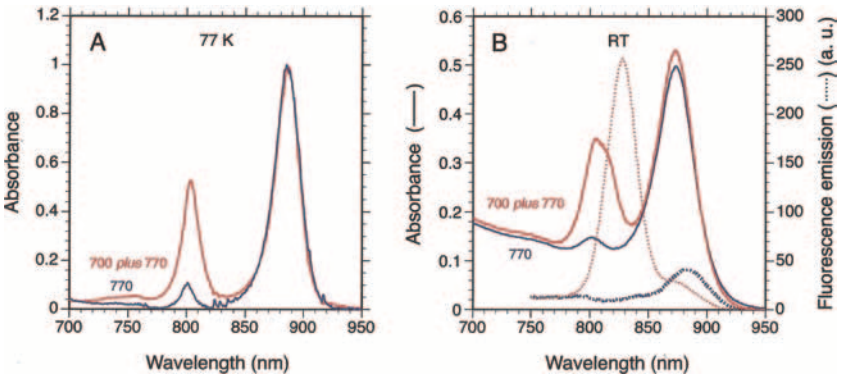

FIG. 5. Absorption and fluorescence emission spectra of intact cells of *Bradyrhizobium* BTAi1 grown under two different light conditions. (A) Low-temperature (77 K) absorption spectra of intact cells of *Bradyrhizobium* BTAi1 grown under 770-nm light (blue) or 700- plus 770-nm lights (red). The absorption spectra have been normalized to the same concentration of RCs. (B) Absorption spectra (continuous lines) and emission spectra (dashed lines), recorded at room temperature, of cells grown under 770-nm light or 700- plus 770-nm light are in blue and red, respectively. Recording of the fluorescence emission spectra was performed under 380-nm excitation. The absorption and fluorescence spectra have been normalized to the same concentration of RCs.

complex. However, low-temperature measurement of the absorption spectrum of the extra BTAi1 LH complex in intact cells reveals a single band centered at 803 nm, typical of the LH4 complex (Fig. 5A). Therefore, the definite assignment of the peripheral LH complex of BTAi1 as an LH3 or LH4 complex requires further experiments.

The *pucA* gene product presents a long carboxy-terminal extension of 65 amino acids starting at amino acid 59. BLAST analysis shows that this C-terminal region does not present any similarity to known proteins or functional domains. A search for transmembrane helices based on a hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM/) permits identification of only one helix domain between Thr13 and Leu35, indicating that the C-terminal extension should be located outside the membrane. Such extension has already been observed for the PucA product of several other photosynthetic bacteria. The *pucA* gene product of *Rubrivivax gelatinosus* possesses a short alanine- and proline-rich extension, which can be partially reduced without affecting the assembly of the complex (26). On the other hand, the *puc2A* gene product found in *Rhodobacter sphaeroides*, which presents a long extension of 209 amino acids, is not functional (35). Of particular interest in the context of the present work is the *pucA* gene from the AAP *Roseobacter denitrificans* OCh114, initially named *Erythrobacter denitrificans*. The recent sequence analysis of the genome of this bacterium revealed that the product of this gene presents an extension of 58 amino acids (27). The corresponding LH presents a single absorption band in the near-infrared region centered around 806 nm, similar to the LH4 complex of *R. palustris* (16). The molecular masses of the two polypeptides of the LH complex purified from *Roseobacter denitrificans* OCh114 are around 6 kDa, implying that the PucA polypeptide is processed before insertion into the membrane (24). Several attempts to extract and purify the peripheral LH complex of *Bradyrhizobium* BTAi1 using various detergents have been unsuccessful. For example, only a supramolecular complex containing both the RC-LH1 complex and the peripheral 805-nm LH complex could be isolated by addition of 1.5% lauryldimethylamine-oxide. Therefore, we do not know presently if the PucA subunit of this peripheral LH complex is assembled in the membrane with or without the extension.

We ascertained the efficiency of energy trapping and transfer to the RC by the peripheral LH complex of *Bradyrhizobium* BTAi1 by measuring the fluorescence emission spectrum of intact cells of *Bradyrhizobium* BTAi1 grown under conditions where the peripheral LH complex has been expressed or not (Fig. 5B). In the absence of the peripheral LH complex, the fluorescence emission spectrum peaks around 880 nm, corresponding to fluorescence emitted by LH1 complexes. The fluorescence emission spectrum of membranes containing the peripheral LH complex presents an intense fluorescence emission around 825 nm in addition to that of the LH1 complex. The high yield of the 825-nm fluorescence and the relative low concentration of the peripheral LH complex imply an inefficient energy transfer between these complexes and the LH1 complex. This inefficient energy transfer between these two LH complexes could be related to the small overlap of their associated absorption bands in the near-infrared region. This contrasts with what has been observed for *R. palustris*, *R. acidophila*, and *Roseobacter denitrificans* OCh114, for which an efficient excitonic energy transfer between peripheral LH3/LH4 complexes and LH1 complexes has been reported (4, 8, 25).

Since this peripheral LH complex does not act as an efficient light antenna, what could its physiological role be? As already underlined, the photosystem of AAP bacteria is active only under aerobic conditions. In the presence of oxygen and light, the photosynthetic activity generates triplet states of bacteriochlorophyll molecules. These excited states can react with singlet oxygen and form harmful reactive oxygen species. To cope with this problem, various strains of photosynthetic *Bradyrhizobium*, symbionts of *Aeschynomene*, synthesize, in addition to spirilloxanthin, large amounts of canthaxanthin (21). In a previous study, we showed that *Bradyrhizobium* ORS278 possesses two distinct *crt* gene clusters for the synthesis of spirilloxanthin and canthaxanthin, respectively (12). While spirilloxanthin is coupled to the photosynthesis activity, canthaxanthin protects the bacteria from oxidative stress (12). The canthaxanthin *crt* gene cluster, acquired by a lateral gene transfer in *Bradyrhizo-*

bium ORS278, is absent in *Bradyrhizobium* BTAi1. One putative function of the peripheral LH complexes of *Bradyrhizobium* BTAi1, whose genes were also acquired by lateral gene transfer, would also be to evacuate excess light energy in order to protect the bacteria against (photo)oxidative damage during photosynthesis. This hypothesis is reinforced by the fact that the products of the *pucBA* genes, acquired by lateral transfer probably from *R. palustris*, have evolved from an LH2-type complex that transfers energy very efficiently to the LH1 complexes to an LH complex, much less efficient in energy transfer.

For both the *Bradyrhizobium* ORS278 and *Bradyrhizobium* BTAi1 strains, which photosynthesize under conditions where harmful reactive oxygen species are generated, these two different gene acquisitions may constitute a major selective advantage.

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