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Dual role for a bacteriophytochrome in the bioenergetic control of *Rhodopsdeudomonas palustris*: Enhancement of photosystem synthesis and limitation of respiration

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Abstract

In the purple photosynthetic bacterium *Rhodopseudomonas palustris*, far-red illumination induces photosystem synthesis via the action of the bacteriophytochrome *Rp*BphP1. This bacteriophytochrome antagonizes the repressive effect of the transcriptional regulator PpsR2 under aerobic condition. We show here that, in addition to photosystem synthesis, far-red light induces a significant growth rate limitation, compared to cells grown in the dark, linked to a decrease in the respiratory activity. The phenotypes of mutants inactivated in *Rp*BphP1 and PpsR2 show their involvement in this regulation. Based on enzymatic and transcriptional studies, a 30% decrease in the expression of the alpha-ketoglutarate dehydrogenase complex, a central enzyme of the Krebs cycle, is observed under far-red light. We propose that this decrease is responsible for the down-regulation of respiration in this condition. This regulation mechanism at the Krebs cycle level still allows the formation of the photosynthetic apparatus via the synthesis of key biosynthesis precursors but lowers the production of NADH, i.e. the respiratory activity. Overall, the dual action of *Rp*BphP1 on the regulation of both the photosynthesis genes and the Krebs cycle allows a fine adaptation of bacteria to environmental conditions by enhancement of the most favorable bioenergetic process in the light, photosynthesis versus respiration.

Keywords: Bacteriophytochrome; Photosynthesis; Respiration; Alpha-ketoglutarate; Regulation; *Rhodopseudomonas palustris*

1. Introduction

Due to their extraordinary metabolic versatility, photosynthetic bacteria can grow with or without oxygen, via aerobic or anaerobic respirations and photosynthesis. To benefit from this flexibility and take advantage of available resources, these bacteria have to rapidly adapt and respond to variations in their environment. This requires sensing changes in light and oxygen concentration since the regulation between photosynthesis and respiration is of prime importance for their energetic fitness (for review see [1–3]). This regulation occurs at two different levels.

Short-term interactions between the electron carriers enable photosynthetic bacteria to utilize the most favorable bioenergetic process in the light, i.e. photosynthesis. Consequently, light strongly inhibits respiratory activity [4] by two non-exclusive mechanisms. The first is an indirect effect of the light-induced proton motive force on complexes involved in the respiratory activity in particular at the level of complex I [5–7]. The second mechanism involves a direct competition between electron carriers common to both photosynthetic and respiratory chains (ubiquinone, the cytochrome bc_1 complex or the cytochrome c_2) [8,9].

On the transcriptional level, regulations of the synthesis of the bioenergetic chains in purple bacteria involve several redox and light sensors [3,10,11]. The sensor kinase RegB and its

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response regulator RegA are responsible for the global transcriptional control of aerobic and anaerobic metabolic processes in response to the redox state of the cell (for review see [12]). In addition, specific aerobic regulators of the expression of photosynthesis genes have been described in several photosynthetic bacteria. The transcriptional factors PpsR (or CrtJ) act as repressors of the synthesis of the photosynthetic apparatus under high aeration in *Rhodobacter* (*Rb*.) *sphaeroides* [13], *Rb. capsulatus* [14], and *Rhodopseudomonas* (*Rps.*) *palustris* [15,16]. A more complex role has however been reported in the case of *Rubrivivax gelatinosus* where this transcriptional factor represses some photosynthesis genes or activates others [17]. The aerobic photosynthetic bacterium *Bradyrhizobium* ORS278 possesses two distinct proteins, PpsR1 and PpsR2, which have opposite effect on the synthesis of the photosynthetic apparatus, the O_2-sensitive PpsR1 being an activator whereas the O_2-insensitive PpsR2 acts as a repressor [10,18].

To date, two types of photoreceptors have been documented as regards to the light regulation of the synthesis of the photosynthetic apparatus of purple bacteria. (i) AppA, a blue light receptor, represses the photosynthetic genes expression in *Rb. sphaeroides* [19–21]. (ii) A bacteriophytochrome, present in *Rps. palustris* (*Rp*BphP1) and *Bradyrhizobium* (*Br*BphP), has been shown to trigger the synthesis of the entire photosynthetic apparatus under aerobic conditions by antagonizing the repressing effect of PpsR2 [10,15,22]. In addition to this bacteriophytochrome, two others, *Rp*BphP2 and *Rp*BphP3, act in tandem in *Rps. palustris* to control the synthesis of LH4 complexes [23,24], characterized by a single absorption band in the near infra-red at 800 nm [25].

The sequencing of the complete genome of *Rps. palustris* CGA009 has revealed the presence of 3 other putative bacteriophytochromes and a large number (451) of putative regulatory and signaling genes, corresponding to 9.3% of its genome as compared to the usual 5–6% for free-living bacteria [26]. This makes *Rps. palustris* an excellent model to study the adaptability of a photosynthetic bacterium in response to environmental changes.

In the present work, we demonstrate that *Rp*BphP1, in addition to its up regulation of the photosynthetic units, down-regulates the respiratory activity under far-red illumination. This regulation also involves the transcriptional regulator PpsR2. We propose that this down-regulation of the respiratory activity is linked to a decrease in the expression of the alpha-ketoglutarate dehydrogenase complex, a central enzyme of the Krebs cycle. This regulation at the Krebs cycle level both decreases respiratory activity but still allow the synthesis of key biosynthesis precursors for the formation of the photosynthetic apparatus. This mechanism allows a fine adaptation of bacteria to environmental conditions by enhancement of photosynthesis activity versus respiration, i.e. the most favorable bioenergetic process in the light.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The WT strain CEA001 and the various mutants of *Rps. palustris* were grown in a modified photosynthetic medium (PM) [27] by addition of 20 mM ammonium malate as additional carbon and nitrogen sources, and growth factors containing vitamins in proportion used in Hutner medium [28]. Bacteria were grown either in the dark or under 770 nm illumination provided by light emitting diodes (100 μmol of photon/s/m^2, half-peak bandwidth of 25 nm). When indicated, additional actinic illumination was provided by light emitting diodes (875 nm, 1500 μmol of photon/s/m^2, half-peak bandwidth of 25 nm). Pure N_2 and air were mixed using mass-flow controller (Brooks) in order to obtain the appropriate O_2 tension ranging from 1% and 21%. The gas mixtures were flushed in the gas phase of Erlenmeyer flasks of 250 ml. To obtain a good equilibrium between the gas and the liquid phases, the Erlenmeyer flasks contained only 50 ml of growth medium and were shaken at 140 rpm. Cells, inoculated at an initial concentration of OD_{660}=0.1, were collected at various times ranging from 8 h to 160 h for absorption spectra recording, respiratory activity determination, RNA extraction and alpha-ketoglutarate dehydrogenase complex activity determination.

2.2. Absorption spectra and photosystem synthesis measurements

Absorption spectra of intact cells of the WT strain and of the various mutants were recorded with a Cary 50 spectrophotometer. The relative amount of photosystem was determined by integrating the Qy bacteriochlorophyll absorption bands between 700 and 950 nm for suspension of intact cells collected at different times and growth conditions.

2.3. Bacterial growth

Bacterial growth was determined by recording the optical density at 660 nm (OD_{660}) in function of time. This wavelength was chosen because it only depends upon the bacteria light scattering and not to the presence of bacteriochlorophyll molecules which do not absorb in this spectral range. Correlation between OD_{660} and bacterial concentration was determined by plating serial dilutions of the bacterial suspensions and counting the CFU/ml.

2.4. Respiratory activity measurements

The respiratory activity of intact cells was determined using a Clark electrode (Hansatech, Great Britain). Prior to the measurements the cells were harvested and suspended in fresh modified PM medium. The respiratory activity of each strain was normalized to the same number of cells (determined by recording the OD_{660}). The total cytochrome oxidase activity was tested by the addition of tetramethylparaphenylene diamine (TMPD) at a saturating concentration of 100 μM.

2.5. Construction of RpBphP1, RpBphP5, RpBphP6 and ppsR2 mutant strains

The construction of the *RpBphP1*, and *ppsR2* mutants of *Rps. palustris* CEA001 strain has been previously described [15]. The *RpBphP5.CEA001* null mutant was obtained by inserting the *lac*Z-Km^r cassette directly into the unique *Bam*H1 site of the *RpBphP5* gene. To create the *RpBphP6. CEA001* null mutant, a *Sal*I fragment of 39-bp inside the gene was deleted and replaced by the *lac*Z-Km^r cassette of pKOK5 [29]. These constructs were introduced into the pJQ200 suicide vector [30] and delivered by conjugation into the corresponding *Rps. palustris* strain as described [15]. Double recombinants were selected on sucrose and confirmed by PCR.

2.6. RNA extraction

Bacterial cells were collected during dark and 770 nm exponential phases (between 20 h and 25 h of growth corresponding to $OD_{660\ nm}$=0.6–0.9 for dark-grown bacteria, $OD_{660\ nm}$=0.4–0.5 for 770 nm grown bacteria). Two volumes of RNAprotect™ Bacteria Reagent (Qiagen GmbH, Hilden, Germany) were added to one volume of bacterial culture for RNA stabilization. Cells were harvested (5000×*g*, 10 min, room temperature) and kept at −80 °C. Total RNA extraction was performed from 10^9 bacteria using the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany) following the manufacturer's procedures except for the first lysis step. Indeed, *Rps. palustris* cells being resistant to lysozyme digestion, cells were resuspended in RA1 buffer from the Nucleospin

RNA II kit and broken at 2.3 kbar using a Constant Cell Disruption System (Constant Cell Disruption Systems LTD, Daventry, United Kingdom). Residual genomic DNA was digested using the DNA-free™ kit (Ambion INC, Austin, TX, USA) according to the manufacturer's instructions.

2.7. Real-Time RT-PCR

Reverse transcription was performed using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara Bio INC, Shiga, Japan) in a total volume of 10 μL containing 300 ng total RNA following the manufacturer's instructions. The reaction mixture was subjected to subsequent incubation steps: 10 min at 30 °C, 30 min at 42 °C, 5 min at 99 °C and 5 min at 5 °C. Gene expression analysis was performed on the ABI Prism 7000 Sequence detection system using the SDS 1.0 application software (Applied Biosystems). Primers, summarized in Table 1, were designed using the software eprimer3 (http://bioportal.cgb.indiana.edu/cgi-bin/emboss/eprimer3). Quantitative PCR was performed using the qPCRTM Mastermix Plus for SYBR Green I (Eurogentec, Seraing, Belgium). Assays were performed in a 25-μl final volume with 12.5 μl of 2X Master Mix SYBR Premix, 0.2 μM of each primer and 5 μl of 50 fold diluted cDNA or water for the negative control. Thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C followed by 40 repeats of 15 s at 95 °C and 1 min at 60 °C. Specificity of the PCR product was confirmed by melting curve analysis.

Gene expression was analyzed by relative quantification using the control transcripts of *rpoD* (*rpa1288*, encoding the RNA polymerase sigma subunit), *rnpO* (*rpa2692*, encoding the RNA polymerase omega subunit) and *16s rrna* (encoding 16S ribosomal RNA) used as internal standards in several bacteria [31–34]. Prior to 16S quantification, cDNA were diluted 1/500 to obtain results in the confidence range of the technique [31]. The primer efficiency was determined by performing calibration curves using genomic DNA template over 5 orders of magnitude. Calibration curves with linearity R^2 superior to 0.99 were considered and primers with efficiencies superior to 90% were used. Gene expression was expressed as normalized gene expression under 770 nm relative to normalized gene expression in the dark using the efficiency corrected Ct model [35]. Since the three internal standards gave similar tendencies upon gene expression quantification, only results obtained with *rpoD* are shown in this report. Three to five different experiments were performed for each gene quantification. For each of these experiments, the RT-PCR assays were done in triplicate.

2.8. Alpha-ketoglutarate dehydrogenase complex activity

Cell extracts were prepared following a modified procedure of Beatty and Gest [36]. Bacteria in exponential phase (around 21 h of growth, OD_{660} ~ 0.7 for dark-grown bacteria, OD_{660} ~ 0.45 for 770 nm grown bacteria) were harvested by centrifugation, washed and resuspended in 50 mM phosphate buffer, pH 7. Cells were broken in Constant Cell Disruption System (Constant Cell Disruption Systems LTD, Daventry, United Kingdom) under a 2.3-kbar pressure. Extracts were centrifuged at 30,000×*g* for 10 min and the supernatant was then centrifuged 1 h 30 at 150,000×*g*. The pellet corresponding to membranes was resuspended in phosphate buffer. Alpha-ketoglutarate dehydrogenase complex (KGD) activity was determined in membrane extracts (KGD enzyme complex co-sediments with membranes) following the Beatty and Guest procedure [36]. Increase in absorbance at 340 nm corresponding to NAD^+ reduction was measured. Calculations were performed using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH.

2.9. In silico search for PpsR binding sites

PpsR binding site search was performed using the software PATTERNn (http://bioinfo.hku.hk/services/analyseq/cgi-bin/patternn_in.pl) [37]. We first extracted the 500 nucleotides upstream all *Rps. palustris* CGA009 CDS using query win (a graphical application for retrieving sequences from databases in ACNUC format developed at PRABI (www.prabi.fr) [38,39]. The data here were retrieved from GenBank. The sequences in fasta format were then entered as personal bank in the PATTERNn program. The pattern searched was either one strictly conserved $TGTN_{12}ACA$ motif or one conserved $TGTN_{12}ACA$ motif and one degenerated motif [T,C]G[A,T]N12[T,A]C[G,T,A] (the letters separated by a comma meaning an alternative) [40] separated by 2 to 300 bases. For the degenerated motif, only one alternative was considered at a time and the results were then compiled.

2.10. Gel mobility shift assay

A 421-bp DNA segment corresponding to the *sucA* promoter region containing the PpsR2 binding site identified in silico was obtained by PCR using ^{32}P 5′-end labeled oligonucleotide primers (5′-CCCGGTCAAGGGCACCGAATTC-3′ and 5′-GGTTACCGGATCAGCAACATAG-3′), as previously described [15]. The purified PpsR2 protein (14 μg) was added to 20 μl of reaction buffer composed of 5 fmol of ^{32}P-labeled DNA probe, 1 μg of polydIdC as competitor, 50 mM Tris–HCl (pH 8), 1 mM DTT, 50 mM potassium acetate, 5 μg of bovine serum albumin and 10% glycerol. The reaction mixture was incubated at room temperature for 30 min and was subjected to non-denaturing 5% Tris–glycine–EDTA-buffered polyacrylamide gel electrophoresis at 4 °C for 4 h at 70 V.

3. Results

3.1. Photosystem synthesis is controlled by RpBphP1 in a wide range of oxygen tension

In a previous work, we reported that photosystem synthesis of *Rps. palustris* CEA001 occurs in the dark only at low O_2 tension, below 8%, a behavior frequently observed in photosynthetic bacteria [15]. At higher O_2 tension, this synthesis is repressed by the transcription regulators PpsR. This repression is however antagonized under low intensity far-red light, specifically absorbed by the Pfr form of *Rp*BphP1 [10,15,22]. This is exemplified in Fig. 1 where the absorption spectra of bacteria grown in liquid medium in the dark or under various illumination

Table 1
Primers used in real time RT-PCR

Gene name	Gene number	Encoding	Forward (5′ to 3′)	Reverse (5′ to 3′)	Amplicon length (bp)
rpoD	*rpa1288*	RNA polymerase sigma 70 subunit	CGGTGCACATGATCGAGA	CCTTGGCGATCTTCAGGA	150
16s rrna	*RNA_52*	16S ribosomal RNA	AGCAATACGTCAGTGGCAGA	TTTCGGCGATAAATCTTTCC	131
rnpO	*rpa2692*	RNA polymerase omega subunit	TCGCGACAACGACAAGAA	GAACCAATCAGCGGAACG	153
sucA	*rpa0189*	putative alpha-ketoglutarate dehydrogenase (E1 subunit)	TCCGGCAAGGTCTATTACGA	TCTTCCTGACACCACACCAG	161
nuoB2	*rpa4263*	NADH-ubiquinone dehydrogenase chain B	TGCTACGTCGAACAGGTCAC	GCTCGTACAAACGCAGCAG	157
nuoM1	*rpa2938*	NADH-ubiquinone dehydrogenase chain M	ATCCTCGAAACGCTGATGAT	AGCAGCGTGTACAGGAAGAA	161
–	*rpa1193*	cytochrome *bc1* precursor	CGACCCTCAAGGACGTGT	AGTACCATTCCGGCACGA	150
cycA	*rpa1535*	cytochrome c_2	CGGCTGGCTTCACCTATTC	ACCGCCTGATCAGCCTTAC	148
pufB	*rpa1525*	light-harvesting complex 1 beta chain	AAGCGGAAGCCAAGGAAT	TCAGTTGATGAACGCTGTCC	150

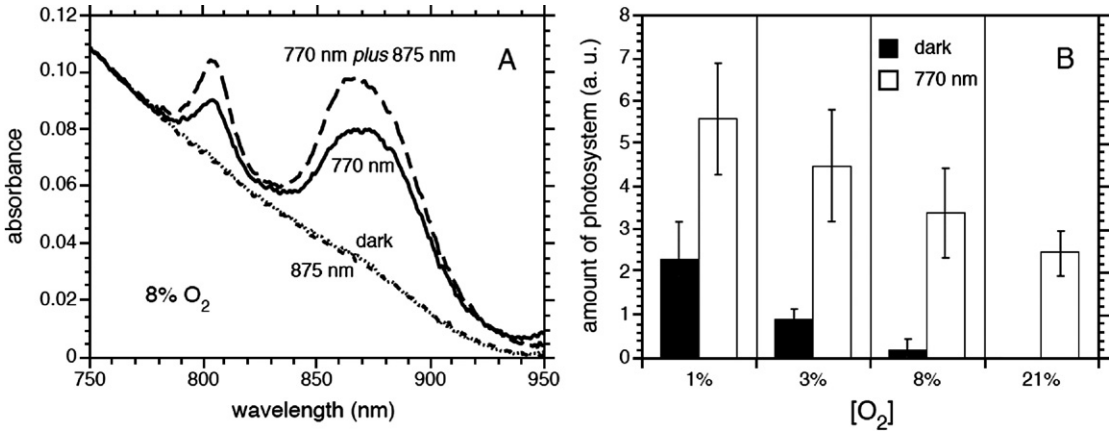

Fig. 1. Effect of O_2 tension and illumination on the synthesis of the photosynthetic apparatus of *Rps. palustris*. (A) Absorption spectra of intact cells harvested in exponential phase (around 21 h of growth) grown in the dark or under various illuminations (770 nm, 875 nm or 875 plus 770 nm) at 8% O_2 tension. (B) Amount of photosystem synthesized in the dark (black) and under 770 nm illumination (white) in function of oxygen tension. This amount was deduced from absorption spectra recorded on intact cells as shown in part A. 770 nm light corresponds to 100 μmol of photon/s/m^2, and 875 nm, light to1500 μmol of photon/s/m^2. In both cases the half-peak bandwidth was 25 nm.

conditions at 8% O_2 tension are reported. In the dark or under strong illumination at 875 nm (1500 μmol of photons/m^2/s), a wavelength absorbed by the bacteriochlorophyll molecules and not by bacteriophytochromes, no synthesis of the photosystem is observed. On the other hand, bacteria grown under 770 nm illumination, preferentially absorbed by *Rp*BphP1, or under 770 nm plus 875 nm illumination present absorption bands around 800 and 860 nm, characteristic of LH2 complexes and LH1 complexes of the photosynthetic apparatus (Fig. 1A). This induction occurs from microaerophilic (1%) to aerophilic (21%) (Fig. 1B) growth conditions, extending the activating role of *Rp*BphP1 in photosystem synthesis in a wide range of O_2 tension [10,15,22].

3.2. 770 nm illumination induces growth rate decrease

Interestingly we noticed that, in addition to the activation of the photosystem synthesis, 770 nm illumination induces a decrease in cell density. This is exemplified in Fig. 2, which compares images of the bacteriochlorophyll fluorescence and of light transmission for *Rps. palustris* cells homogeneously inoculated on a Petri dish and subjected to illumination with different wavelengths between 590 and 870 nm. The wavelength dependence of the decrease in cell density is very similar to the action spectrum of photosystem formation, i.e. corresponds to the Pfr form of a bacteriophytochrome (Fig. 2) [10]. The decrease in growth capability induced by 770 nm illumination was confirmed in liquid medium. An example is shown in Fig. 3 where the growth curves of bacteria under 8% of O_2 tension, placed under 770 nm illumination or in the dark are compared. The 770-nm illumination induces a 2-fold decrease in growth rate compared to dark-grown bacteria. For all O_2 tensions tested (between 1 and 21%), a clear reduction in growth rate was observed when comparing cells grown in these two conditions (Fig. 4A). In the following, we adopted an O_2 tension of 8%, i.e. semi-aerobic conditions.

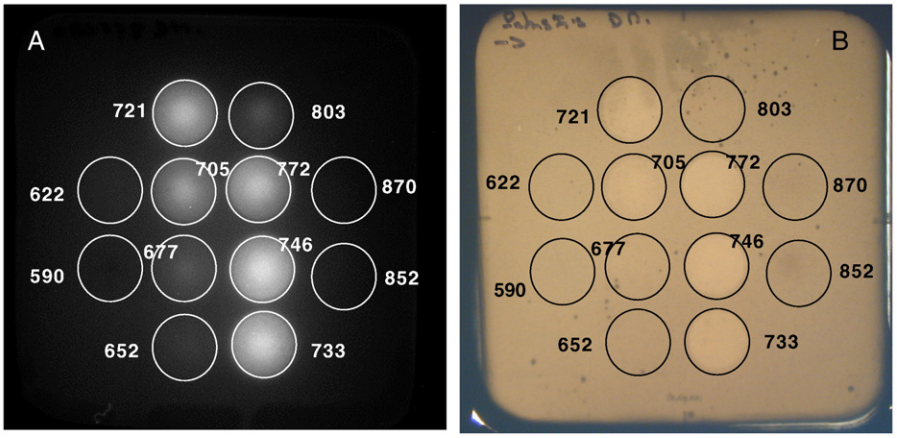

Fig. 2. Effect of light of various wavelengths on the bacteriochlorophyll fluorescence (Part A) and light transmission (Part B) properties of *Rps. palustris* cells. The cells were homogeneously inoculated on a Petri dish and subjected to illumination from 590 to 870 nm in different area (3.5 cm^2) delimited by circles. Irradiance was adjusted to 15 μmol of photon/s/m^2 and the half-peak bandwidth was 25 nm for all wavelengths.

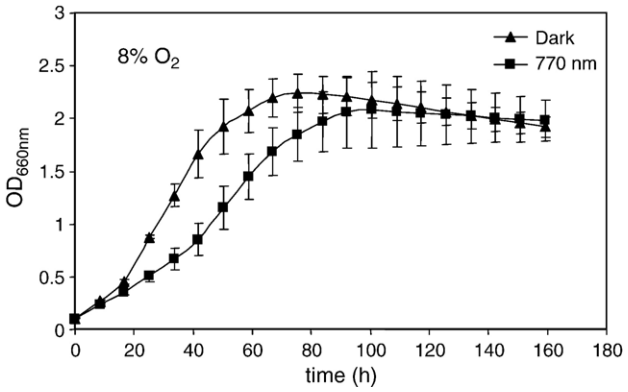

Fig. 3. Growth curves of *Rps. palustris*. Cells were grown in the dark (triangles) or under 770 nm illumination (squares) at 8% of oxygen tension. The curves are the average of three experiments. Bacterial cultures were inoculated at $OD_{660\ nm}$=0.1 with bacteria grown in the dark at 21% oxygen for 60 h.

The suggestion that the photoreceptor responsible for the growth rate limitation is a bacteriophytochrome and not the photosystem bacteriochlorophyll molecules (Fig. 2) was confirmed by the normal growth rate observed for cells subjected to an 875-nm illumination, specifically absorbed by LH complexes, in liquid medium (Fig. 4B). It is important to emphasize at this point that the 770-nm light used in these experiments is not intense enough to produce a significant photosynthetic activity. Photosynthesis could occur only for cells subjected to an additional strong 875 nm light. Under this last condition (770 plus 875 nm), the growth rate limitation induced by the 770-nm light is overcome by the 875-nm illumination, i.e. photosynthetic activity (Fig. 4B). These observations prompted us to test whether the 770-nm induced growth rate decrease could be due to an impaired respiration, the only bioenergetic pathway utilizable for the bacteria to develop under 770 nm illumination and to identify which bacteriophytochrome is involved in this growth rate limitation.

3.3. A respiration restriction is responsible for the 770-nm induced growth rate decrease

In order to test whether the 770-nm induced growth rate decrease is due to a restriction in respiration, the respiratory activities of bacteria grown under different illumination conditions were assessed during bacterial growth. A small but significant decrease of the respiratory activity is observed during the exponential growth phase for both dark-grown bacteria or bacteria grown under 770 nm light (Fig. 5A). But the most striking result is the much lower respiratory activity, for the same numbers of cells, of bacteria grown under 770 nm light compared to dark-grown bacteria all along the first 40 h of growth (exponential and beginning of stationary phases). The difference in respiratory activity observed between these two growth conditions is maximal during the mid-exponential phase reaching a 2-fold difference at 21 h of growth (Fig. 5A). We have also determined the amount of photosystem synthesized in these different growth conditions. Large quantities of photosystem are rapidly synthesized in cells grown under 770 nm light (Fig. 5B). In contrast, dark-grown bacteria synthesized a small amount of photosystem (equivalent to 20–30% of the amount produced by 770 nm grown bacteria) only after the mid-exponential phase (Fig. 5B). This is probably related to the lowering in oxygen tension linked to the respiratory activity of the dense cells suspension. In any case, these results suggest a clear correlation between the decrease in respiratory activity and the synthesis of the photosynthetic apparatus.

To gain further evidence for this correlation, bacteria were subjected to dark–light transitions and analyzed for their respiratory activity and photosystem content. Bacteria grown in the dark until the mid-exponential phase and then illuminated with far-red light, present a well-marked decrease in respiratory activity upon illumination (53±5% decrease after 22 h of growth) and their respiratory activity reaches the respiratory level of 770 nm illuminated bacteria around 41 h of growth. Conversely, the respiratory activity of bacteria subjected to a

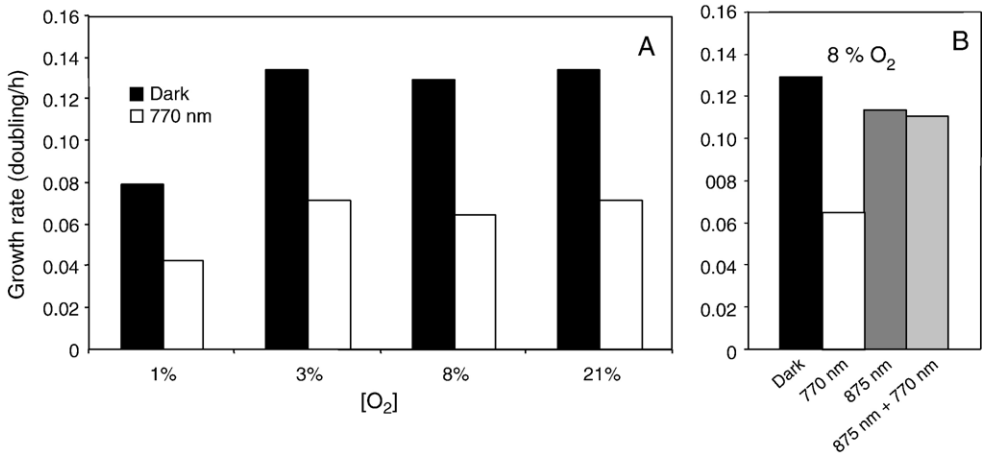

Fig. 4. Growth rate of *Rps. palustris* in function of illumination and oxygen tension. (A) Growth rate of cells grown in the dark (black bars) or under 770 nm illumination (white bars) at various O_2 tensions. (B) Growth rate of cells grown at 8% O_2 tension in the dark (black bar), under 770 nm (white bar), 875 nm (grey bar) or 875 plus 770 nm (light grey bar) illuminations.

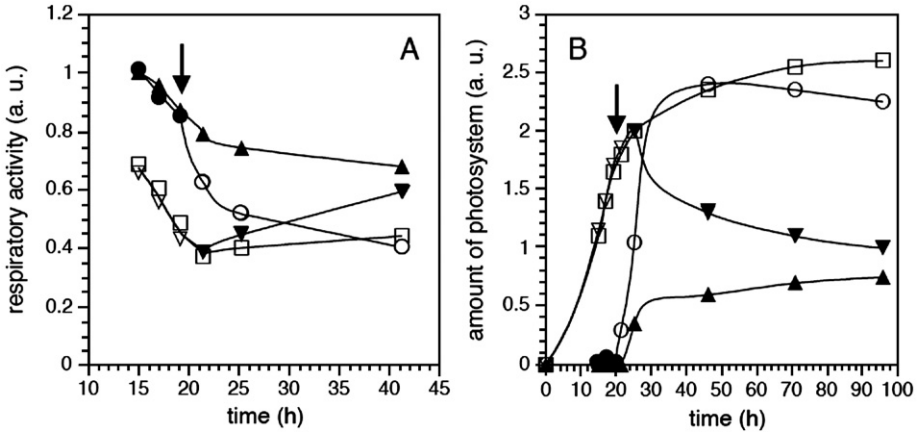

Fig. 5. Respiratory activity and photosystem synthesis during growth in function of illumination under 8% oxygen tension. Cells were grown in the dark (filled triangles), under 770 nm illumination (empty squares), under dark (filled circles) followed by 770 nm illumination (empty circles) or illuminated by 770 nm light (empty reversed triangles) followed by dark conditions (filled reversed triangles). The switch from dark to light (or light to dark) happens at 19 h of growth and is indicated by an arrow. All measurements were normalized to the same cell density. (A) Relative respiratory activity during growth in function of illumination. The results are expressed as the respiratory activity obtained at various times and under different illuminations relative to the respiratory activity of the dark-grown bacteria at 15 h of growth taken equal to 1. This respiratory activity is of about 39 μM O_2/min. (B) Photosystem synthesis during growth in function of illumination. The amount of photosystem corresponds to the area of the bacteriochlorophyll absorption bands in the near infra-red. All measurements are between 5 and 7% interval confidence.

switch from 770 nm illumination to darkness increased about 30±5% after 41 h of growth, reaching a level nearly similar to the one measured for bacteria that were continuously under dark condition. These changes in respiratory activity are correlated to changes in photosystem synthesis. Bacteria grown in the dark until mid-exponential phase (19 h) and then submitted to 770 nm light rapidly produced large amounts of photosystem, reaching the level equivalent to continuously illuminated bacteria within 27 h of growth (Fig. 5B). Conversely, bacteria grown under 770 nm illumination during 19 h and then placed in the dark stopped producing photosystem within 6 h (Fig. 5B). Since the maximal effect of light on respiratory activity is observed in the mid-exponential phase (Fig. 5A), all following experiments were performed with cells collected at this stage.

When bacteria were grown under both 770 nm light (to induce photosystem synthesis) and strong 875 nm light (to perform photosynthetic activity), the respiratory activity of bacteria is about 2-fold decreased compared to the dark control, i.e. similar to effect observed for bacteria illuminated with only the 770-nm light (Fig. 6). Moreover, their growth rate is significantly higher than that of cells subjected to only 770 nm light and represents more than 85% of the dark control (Fig. 6). This indicates that the respiration limitation induced by the 770-nm illumination is overcome by the photosynthetic activity provided by the 875-nm actinic light.

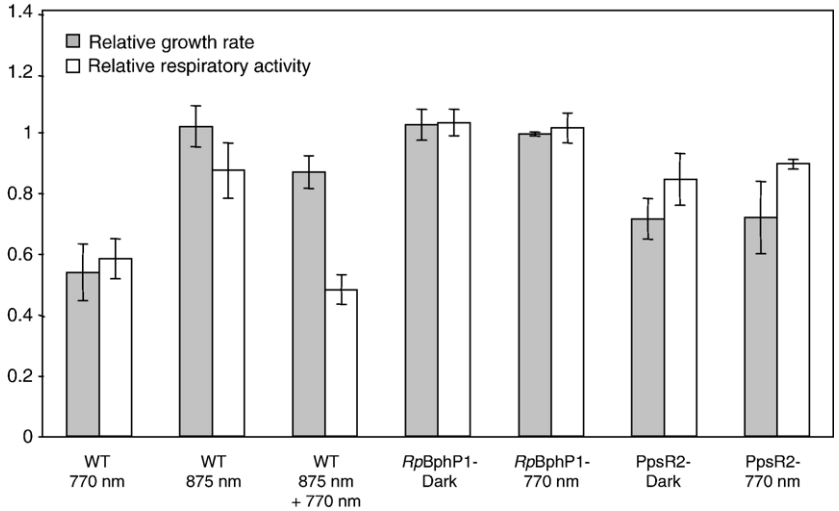

Fig. 6. Growth rate and respiratory activity of WT and mutants of *Rps. palustris* in function of illumination under 8% of oxygen tension. The results are expressed as the growth rate or respiratory activity of the considered strain relative to the growth rate or respiratory activity of the dark-grown WT strain. Each ratio is an average of at least three experimental replicates. The average dark-grown WT strain growth rate is of 0.12±0.2 (corresponding to a doubling time of 8.26 h±1.4). The dark grown WT strain respiratory activity is of about 39 μM O_2/min for bacteria in exponential phase (OD_{660nm} ~0.7 corresponding to about 2.6×10^9 bacteria/ml).

From this series of experiments we conclude that 770 nm light absorbed by the Pfr form of a bacteriophytochrome induces a decrease of about two-fold in respiratory activity. This regulation is reversible and concomitant to the synthesis of the photosynthesis apparatus via the action of *Rp*BphP1 [10,15,22]. This leads to a significant slowing of growth when respiration is the sole available bioenergetic pathway. We cannot exclude that in addition to the decrease in respiratory activity a small part of the growth limitation is due to the burden linked to the synthesis of the photosynthetic apparatus.

Is the same bacteriophytochrome responsible for both the enhancement of the synthesis of the photosynthetic apparatus and the limitation of respiration? Or are two different bacteriophytochromes involved in this dual regulation? This also raises the question of the participation of the transcriptional regulator PpsR2, known to be involved, with *Rp*BphP1, in the regulation of photosystem synthesis. These questions are addressed below.

3.4. The bacteriophytochrome RpBphP1 and the transcription factor PpsR2 are involved in the respiration limitation

Out of the six bacteriophytochromes present in *Rps. palustris*, only three, *Rp*BphP1, *Rp*BphP5 (Rpa0122) and *Rp*BphP6 (Rpa0990) are under their Pfr form after dark adaptation and are therefore susceptible to be activated by 770 nm illumination [41]. Therefore, we measured the growth and the respiratory activities as a function of illumination for *Rps. palustris* mutants inactivated in these three bacteriophytrochromes. The *Rp*BphP5$^-$ and *Rp*BphP6$^-$ mutants showed the same behavior as the wild type strain CEA001: 770 nm illumination induced an important decrease both in the growth rate and the respiratory activity compared to dark growth conditions (not shown). On the contrary, the *Rp*BphP1 mutant had the same growth and respiratory activity as the dark-grown WT strain independently of the illumination conditions (Fig. 6). We therefore conclude that only the bacteriophytochrome *Rp*BphP1 is involved in the light-induced respiration limitation. Since the level of respiratory activity is similar for the 770-nm illuminated *Rp*BphP1 mutant and for WT cells grown in the dark, we conclude that *Rp*BphP1 is a repressor of the respiratory activity. A similar experiment was performed with a mutant inactivated in the transcription factor PpsR2, implicated in the *Rp*BphP1 transduction pathway [10,15,22]. Compared to dark-grown cells, illumination affects neither the growth rate nor the respiratory activity in the PpsR2$^-$ mutant (Fig. 6). This implies that both *Rp*BphP1 and PpsR2 are implicated in the regulation of the respiratory activity. The growth and respiratory levels of the PpsR2$^-$ mutant grown in the dark are lower, by about 30% and 15% respectively, than for the WT strain (Fig. 6) indicating that this transcriptional factor could be an activator of the respiration activity. We therefore propose that the light-induced Pr form of *Rp*BphP1 not only enhances the synthesis of the entire photosynthetic apparatus by counterbalancing the repressive effect of PpsR2 [10,15,22] but also down-regulates the respiratory activity by antagonizing the activation of this transcriptional factor on the respiratory activity. The points where this regulation on the respiratory activity is exerted are addressed in the following paragraphs.

3.5. Decrease in respiratory activity and expression of related genes

Since the transcription regulator PpsR2 is involved in respiration decrease, we searched in silico for genes of *Rps. palustris* that could be affected by this regulator. It has been established that PpsR binds to two adjacent palindromic motifs $TGTN_{12}$-ACA [14,42]. Some variations in the palindromic sequences are possible depending upon the considered species, except for the G and C nucleotides, which appear strictly conserved [40]. A first class of PpsR binding sites consists of two palindromic motifs spaced by 7 or 8 base pairs [43]. For a second class of PpsR, the two palindromic sequences are spaced by a large number of base pairs up to 240 bp as for example in the case of the *pucBA* promoter of *Rb. capsulatus* [44]. In order to identify all the genes potentially controlled by PpsR, we first looked for one strictly conserved motif $TGTN_{12}ACA$ in each promoter region (500 bp) of *Rps. palustris* strain CGA009 genes. 238 genes presenting one such conserved motif in their promoter region were identified. We further searched for an additional possibly degenerated palindromic sequence ([T,C]G[A,T]N_{12} [T,A]C[G,T,A], the letters separated by a comma meaning an alternative) [40] spaced by 2 to 300 bp around these previously identified palindromes. This leads to 104 genes potentially regulated by PpsR (see Table 1 supplementary material). Some of them, located in the Photosynthetic Gene Cluster (PGC), are known to be regulated by PpsR, in *Rps. palustris* CEA001 [15] and CGA009 [16,22], in the closely related bacterium *Bradyrhizobium* ORS278 [18], in *Rb. sphaeroides* [32], and in *Rb. capsulatus* [43,44]. Several genes encoding complexes involved in the respiratory chain or an enzyme of the Krebs cycle are of particular interest: (i) *rpa0831 (coxB)* encoding the subunit II of the cytochrome oxidase aa_3, (ii) *rpa1535* (*cycA*) encoding the cytochrome c_2, (iii) *rpa4263 (nuoB2)* encoding the NADH-ubiquinone dehydrogenase chain B, a subunit of one of the two NADH-dehydrogenases of *Rps. palustris*, (iv) *rpa 2938* (*nuoM1*) encoding the NADH-ubiquinone dehydrogenase chain M, a subunit of the other NADH dehydrogenase of *Rps. palustris*, and (v) *rpa0189* (*sucA*) encoding the E1 component of the alpha-ketoglutarate dehydrogenase complex (KGD), which catalyzes a key reaction in the Krebs cycle. The promoter region of these various genes are shown in Table 2 of supplementary material.

The regulation of the synthesis of these various complexes and enzymes could therefore be responsible for the 2-fold decrease in respiratory activity via *Rp*BphP1 and PpsR2. We first examined the expression of the enzymes involved in the entry (NADH-ubiquinone dehydrogenase) and the output (cytochrome oxidase) of electrons in the respiratory chain by enzymatic activity assays or quantitative real time RT-PCR measurements.

Difference in the cytochrome oxidases expression for cells grown either in the dark or under 770 nm illumination in exponential phase was probed by measuring their respiration rate. These measurements were performed in the presence of saturating concentration of tetramethylparaphenylene diamine (TMPD), an efficient electron donor to cytochrome oxidases. This procedure overcomes the possible limitation of the respiratory activity

upstream these enzymes. Under such conditions, the levels of respiratory activity are identical for the two growth conditions (data not shown) indicating that the limitation of the respiration activity induced by the 770-nm illumination is not occurring at the cytochrome oxidases level. Looking at the expression of electron carriers upstream the cytochrome oxidases, measurements by real time RT-PCR (average of 3 to 5 experiments) revealed that the expression of *rpa1535*, encoding the cytochrome c_2 was slightly up-regulated (1.2 fold±0.3) by 770 nm illumination (Fig. 7A). Similarly, the expression of *rpa2938* and *rpa4263,* encoding subunits of the two NADH dehydrogenases, were up-regulated 2.5±0.4 and 2.1±0.5 fold respectively (Fig. 7A). These results do not explain the 770-nm induced respiration limitation and will be discussed later. For comparison, as expected, the expression of *pufB* (*rpa1525*), a membranous component of the photosynthetic chain encoding the beta chain of the LH1, is strongly up-regulated (up to 13fold, not shown) under 770 nm illumination.

Looking now at the enzyme involved in the Krebs cycle, the expression of *sucA*, measured by real time RT-PCR, was about 30% down-regulated during the mid-exponential phase (~22 h of growth) and the beginning of the stationary phase (~39 h of growth) under 770 nm illumination compared to dark-grown cells (Fig. 7A). Such difference in *sucA* expression was also observed in the beginning of the stationary phase (39 h of growth). To confirm the alpha ketoglutarate dehydrogenase complex (KGD) down-regulation under 770 nm light, this activity was tested for membrane fractions and checked for its sensitivity to the addition of 1 mM arsenite as described by Beatty et al. [36]. KGD complex activity is 35%±6% down-regulated in 770 nm illuminated bacteria versus dark-grown bacteria on the cell number basis, in agreement with the results obtained in quantitative real time RT-PCR.

To provide further proofs of the involvement of PpsR2 in the regulation of *sucA* expression we tested by mobility shift assay its capability to bind to the *sucA* promoter region. As shown in Fig. 7B, PpsR2 binds to *sucA* promoter in agreement with our in silico analysis. This result strongly suggests that PpsR2 activates *sucA* transcription in the dark. This activation is partially repressed under 770 nm illumination via *Rp*BphP1.

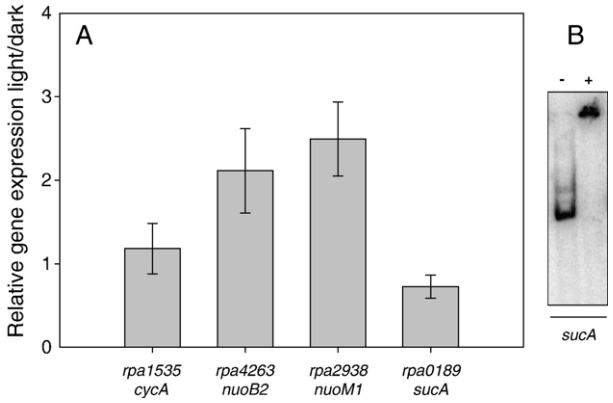

Fig. 7. (A) Relative gene expression of *cycA*, *nuoB2*, *nuoM1* and *sucA* under 770 nm illumination compared to dark growth conditions. Gene expression was normalized using the control transcript of *rpoD*. The results are the average of three to five independent experiments. (B) Gel mobility shift assay with purified PpsR2. The left lane (−) contained only ^{32}P-labeled *sucA* promoter region, while the right lane (+) contained in addition 14 µg of PpsR2.

4. Discussion

Our study demonstrates a dual effect of the bacteriophytochrome *Rp*BphP1 on the bioenergetic status of *Rps. palustris*. Activation of *Rp*BphP1 with 770 nm light induces, on the one hand, the synthesis of the photosynthetic apparatus and limits the respiratory activity on the other hand as discussed in more details below.

4.1. Activation of the photosystem synthesis

*Rp*BphP1 plays an essential role in the light activation on the photosystem synthesis in a large range of O_2 tension ranging from 21% down to 1% (Fig. 1). As already reported [10], this activation is due to the antagonizing effect of *Rp*BphP1 on the repression exerted by PpsR2 under aerobic condition on the transcription of several genes involved in pigments and photosystem synthesis (i.e. *bch*, *crt* and *puf*) [15,16].

In addition to the formation of the photosynthetic apparatus, we found that the *nuoB2* and *nuoM1* genes of the two NADH dehydrogenases of *Rps. palustris* are up-regulated more than two fold under 770 nm illumination. This two-fold increase in mRNA level, which may result in an increase of the NADH dehydrogenase enzymatic activity, might seem at odds with the limitation of the respiratory activity observed under the same illumination condition. It should be recalled, however, that these enzymes are not only involved in the respiratory pathway but are also essential for photosynthesis. Indeed, under photosynthetic activity the proton motive force formed by the light-induced cyclic electron transfer is responsible for a “reverse” electron flow (i.e. reverse with respect to the respiratory pathway) between the reduced quinone pool and NAD^+ at the level of NADH dehydrogenase [6,7,45]. The reducing power produced during this electron transfer is essential for biosynthesis syntheses and carbon assimilation. Therefore it is not surprising that the switch to photosynthetic conditions is accompanied by an enhanced synthesis of NADH dehydrogenases. A similar explanation can be proposed for the slight up-regulation (1.2 fold) we observed for the transcription of genes encoding the cytochrome c_2 because this electron carrier is involved in the photosynthetic chain.

4.2. Limitation of the respiratory activity

As discussed above, the limitation in respiratory activity leading to a decrease in growth rate under 770 nm illumination is not related to changes in the amount of the membrane or soluble electron carriers complexes of the respiratory chain. We propose that the down-regulation of the KGD complex activity is most probably responsible for the observed decrease in respiratory activity. The regulation of the Krebs cycle is essentially controlled at the KGD level. Such a control is exemplified in the case of the photosynthetic bacterium *Rb. capsulatus*, which presents 10-fold more KGD activity when grown aerobically

than photosynthetically [46]. Indeed under aerobic conditions, cells development requires an active Krebs cycle to produce reducing equivalents (NADH) for ATP synthesis by oxidative phosphorylation [46,47] and biosynthesis intermediates, such as alpha-ketoglutarate, oxaloacetate and succinyl-CoA. Due to the production of reducing power under photosynthetic activity, the Krebs cycle activity is only required for the generation of biosynthesis precursors [46,47]. A decrease of the KGD complex activity limits the overall Krebs cycle and consequently the respiratory activity, which is dependent on the reducing power produced by this cycle [47,48]. Our proposal is that the decrease in KGD activity observed for cells placed under 770 nm illumination induces a decrease in respiration and a concomitant limitation of the growth rate since respiration is the only bioenergetic pathway utilizable for the bacteria to develop under this condition.

The KGD expression is modulated by both *Rp*BphP1 and PpsR2. The lower respiratory activity of the PpsR2^{-} mutant compared to the WT and its ability to bind to the promoter of *sucA* suggests that this transcriptional factor acts as an activator of *sucA* transcription in the dark. This activation is repressed under far-illumination by the action of *Rp*BphP1. PpsR2 would therefore be both a transcriptional repressor (of the photosynthetic genes) and an activator (of *sucA*). A dual role of PpsR transcriptional regulators was already observed in *R. gelatinosus* where PpsR acts as a repressor of *crtI* expression and as an activator of *pucBA* expression [17]. In the case of *Rps. palustris*, PspR1 has also been reported to be both a repressor of photosystem synthesis [16] and an activator of LH2 antennae [49]. In the closely related species *Bradyrhizobium* ORS278, the two PpsRs have antagonistic roles, PpsR2 being a repressor of the photosynthetic genes whereas PpsR1 seems to act as an activator of the photosystem formation [18]. Altogether these studies suggest that the dual role of PpsR could be a general characteristic of this regulator family.

4.3. Photosynthesis versus respiration

Light absorbed by the photosynthetic apparatus is known to strongly inhibit the respiratory activity in photosynthetic bacteria [4]. This inhibition is due to interactions between the photosynthetic and respiratory chains present in the intracytoplasmic membrane. A first indirect interaction is linked to the proton motive force, produced by the light-induced cyclic electron transfer. This light-induced proton motive force initiates a "reverse" electron flow from the reduced quinone to NAD^{+} via the NADH dehydrogenases as already mentioned. A direct interaction occurs at the level of the cytochrome c_2, which reduces the photooxidized reaction center preferentially to the cytochrome oxidases [1]. Here we reveal an additional regulation by light, via the bacteriophytochrome *Rp*BphP1, of the alpha-ketoglutarate dehydrogenase enzymatic complex, a key enzyme of the Krebs cycle. This system allows the integration of the two essential parameters, light and oxygen, for the bacteria to adapt to their environment. On the one hand, *Rp*BphP1 detects the presence of light and induces the synthesis of the photosynthetic apparatus even under aerobic conditions. On the other hand, the light activation of *Rp*BphP1 decreases the synthesis of KGD, which limits the production of reducing power and a concomitant decrease in the respiratory activity. This dual regulation at the transcriptional level favors the photosynthetic activity over the respiratory activity, i.e. the most favorable bioenergetic process in the light.

The repression of the respiratory activity via *Rp*BphP1 is far to be total and does not exceed a factor of 2. Similarly, the repressive effect of O_2 on the synthesis of the photosynthetic apparatus in the dark is incomplete at low tension. The strategy of maintaining a low level of both photosynthetic and respiratory chains allows the bacteria to rapidly switch between their two related bioenergetic processes for a fast adaptation to changes in their environment. The additional predominance of photosynthesis upon respiration at the electrons carriers level ensures an optimal use of their available energy [1].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2007.09.003.

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