Studies on a bacterial photosensor

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Chapter 3
Light-induced motility and adaptation responses in
Rhodobacter sphaeroides

The computer-assisted single-cell motion analyses and the methylation assays described in this chapter have been carried out at the Department of Microbiology and Molecular Genetics, University of Texas Medical Center, Houston, USA, in the laboratory of Prof. dr. J. L. Spudich with support of a collaborative research grant No. 960237 from NATO to J.L. Spudich and SIR-travel grant 14-1779 from the Dutch Organization for Scientific Research (NWO) to R. Kort.
3.1 Physiological and genetic characterization of blue-light responses

The recent identification of a photoactive yellow protein gene (\(pyp\)) in the well-characterized bacterium \(Rb. sphaeroides\) incited us to investigate the \textit{in vivo} role of this photosensor, which is proposed to mediate a photophobic tactic response towards blue-light. We identified a blue-light photophobic response in swimming \(Rb. sphaeroides\) cells and characterized this response by computer-assisted motion analysis. Subsequently, mutants of \(Rb. sphaeroides\) were constructed, where the \(pyp\) gene was deleted or inactivated by insertion of an antibiotic resistance cassette. Neither of these mutants was affected with respect to its blue-light response. In addition, these mutants showed wild-type like blue-light induced release of methanol groups, indicative for demethylation involved in adaptation. These results indicate that the blue-light responses analyzed so far are mediated by a blue-light photosensor other than PYP. To further investigate the role of PYP, we carried out a Northern analysis, which suggests that the \(pyp\) gene is co-transcribed with a \(pel\) gene. The latter gene has been proposed to be involved in the 4-hydroxy cinnamic acid chromophore activation. In addition, the presence of two large \(pyp\) transcripts (~15 and ~25 kb) may indicate the involvement of a relatively large number of genes in the photosensory system mediated by PYP.

Introduction

The photosensor photoactive yellow protein (PYP) is a 14 kDa water-soluble protein, which has been purified from a number of halophilic purple bacteria, including \textit{Ectothiorhodospira halophila}, \textit{Rhodospirillum salexigens} and \textit{Chromatium salexigens} (Koh \textit{et al.}, 1996; Meyer, 1985; Meyer \textit{et al.}, 1990). After absorption of a blue photon, PYP displays a photocycle that resembles that of the archaebacterial sensory rhodopsins (Hoff \textit{et al.}, 1994c; Meyer \textit{et al.}, 1987). The chromophore in PYP is 4-hydroxy cinnamic acid, which is covalently linked to the unique cysteine of the protein via a thiol ester bond (Baca \textit{et al.}, 1994; Hoff \textit{et al.}, 1994a). The identification of this new chromophore and the presumed wide distribution of this type of chromoprotein among eubacteria (Hoff \textit{et al.}, 1994b) led to the proposal to group all photoactive yellow proteins into a new protein family, 4-hydroxy cinnamic acid-containing photosensory proteins, the xanthopsins (chapter 2), in addition to the retinal-containing rhodopsins in archaeabacteria. The crystal structure of PYP has been solved at 1.4 Å resolution (Borgstahl, Williams & Getzoff, 1995), showing an \(\alpha/\beta\)-fold that has been hypothesized to be the structural basis of a PAS-domain (Pellequer \textit{et al.}, 1998). This domain is present in a large set of multidomain protein sensors and transcription factors involved in signal transduction. The intrinsic photoactivity of PYP makes this protein an excellent model system for time-resolved X-ray crystallography and NMR studies. These studies provide structural information about photocycle intermediates, formed on the nanosecond to millisecond time scale, after reaction initiation (Genick \textit{et al.}, 1997; Perman \textit{et al.}, 1998; Rubinstein \textit{et al.}, 1998).

The detailed insight in the photocycle of PYP and its associated structural changes upon light absorption, is in strong contrast with the poor understanding of its physiological role in the living cell. Studies on phototactic behavior of \textit{E. halophila} revealed a light-induced increase in reversal frequency of swimming cells, with a wavelength dependence that matches the absorbance spectrum of PYP (Sprenger \textit{et al.}, 1993). This observation led to the hypothesis that PYP acts as a photosensor for phototaxis, mediating this blue-light repellent response. In 1996, the \(pyp\) genes from \textit{E. halophila} and \textit{Rs. salexigens} were cloned (chapter 2.1), allowing in principle the experiments to obtain the genetic proof for this presumed function of PYP by gene replacement in one of these organisms. Since \textit{E. halophila} shows, in contrast to \textit{Rs. salexigens} (Sprenger and Hellingwerf, unpublished observations), a clear phototactic blue-light response (Sprenger \textit{et al.}, 1993), this purple sulfur bacterium
was initially chosen as the candidate to obtain this genetic proof. However, the development of a genetic system in *E. halophila* has been hampered by (i) its low growth rate (colonies on plates appear only after 2 weeks) (ii) the need for strictly anaerobic conditions in the light during growth, (iii) the inefficiency of many antibiotics at high salt concentrations and (iv) the instability of tested plasmids (IncPα incompatibility group), needed to test the feasibility of markers and for the complementation of mutants. In spite of their large difference in tolerance against low and high salt concentrations, conditions could be found for mating experiments of *E. coli* and *E. halophila* and conjugal transfer of DNA from *E. coli* to *E. halophila* has been demonstrated by Southern blot analyses (Hoffer, Hellingwerf & Kelly, unpublished observations). Lowering the salt concentration in general increased the susceptibility of *E. halophila* for antibiotics, a trend also evident from previous studies with other, moderately halophilic bacteria (Coronado et al., 1995). In agreement with our observations in *E. halophila*, it was demonstrated that IncPα plasmids are not stably maintained in the closely related purple sulfur bacterium *Chromatium vinosum* (Pattaragulwanit & Dahl, 1995). However, an IncQ vector was stable in this bacterium, making this type of plasmid the perfect candidate for future attempts to express genes in *E. halophila*.

Additional evidence about the function of *E. halophila* PYP may be obtained by sequencing and subsequent analysis of flanking regions of the *pyp* gene, since functionally related genes are often located in each other’s vicinity in prokaryotes. The analysis of these regions however, did not lead to the identification of genes encoding additional components of a photosensory signal transduction system. This indicates an organization of genes that is different from that in the well-studied photosensory system of *Halobacterium salinarum*, where the genes encoding the two photosensors are directly downstream from the genes encoding their corresponding transducer (Yao & Spudich, 1992; Zhang et al., 1996). Instead, upstream from *pyp*, a gene (*dada*) was found that encodes a protein that is homologous to the small subunit of the membrane-bound iron-sulfur flavoenzyme D-4-amino acid dehydrogenase (37% identity, 61% similarity) (Baca et al., 1994). Directly downstream from *pyp*, a gene (*pcl*) was identified that encodes a protein homologous to CoA ligases (20-26% identity, 45-52% similarity) (chapter 2.1). The latter finding led to the proposal that this putative *E. halophila* protein could function as a 4-hydroxy cinnamyl CoA ligase, activating the 4-hydroxy cinnamic acid chromophore of PYP by CoA esterification, before covalent linkage to the apoprotein (chapter 2.1).

Recently, we identified a *pyp* gene in the well-characterized purple non-sulfur bacterium *Rh. sphaeroides*, by screening chromosomal DNA from a number of microorganisms by sequencing synthesized DNA fragments, resulting from a polymerase chain reaction with specifically designed oligonucleotides (chapter 2.1). *Rh. sphaeroides* is a non-halophilic anoxygenic photosynthetic bacterium, which also grows under aerobic conditions in the dark, and for which the genetic techniques for inactivation and expression of genes have been well-established (Donohue & Kaplan, 1991). Thus, the identification of this *pyp* gene shifted our focus from *E. halophila* to *Rh. sphaeroides* as the most suitable organism to prove the physiological function of PYP. The *Rh. sphaeroides* *pyp* gene was cloned and sequenced and putative genes were identified upstream and downstream from *pyp* (chapter 2.2). This sequence analysis revealed two points of primary interest. First, a *pcl* gene is present in *Rh. sphaeroides* 1 kb downstream of *pyp*. This putative gene encodes a protein that is most similar to the PCL homologue from *E. halophila* (36% identity, 55% similarity). This finding makes a functional correlation between this gene and *pyp* more likely. Second, an open reading frame downstream from *pyp*, designated *orfF*, encodes a putative membrane-spanning protein that shows homology to sensory rhodopsin I from *Halobacterium salinarum* (22% identity, 47% similarity), be it that the retinal-binding site sequence is not conserved in OrfF. Besides the function of *pyp*, also those of *pcl* and *orfF* have been investigated in this study, by Northern analysis and gene disruption, respectively.

In order to characterize the photoactive
yellow protein from *Rhodobacter sphaeroides*, the *pyp* gene was inserted into the vector pQE30 for overexpression in *E. coli*. In contrast to results obtained for *E. halophila* PYP (chapter 2.1), this approach did not lead to the overproduction of high amounts of *Rhodobacter sphaeroides* apoPYP. Only very small amounts of recombinant protein were detected in cell-free extracts of *E. coli*, which only could be identified by Western blots with a polyclonal antiserum raised against *E. halophila* PYP (Los, Kort & Hellingwerf, unpublished observations) These amounts were not sufficient to demonstrate the *in vitro* formation of a yellow-colored protein with activated chromophore, according to methods described by Imamoto et al., (1995). Nevertheless, we were able to demonstrate the presence of the 4-hydroxy cinnamic chromophore in cell-free extracts of *Rhodobacter sphaeroides* by capillary zone electrophoresis (chapter 2.2). We assume that this compound was released from PYP, present in the *Rhodobacter sphaeroides* extracts, during high-pH treatment, which is part of the chromophore-extraction procedure. The conservation of all amino acids in *Rhodobacter sphaeroides* PYP, that play an essential role in the chromophore binding pocket, is a second indication that the *Rhodobacter sphaeroides* *pyp* gene product is a 4-hydroxy cinnamic acid-containing chromoprotein.

Taxis in *Rhodobacter sphaeroides* (especially chemotaxis) has been subject of extensive studies over the last two decades and has been shown to deviate in many ways from the prototype system in enteric bacteria; for a recent review see Armitage & Schmitt (1997). A phototactic response has been reported in *Rhodobacter sphaeroides* WS8-N: the bacterium responds to a step-down in yellow-green light (530-600 nm) and to near infrared light in a background of red monitoring light (650 +/- 10 nm) by an increase of the stop or reorientation frequency, with adaptation taking 40 s (Grishanin, Gauden & Armitage, 1997). Several lines of evidence indicate that the photosynthetic apparatus is the primary photoreceptor for this response (Grishanin et al., 1997). Photoresponses to increases in light intensity, as reported here to be present in *Rhodobacter sphaeroides* RK1, have not been observed in the *Rhodobacter* strain WS8-N.

This study is aimed at the identification and characterization of blue-light responses in *Rhodobacter sphaeroides* and the subsequent genetic characterization of *pyp* mutants. Tracks of single cells were followed before, and during blue-light exposure, by computer-assisted motion analysis. In addition, adaptive demethylation was analyzed in these mutants by measuring release of methanol groups in blue-light exposed, intact *Rhodobacter sphaeroides* cells. The involvement of the photosensor photoactive yellow protein in these blue-light responses was investigated by the construction and analysis of two *Rhodobacter sphaeroides* mutants, which lack the *pyp* gene by insertional inactivation and deletion, respectively.

**Materials and Methods**

**Strains, plasmids and primers.** The bacterial strains, plasmids and primers used are listed in table 1. *E. coli* strains were cultured at 37°C in Luria Bertani medium. *Rhodobacter sphaeroides* strains were cultured at 30°C degrees under anaerobic conditions in the light (15 W/m²) in Sistrom's minimal medium A, supplemented with succinate as the carbon source (Sistrom, 1962).

**Construction of pyp mutants.** In order to make a *pyp* deletion, a 10 kb DNA fragment was amplified, using the plasmid pAMRO as the template, the oligonucleotides MIRJAM1 and MIRJAM2, annealing at the 5' and 3' ends of *pyp* in opposite directions, and the Expand PCR kit (Boehringer Mannheim). PCR was carried out in 30 cycles of 10 s denaturation at 94°C, 30 s annealing at 60°C and 600 s elongation at 68°C. The PCR-product was circularized by T4 DNA ligase after digestion with *Xba*I, and removal of template DNA with *Dpn*I, cutting only the *in vivo* methylated 5'-GAATTC-3' sites. The ligation mixture was transformed to *E. coli* and transformants were checked for the presence of a 10-kb plasmid, containing a unique *Xba*I site. This plasmid, designated pAMBI was retransformed to *E. coli* S17-1. In addition, the *pyp* gene was interrupted by insertion of a Km cassette into the unique Eco47III site, yielding pAMMI-980. Besides, a plasmid was constructed for inactivation of
the putative gene orfF by insertion of a PCR-product of 360 bp, from the 5' prime part of this 681 bp-gene lacking the initiation signals, in pSUP202. A single crossing-over event with this plasmid, designated to pAMMI-973, will lead to 2 inactive copies of orfF in the chromosome of Rb. sphaeroides. Approximately 10^10 cells of E. coli S17-1/pAMBI (conjugation 1), E. coli S17-1/pAMMI-980 (conjugation 2), E. coli S17-1/pAMMI-973 (conjugation 3) and E. coli S17-1/pLA2917 (conjugation 4) were incubated overnight at 32°C on Sistrom plates for conjugal transfer of plasmids to Rb. sphaeroides RK1, using a donor:recipient ratio of 1:10. Selection for trans-conjugants was carried out by transfer of cell mixtures to Sistrom plates containing Km (conjugation 2) or Tc (conjugations 1, 3 and 4). After incubation for three days at 30°C single Rb. sphaeroides colonies were visible on selective plates. A single Rb. sphaeroides Tc resistant colony, resulting from conjugation 1, was used as an inoculum for 2 cycles of growth in Sistrom medium (1:50 dilution) of 24 hours each without antibiotic pressure, to allow excision of the integrated plasmid.

Table 1. Bacteria, plasmids and primers used in this study.
Subsequently, cells were plated on Sistrom plates and screened for Tc-sensitivity. As a negative control in these experiments, matings with *E. coli* strain S17-1 were carried out, which does not harbor any plasmid. All mutants were further checked by Southern blots.

**Single-cell motion analysis.** *Rhodobacter sphaeroides* cells were cultured under anaerobic conditions in the light and analyzed at OD$_{660}$ ~0.8. The optical arrangement used in this study is described by Zacks et al., (1993) with a few modifications (figure 1). Cells were monitored by dark field microscopy with a 150 W tungsten-halogen lamp (Ushio Inc.), using infrared light and a 600 nm long-pass filter with a light intensity of $8.3 \times 10^3$ erg cm$^{-2}$ s$^{-1}$, determined with a Kettering Radiant Power Meter (Scientific Instruments). Blue-light photophobic stimuli were 3 seconds in duration and were delivered

![Figure 1. Optical arrangement for measurement of phototaxis and photophobic responses. Figure adapted from Zacks et al. (1993).](image-url)
via a HBO 103W/2 mercury short arc lamp (Osram) with use of 400, 450 and 500 nm broad band interference filters (+/- 20 nm). The surface of the photophobic light spot is smaller than the surface of the sensor of the light intensity meter. Thus, to overcome an underestimation of the light intensities used, a correction factor should be applied. The surface of the light spot was determined by exposure of a film to photophobic stimulating light. The radius of the light spot was 5 mm. The surface of the sensor of the light-intensity meter is 8 x 14 mm², resulting in a correction factor of 1.6. The corrected light intensities used were 1.1x10⁴ ergscm⁻²s⁻¹, 8.3x10⁴ ergscm⁻²s⁻¹ and 5.1x10⁴ ergscm⁻²s⁻¹ for 400, 450 and 500-nm light, respectively. The motion analysis system was run on a SPARC IPC workstation. The average linear speed (spd) was obtained by the combination of two data sets, in which the paths of single, motile cells were tracked for a period of 4 seconds. The first data set was obtained 1 second before the blue-light pulse and 3 seconds during the pulse and the second data set 1 second during the pulse and 3 seconds after the pulse, both with a frame rate of 15 frames per second (i.e 67 ms/frame). The settings used for the calculation of the centroids were: neighbor width/height 2/2, minimum number of pixels 1, maximum 4096; the settings for the calculation of the paths were: search mask size 15, minimum path duration 40, average minimum movement 1. All calculated paths were inspected and paths of immotile cells were removed with the path editor. About 150 paths, obtained from 10 independent recordings, were merged into a single file and used for the calculation of the average linear speed.

**Methanol release assay.** This assay was developed by Kehry, Doak & Dahlquist (1985). *Rh. sphaeroides* cells used for this assay were cultured under anaerobic conditions in the light (OD₆₅₀ = 0.6), with use of the RNeasy mini kit (Qiagen, Santa Clarita, CA). To determine the size of *pyp* transcripts and to check for co-transcription with the *pel* gene, 15 µg of *Rh. sphaeroides* RNA was equally divided over 3 slots and an RNA marker, ranging from 0.28 to 6.58 kb (Promega, Madison, WI), was loaded in a fourth slot of a 1% agarose gel, containing 6% formaldehyde. After electrophoresis for 3 hours at 30 V, the gel was divided in 2 parts. The first part, containing the marker and one of the RNA lanes, was stained with ethidium bromide and photographed for size-estimation. The second part, containing the 2 remaining RNA lanes, was blotted by capillary transfer on a Hybond-N filter (Amersham, Life Science Inc). After blotting, the filter was cut in 2 parts, with 1 lane of RNA each: one for probing with the *pyp* gene, the other for probing with the *pel* gene. Probes were obtained by PCR with oligonucleotides SPHR, SPHF for the *pyp* probe and PCLR, PCLF for the *pel* probe, yielding products of 180 bp and 616 bp, respectively (table 1). Labeling of these probes was carried out as described for Southern blotting. Hybridization was performed at 42°C and washing steps at high stringency were carried out according to standard methods (Sambrook et al., 1989).

**Southern blotting.** Chromosomal DNA was isolated as described (Sambrook, Fritsch & Maniatis, 1989). DNA was digested with *PstI* and fragments were separated on a 0.9% agarose gel. DNA was transferred by vacuum blotting to a nitrocellulose membrane. Labeling of the probes (50 ng DNA) was carried out for 3 hours at 37°C with a random hexanucleotide mixture (Boehringer Mannheim), priming the DNA polymerase reaction with use of Klenow, dNTPs and labeled α-³²P-dCTP (75 µCi). Unincorporated label was removed by purification with a Sephadex G50 column. Hybridization was performed at 65°C and washing at high stringency were carried out according to standard methods (Sambrook et al., 1989).

**Northern blotting.** RNA was extracted from *Rh. sphaeroides* cells, cultured under anaerobic conditions in the light (OD₆₅₀ = 0.6), with use of the RNeasy mini kit (Qiagen, Santa Clarita, CA). To determine the size of *pyp* transcripts and to check for co-transcription with the *pel* gene, 15 µg of *Rh. sphaeroides* RNA was equally divided over 3 slots and an RNA marker, ranging from 0.28 to 6.58 kb (Promega, Madison, WI), was loaded in a fourth slot of a 1% agarose gel, containing 6% formaldehyde. After electrophoresis for 3 hours at 30 V, the gel was divided in 2 parts. The first part, containing the marker and one of the RNA lanes, was stained with ethidium bromide and photographed for size-estimation. The second part, containing the 2 remaining RNA lanes, was blotted by capillary transfer on a Hybond-N filter (Amersham, Life Science Inc). After blotting, the filter was cut in 2 parts, with 1 lane of RNA each: one for probing with the *pyp* gene, the other for probing with the *pel* gene. Probes were obtained by PCR with oligonucleotides SPHR, SPHF for the *pyp* probe and PCLR, PCLF for the *pel* probe, yielding products of 180 bp and 616 bp, respectively (table 1). Labeling of these probes was carried out as described for Southern blotting. Hybridization was performed at 42°C and washing steps at high stringency were carried out according to standard methods (Sambrook et al., 1989).
Results

Selection of mutants. In conjugation 1 (see materials and method) Tc resistant colonies, resulting from a single crossing-over event, were obtained with a frequency of 1 conjugant per 4x10^8 recipients. One of these colonies was further cultured for two cycles without antibiotic pressure to allow excision of the plasmid for a pyp deletion. The resulting culture was diluted and spread on Sistrom plates, followed by screening for Tc sensitivity, yielding 3 out of 288 colonies. In conjugation 2 (for the insertional inactivation of pyp), selection for Km resistant colonies was followed by screening for Tc sensitive colonies, yielding 1 Tc sensitive colony out of 60 Tc resistant and Km resistant colonies, resulting in a final frequency for double crossing-over conjugants of 1 per 7x10^10 recipients. Conjugation 3, carried out for inactivation of ofr by a single crossing-over event, yielded Tc resistant colonies with a frequency of 1 per 2x10^10 recipients. Conjugation 4 with plasmid pLA2917, which can be considered as the positive control for conjugal transfer of DNA in this experiment, yielded 1 Tc resistant conjugant per 5 10^9 recipients.

Characterization of motility response. A step-up in blue-light in a background of infrared light causes a motility response in swimming Rb. sphaeroides cells, cultured under anaerobic conditions in the light (a single-cell track is shown in figure 2). The average linear speed and of 150 of these cell paths was determined (figure 3). The cells start to respond by a stop after a delay of 0.27 s, detailed inspection of the recorded response (15 frames per second) reveals the start of the blue-light pulse at frame 16 and a decrease of the average speed after frame 20. During this delay the speed of individual cells slightly increases, which may be due to the increase in light intensity, causing a higher proton motive force, affecting the swimming speed of cells (this is called photokinesis). After 1.27 s, the cells reach the lowest average speed. This is not caused by a decrease in swimming speed, but by the fact that the cells stop for reorientation (equivalent to tumbling in E. coli). After the pulse (duration 3 s), the cells start swimming again after a delay of 0.07 s. The cells recover to their pre-stimulus swimming speed. When the blue-light pulse duration is extended for several minutes, full adaptation to pre-stimulus level was never observed, but cells continued swimming with increased stop frequency during blue light exposure. When the 450 +/- 20 nm interference filter was replaced by a 500 +/- 20 nm interference filter, no motility response after a light step-up was observed. On the other hand, a step-up in light of 400 nm +/- 20 nm or white light (without any interference filter) did result in a stop response as well (data not shown). A decrease of the 450 nm +/- 20 nm pulse duration from 3 seconds to 1 second did not significantly affect the amplitude of the response (calculated by the slope of the plot between 1.3 and 2.3 seconds; see also figure 3), but a decrease to 100 ms did result in a reduction of the response to approximately 20%. Exposure of anaerobically cultured Rb. sphaeroides RK1 cells to oxygen also results in an increased stop frequency, as observed previously for Rb. sphaeroides WS8-N (Gauden & Armitage, 1995). No effect of blue-light on the motility of cells was observed, when the cells respond to oxygen, thus the cells need to be kept under anaerobic conditions.
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(100 W/m²) or low-light (3W/m²) intensity did not show significant changes in amplitude of the blue-light motility response. Cells grown at low-light intensities have an increased capacity of the photosynthetic light-harvesting apparatus. Thus, this result argues against a role for the photosynthetic pigment in this motility response. In contrast, the response to a decrease in photosynthetic light is strongly dependent on the capacity of the photosynthetic light-harvesting apparatus: cells grown at high-light intensities show photoreponses to a much greater range of step-down intensities than cells grown at low-light intensities, because photosynthesis remains saturated at relatively low-light intensities in low-light grown cells (Grishanin et al., 1997).

A determination of the blue-light response as a function of the light intensity with use of neutral density filters showed that, when stimulating light was reduced to 70% and 30%, the amplitude of the response was reduced to approximately 70% and 30%, respectively. Furthermore, it was observed that sometimes only part of the cell population responds and that sometimes, after a period of 10 to 15 minutes all the cells completely lose their response to blue-light, while the motility of these cells is not affected.

Genotypic analysis. A Southern blot with digested chromosomal DNA, probed with a

Figure 3. The effect of blue-light on the average speed of free-swimming *Rb. sphaeroides* cells as a function of time. Delay is 1 second, followed by 3 seconds step-up in blue-light. Background monitoring light is infrared. The figure shows an average of 150 single-cell tracks.
180 bp DNA fragment, which is part of the pyp gene, obtained by PCR with oligonucleotides SPHR and SPHF (table 1), confirmed the deletion of the pyp gene in all 3 Rh. sphaeroides Tc sensitive trans-conjugants (data not shown). A Southern blot with PstI digested chromosomal DNA, isolated from Rh. sphaeroides cells originating from 2 single Km , Tc colonies (genotypes pyp-A and pyp-B), showed that the 2.4 kb wild-type hybridizing signal with the pyp probe was absent in both mutants (figure 4A). Instead, these mutants showed two separate hybridizing bands of 2.8 and 0.9 kb, in accordance with the introduction of an additional PstI site via insertion of the Km cassette. The sum of the estimated size of the two hybridizing fragments observed in these mutants (3.7 kb) should be similar to that of the wild-type signal plus the Km cassette (3.8 kb). The blot, presented in figure 4A, was stripped and probed with the Tc resistance cassette (1 kb XmaIII fragment from pSUP202), as indicated in figure 4B, in order to be sure that no further plasmid DNA was integrated in the chromosome of these strains. Indeed, no signals were observed in the mutants pyp-A and pyp-B. The orff-A mutant however, shows a clear hybridizing signal in the blot in figure 4B, in agreement with the integration of the plasmid into the chromosome, which is the strategy for the inactivation of this putative orff gene. Hybridization with the orff probe (a 360 bp PCR-product obtained with primers ORFF and ORFFR; see table 1) shows the expected 2.8 kb signal in wild-type, and signals of 11.5 kb and 1.8 kb in the orff-A and orff-B mutants (figure 4C). This is in agreement with the expected size of the two PstI fragments, which both contain an inactivated copy of the orff gene. In addition to the analysis of these mutants, we checked several different Rhodobacter strains for the presence of the pyp gene with Southern blots. The corresponding results are listed in table 2.

Phenotypic analysis. The selected Rh. sphaeroides pyp mutants RK1PI and RK1DP were subjected to single-cell motion analysis, as described in materials and methods. No differences were observed: blue-light motility responses were virtually the same as those of wild-type cells under all conditions tested (see also above). In addition, the blue-light induced

Figure 4. Southern blots of PstI digested chromosomal DNA of Rh. sphaeroides RK1 wild-type and mutants, probed with A the pyp gene, B the tetracycline antibiotic resistance cassette and C orff.
release of methanol groups was tested in the two *pyp* mutants and was not found to be significantly different from that in *Rhodobacter sphaeroides* wild-type cells (figure 5). The inactivation of *orfF* (encoding a putative membrane-spanning protein) by plasmid integration did not lead to significant changes in the blue-light motility response either (table 2). Besides these mutants, we tested several *Rhodobacter* strains for the presence of a *pyp* gene by Southern blots. Surprisingly, we found that the motile strain WS8-N showed no signals, while the type strain 2.4.1 did show specific signals (the negative control was *Paracoccus denitrificans* chromosomal DNA in this experiment), which disappeared at high stringency (68°C, 0.1xSSC) (Gomelski & Kaplan, unpublished observations). In addition, among the three other purple bacteria known to contain *pyp*, only *E. halophila* shows a clear blue-light induced tactic response. It should be noted that the *Rs. saleigens* and *E. halophila* blue-light tactic responses were tested in an assay described by Sprenger *et al.* (1993), which is different from the assay described here. Among these three organisms, only *Rs. saleigens* is known to show blue-light induced release of methanol groups (data not shown).

**Northern analysis of *pyp* transcripts.** Two Northern blots, containing RNA from anaerobically grown *Rhodobacter sphaeroides* RK1 cells, were probed in two independent experiments with a *pyp* probe (figure 6A) and a *pcl* probe (figure 6B). Both blots show three transcripts of very similar sizes: 3 kb, ~15 kb and ~25 kb. RNA from *Rhodobacter sphaeroides* RK1 cells, cultured under aerobic conditions in the dark, does not show any signals, when probed with the *pyp* probe (data not shown).

**Discussion**

**Construction of mutants.** The highest conjugation frequencies (*i.e.* the number of trans-conjugants per number of recipients) were obtained for conjugation 4 with the *E. coli* S17-1/pLA2917 donor cells (the positive control). This high frequency (1 per 5x10⁴) can be explained by the notion that in this experiment one only asks for plasmid transfer

![Figure 5. Release of ³H-labeled methanol by *Rhodobacter sphaeroides* wild-type cells and *pyp* mutants, grown under anaerobic conditions in the light, after a step-up in blue light, starting at the position of the arrow. Each point shows the counts per minute (CPM) of a collected 0.5 ml fraction; the speed of the flow assay is 1 ml/min.](image-url)
Motility and adaptation responses

Motility and adaptation responses to *Rhodobacter sphaeroides* and subsequent expression of an antibiotic resistance gene, but not for any crossing-over events by homologous recombination. Subsequently, for single crossing-over events in conjugations 1 and 2 similar frequencies were obtained, 1 transconjugant per $4 \times 10^8$ recipients and 1 transconjugant per $10^9$ recipients, respectively. In conjugation 3 however, only 1 transconjugant per $2 \times 10^{10}$ recipients was obtained, while also here selection for a single-crossing over event took place. This can be explained by taking into account the length of homologous DNA for recombination. In conjugation 1 and 2, these lengths are ~1 kb on either sides of the (deleted) *pyp* gene, while in conjugation 3 only 360 bp of homologous DNA is available. Thus, as expected, the frequency of homologous recombination increases, when the length of the homologous DNA fragment is increased. The 360 bp-DNA fragment used for conjugation 3 was obtained by PCR with oligonucleotides, which were designed such, that (i) the initiation signals for translation were deleted (the ribosome binding site and the start codon), (ii) the 3’ half of the gene was deleted and (iii) that *orfF* was out of frame with the chloramphenicol acetyltransferase (*cat*) gene, present on pSUP202. This to make sure that a single-crossing over event would lead to two inactive copies of *orfF* on the chromosome and that there would be no active product, resulting from a fusion to the *cat* gene, transcribed from the integrated plasmid. Besides the risk of polar effects, the disadvantage of the gene disruption method in conjugation 3 is that culturing of mutants without antibiotic pressure could lead to excision of the plasmid, resulting in reformation of the wild-type genotype, while the other two methods lead to stable mutants.

The blue-light motility response. Strictly spoken, it is not correct to describe the blue-light response reported here as a phototactic response, because it has not been demonstrated that *Rhodobacter sphaeroides* cells migrate away from high blue-light intensities. Upon exposure of swimming *Rhodobacter sphaeroides* cells to blue-light, they stop, most probably followed by adaptation (see below). Would such a response lead to migration away from blue-light? The answer is probably not. For comparison one could consider the very similar motility response, displayed by swimming *Rhodobacter sphaeroides* strain WS8-N (as well as strain RK1; data not shown), towards a decrease in photosynthetic light (Grishanin et al., 1997). These cells respond under anaerobic conditions to a step-down of photosynthetic light by a transient stop, followed by adaptation. It was reported however, that swimming *Rhodobacter sphaeroides* WS8-N cells exposed to a light beam for a few minutes, accumulate outside the light beam in the dark (Sackett et al., 1997). For these photosynthetic bacteria this would not make sense, because they are dependent on light for growth under anaerobic conditions. Probably, in nature *Rhodobacter sphaeroides* does not face such strong changes in light intensity, and one could explain the accumulation in the dark by overreacting of the cells. This means that what should actually be an increase in stop or reorientation frequency, results in this case in a complete stop, leading to an unfavorable accumulation pattern. This could also be true.

![Figure 6. Northern blots of RNA extracted from anaerobically cultured Rhodobacter sphaeroides RK1, with use of probes against A the *pyp* gene and B the *pel* gene.](image-url)
for the blue-light motility response reported here.

So far, all Rb. sphaeroides tactic responses show adaptation on relatively long time scales. This seems also to be true for the blue-light motility response reported here. This complicates the observation of adaptation. Free-swimming cells cannot be tracked on these time scales, because they leave the image. Cells tethered to the glass (without the use of antibodies against flagellar filaments) do not provide a solution to this problem, because they may leave their temporarily fixed position after a while or become completely immotile. However, our impression is that cells continue swimming with short intervals (or a high stop frequency) during blue-light exposure. When blue-light is turned off, even after minutes of exposure, cells still show a decrease in stop frequency, indicating that the cells were not fully adapted. This may also be due to the strong change in light intensity, whereas more subtle changes may result in full adaptation on relatively short time scales.

The Rb. sphaeroides RK1 strain shows motility responses towards an increase in blue-light, as well as a decrease of photosynthetic light (this report), whereas strain WS8-N only responds to a decrease in photosynthetic light (Grishanin et al., 1997, table 2). Although the response to light of 450 nm was most pronounced, this wavelength cannot be unambiguously considered as \( \lambda_{\text{max}} \) for the motility response described here, since the light intensity decreases when a shorter-wavelength interference filter is used (see material and methods). As blue-light is also used for photosynthesis, Rb. sphaeroides RK1 shows an increase in stop frequency towards a decrease of blue-light (and other wavelengths of photosynthetic light), while it, as reported, also shows an increase in stop frequency towards high blue-light intensities. These two responses may bias the swimming pattern of Rb. sphaeroides RK1 towards the most favorable light climate for photosynthesis, avoiding radiation damage. The differences found among Rb. sphaeroides strains may be due to their needs, associated to their specific natural habitats or may be caused by the loss of physiological responses due to repeated sub-culturing under lab conditions in very rich media.

The involved photosensors. The finding that Rb. sphaeroides pyp mutants did not show any phenotype in blue-light motility response under all conditions tested, and that no changes were observed in blue-light induced release of methanol groups raises questions with respect to the nature of the photosensors involved in these responses, a topic which is addressed in this part of the discussion. First, the assumption is made that both the motility response and the methyl release response for a specific wavelength are mediated by the same photosensor (see also chapter 3.2). Second, it is known that the

<table>
<thead>
<tr>
<th>Strains</th>
<th>pyp gene</th>
<th>Motility response</th>
<th>Methanol release</th>
</tr>
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<tbody>
<tr>
<td>Rb. sphaeroides RK1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Rb. sphaeroides RK1PI</td>
<td>no</td>
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<td>yes</td>
</tr>
<tr>
<td>Rb. sphaeroides RK1DP</td>
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<td>Rb. sphaeroides RK1FI</td>
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<td>yes</td>
<td>not determined</td>
</tr>
<tr>
<td>Rb. sphaeroides NCIB8253</td>
<td>yes</td>
<td>not motile</td>
<td>not determined</td>
</tr>
<tr>
<td>Rb. sphaeroides WS8-N</td>
<td>no</td>
<td>no</td>
<td>not determined</td>
</tr>
<tr>
<td>Rb. sphaeroides 2.4.1</td>
<td>?</td>
<td>not motile</td>
<td>yes</td>
</tr>
<tr>
<td>Rb. capsulatus SB1003</td>
<td>yes*</td>
<td>no</td>
<td>not determined</td>
</tr>
<tr>
<td>Rs. saleigens WS68</td>
<td>yes</td>
<td>no</td>
<td>yes*</td>
</tr>
<tr>
<td>E. halophila BN9626</td>
<td>yes*</td>
<td>yes*</td>
<td>not determined</td>
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<tr>
<td>E. halophila SL-1</td>
<td>yes*</td>
<td>yes*</td>
<td>not determined</td>
</tr>
</tbody>
</table>

* Southern blots show a specific signal, which disappears at high washing stringency, Gomelski & Kaplan, unpublished results
*\* Jiang & Bauer, unpublished results
*\*\* assay conditions used were those as described by Sprenger et al. (1993)
*\*\*\* Kort, Hellingwerf & Spudich, unpublished results
*\*\*\*\* Dzhambekova, Perman & Moffat, unpublished results

Table 2. Strains which have been tested for the presence of the pyp gene and blue-light responses.
Motility and adaptation responses

photosynthetic apparatus most probably mediates motility and methanol release responses to a step-down in photosynthetic light (Grishanin et al., 1997; chapter 3.2). The hypothesis that both the light step-up and the step-down response are mediated by a single photosensor, in this case the photosynthetic apparatus, cannot be excluded, but we found evidence that argues against this (see results) and it is hard to picture a mechanism. There is however, an archaeobacterial photosensor, sensory rhodopsin I (SRI), that mediates an attractant response towards orange light, as well as a repellent towards blue and UV-light (Spudich & Bogomolni, 1984). The mechanism for this type of color discrimination involves (i) a photocycle with a SRI<sub>373</sub> ground state and SRI<sub>373</sub> metastable state and (ii) a two-photon reaction. The blue-light motility response could be induced by a two-photon reaction, because this experiment was carried out in a background of infrared light, but this is not true for blue-light induced methyl release, where no background light was provided. Thus at first sight, an unknown, dedicated blue-light photosensor, mediating both motility and methyl release repellent responses, seems most plausible. It cannot be excluded that a PYP-like protein, encoded by a second copy of the pyp gene, mediates these blue-light responses in Rb. sphaeroides, although Southern blots have not revealed any additional signals. But Southern blots do not provide a reliable method for the elucidation of the number of copies of a specific gene in the genome of Rb. sphaeroides, as examples with cheY and cheA genes have shown convincingly (Hamblin et al., 1997).

Several aspects of the blue-light motility response cast doubt on the involvement of a dedicated blue-light photo-sensor. First of all, no response is observed for light pulses shorter than 100 ms. Second, the motility response only occurs at relatively high-light intensities; it is difficult to measure a response for light intensities, which are lower than 30% of the initial intensity (see materials and methods). Third, the response is gone after exposure of cells to oxygen, but also sometimes when cells are exposed to blue-light for a period of ~15 min. These observations clearly demonstrate that in these experiments multiple signals feed into the signal transduction pathway, controlling the motility response described here. In addition, the need for high light-intensities and the absence of a response to short light pulses favors an energetic effect, rather than an effect, mediated by a specific photosensor. The best indication for the physiological relevance of this motility response is the strong correlation with blue-light induced release of methanol (see also chapter 3.2). Additional evidence for the nature of the involved photosensor may be provided by an Rb. sphaeroides mutant lacking the photosynthetic apparatus. Such a mutant is currently under construction. In addition, the Rb. sphaeroides cheB gene, encoding the methyl-esterase, can be knocked out to study its phenotype with respect to blue-light motility and methanol release responses.

So, what is the function of PYP? The results reported in this paper have not brought us any closer to the answer. As photoactive yellow protein appears to be the structural prototype for a PAS domain (see chapter 1.3), it may be involved in circadian rhythms, although they have never been demonstrated in purple bacteria. Besides, one could think of a function as a light sensor for light-induced modulation of gene expression. The photocycle of photoactive yellow protein, which is completed in about a second, points to a function, for which it is important to constantly inform the cell about its ambient light climate. The latter point would argue in favour of phototaxis rather than gene expression.

It cannot be totally ruled out on the basis of the data presented here that PYP mediates a phototactic response. One could focus again on E. halophila to test the effect of a pyp in-activation, in spite of the difficulties with respect to the experimental procedures (see introduction). It has been shown that this organism shows a blue-light phototactic response, i.e. an increase in the reversal frequency, which leads to migration away from blue-light. In addition, a more accurate determination of the wavelength dependence of this tactic response, displayed by E. halophila, would make the current evidence for the function of PYP more solid.
The strategy described here to obtain the function of photoactive yellow protein, can be considered as reverse genetics. Starting with a protein one follows the route via N-terminal sequencing, cloning and inactivation of the gene to identify a specific phenotype. It is also possible to start with the selection for a specific phenotype and identify the genes involved, in other words forward genetics. To carry out the latter strategy, *Rhodobacter sphaeroides* cells have been randomly mutagenized by Tn5 transposon mutagenesis, followed by selection of several rounds for mutants that were able to swim through a barrier of bright blue-light in glass tubes (Hoefkens, Kort & Hellingwerf, unpublished results). Wild-type *Rhodobacter sphaeroides* cells accumulated in front of this blue-light barrier. Southern blots showed that the selected mutants contained a transposon integrated in the chromosome, but not in *pyp* or within 5 kb of either flanking regions. Additional analysis of the Tn5 mutants showed that did they did not had lost the wild-type accumulation pattern in front of the light barrier (neither did *Rhodobacter sphaeroides pyp* mutants). It may be worthwhile to carry out the selection again in small capillary tubes instead of glass tubes to decrease the number of cells crossing the light barrier as a result of convection.

The *pyp* gene transcripts. Northern blots show three transcripts that hybridize to the *pyp* gene. The size of one of these transcripts could be determined by interpolation with the use of an RNA marker, resulting in an estimated size of 3 kb. The size of the other two transcripts could only be determined by extrapolation, which is much more inaccurate (figure 6). We estimate the size of these large transcripts to be ~15 kb and ~25 kb. A Northern blot probed with the downstream *pel* gene shows a very similar pattern, suggesting that the *pyp* gene and the *pel* gene are co-transcribed. The size of the *pyp* gene is 372 bp and the size of the *pel* gene is 1233 bp and their intergenic region is 1090 bp, allowing the possibility of co-transcription on a 3 kb transcript. The presence of the two large transcripts raises the question whether transcription takes places from a single promoter or multiple promoters. This can be investigated in Northern blots by the use of probes further upstream and downstream from *pyp*.

Recently, a *pyp* gene from *Rb. capsulatus* was cloned and sequenced (Jiang and Bauer, unpublished results; GenBank accession number af064095). Comparison of this sequence and its flanking regions with that of the *Rb. sphaeroides* RK1 sequence (chapter 2.2) shows that: (i) the PYP proteins are highly similar (78% identity and 87% similarity over 124 AA), (ii) the sequenced 3'-end of orfX in *Rb. capsulatus* (start of the sequence) encodes a protein that is similar to that encoded by orfA, upstream of *pyp* in *Rb. sphaeroides* (72% identity and 92% similarity over 25 AA), (iii) OrfY in *Rb. capsulatus* is similar to OrfC (56% identity and 76% similarity over 134 AA) and (iv) both sequences contain a *pel* gene, encoding a CoA ligase homologue (39% identity and 59% similarity over 422 AA). This indicates that upstream as well downstream of *pyp*, conserved genes are present. A functional involvement of these genes in the photosensory system, mediated by PYP, remains to be investigated.

**Acknowledgements**

I would like to thank Kevin Jung, Xue-Nong Zhang, Bastianella Perazzona, Elena and John Spudich for assistance and a very inspiring time during my visits in Houston. Also many thanks to Mirjam Hoefkens and Michael van der Horst for work on the construction of *pyp* mutants and Betsie Voetdijk for RNA work.

**References**


Sistrom, W. R. (1962). The kinetics of the synthesis


Motility and adaptation responses

3.2 Light-induced modulation of the release of \(^3\)H-methanol

In this study we report light-induced adaptive demethylation responses of the purple non-sulfur bacterium \(Rb.\ sphaeroides\). The results of a flow assay for the release of volatile tritiated compounds from intact \(Rb.\ sphaeroides\) cells, labeled with \(^3\)H-methionine, suggest a correlation between light-induced motility responses and the observed adaptive demethylation responses. An increase in blue-light intensity and a decrease in infrared light intensity can both be considered as repellent signals and cause an increase in stop or reorientation frequency of swimming \(Rb.\ sphaeroides\) cells and an increase of the rate of the release of methanol. The wavelength-dependence of the magnitude of the methanol release response matches the absorbance spectrum of the photosynthetic pigments for wavelengths of 500 nm and above, suggesting that this pigment complex is the primary photosensor for this response. The methanol release responses in \(Rb.\ sphaeroides\) are reminiscent of those in \(Escherichia\ coli\), where an attractant stimulus decreases methyl esterase activity and recovers during the course of several minutes to prestimulus rate and a negative stimulus produces a transient increase in methyl esterase activity.

Introduction

Bacteria respond to a sudden change in chemoeffector concentration by a change in the tumble, reversal or stop frequency for reorientation, biasing their random walk in a favorable direction. However, after a period ranging from seconds to minutes, the cells return to their initial behavioral pattern of runs and reorientations, even though the effector is still present. Thus, bacteria constantly adapt to their changing environment. The biochemical mechanism of adaptation includes the covalent modification of the relevant transducer protein by methylation. A protein methylation reaction, involved in chemotaxis of \(Escherichia\ coli\), has been discovered by Kort et al. (1975). In the absence of stimuli, the absolute methylation level of the transducer proteins remains constant, but methyl groups constantly turn over as a result of continuous methylation and demethylation. Transient changes in the demethylation rate, in response to stimuli, result in changes in the transducer methylation level, that feeds back on the tactic excitation pathway (Kehry, Doak & Dahlquist, 1984; Toews et al., 1979). Several glutamate residues in the cytoplasmic domain of a transducer can be methylated by the constitutively active methyltransferase CheR, while the hydrolysis of methyl groups is mediated by the protein methyl esterase CheB. The \textit{in vitro} activity of the latter enzyme is increased more than 10-fold upon phosphorylation of its N-terminal domain by the autokinase CheA (Lupas & Stock, 1989; Stewart & Dahlquist, 1988). The methyl ester hydrolysis catalyzed by CheB yields a demethylated glutamic acid residue and methanol (Stock & Koshland, 1978).

It has been shown that in \(Escherichia\ coli\), an attractant stimulus decreases methyl esterase activity and a repellent stimulus increases methyl esterase activity, corresponding to behavioral adaptation (Kehry et al., 1984). These stimuli-induced changes in methyl esterase activity result in changes in the amount of methanol released. The effect of stimuli on methanol release is different in the archaeon \(Halobacterium\ salinarum\) and the Gram-positive bacterium \(Bacillus\ subtilis\), where both attractant and repellent stimuli lead to a transient increase of methanol release (Alam et al., 1989; Spudich, Takahashi & Spudich, 1989, Thoelke, Kirby & Ordal, 1989). Recently, assays with \(B.\ subtilis\) showed that methanol formation due to negative stimuli are dependent on the presence of the response regulator CheY, whereas positive stimuli are CheY-independent, indicating that a unique adaptational mechanism exists in this organism (Kirby et al., 1997). In \(H.\ salinarum\) it has been found that the released methyl groups consist of two different chemical species: methanol and methanethiol (Nordmann et al., 1994); only the first compound is involved in adaptive demethylation, analogous to the chemotactic system of \(E.\ coli\).

The most widely studied environmental changes, inducing the adaptation pathway in
prokaryotes by modulation of methylesterase activity, are changes in chemoeffector concentration (chemotaxis), including oxygen (aerotaxis). The latter is exceptional with respect to its mechanism of adaptation among prokaryotes, because aerotaxis appears methylation-independent in *E. coli* and *Salmonella typhi­murium*, but dependent on methylation of methyl-accepting chemotaxis proteins (MCPs) in *B. subtilis* and *H. salinarum* (Lindbeck et al., 1995; Wong et al., 1995). Besides chemoeffectors, also light can induce a methylation-dependent adaptation pathway as found in the photosynthetic archaeon *H. salinarum* (Alam et al., 1989; Spudich, Takahashi & Spudich, 1989). Phototactic behavior in this prokaryote is mediated by two retinal-containing proteins, sensory rhodopsin I (SR-I) and sensory rhodopsin II (SR-II) (Spudich & Bogomolni, 1988). This photosensory system allows accumulation of cells in yellow-red regions of the spectrum (attractant light) and avoidance of UV-blue repellent light (Hildebrand & Dencher, 1975).

Recently, a gene encoding a blue-light photosensor photoactive yellow protein (PYP) has been identified in the anoxygenic photosynthetic bacterium *Rhodobacter sphaeroides* (chapter 2). This 4-hydroxy coumaric acid-containing protein has been proposed to mediate a negative phototactic response towards blue light in the purple sulfur bacterium *Ectothiorhodospira halophila* (Sprenger et al., 1993). The finding of a *pyp* gene in *Rb. sphaeroides* prompted us to carry out a detailed study on the phototactic behavior of this organism, as described in the previous section of this thesis, and on the mechanism of adaptation to photostimuli, as reported here.

Previous *in vivo* and *in vitro* methyla­tion studies, methanol release assays, and antibodies raised against Tar from *E. coli*, all demonstrated the absence of methyl-accepting chemotaxis proteins in *Rb. sphaeroides*, while the same studies confirmed the presence of these proteins in *Rhodospirillum rubrum* (Sockett, Armitage & Evans, 1987). Recently, however, the identification of two methyltransferase genes (*cheR1* and *CheR2*), one methylesterase gene (*cheB*) and at least four *mcp*-like genes has provided strong evidence supporting a methylation-dependent adapta­tion pathway in this purple non-sulphur bacterium (Choudhary et al., 1997; Hamblin et al., 1997; Ward et al., 1995). In addition, it has been shown that CheR-dependent methylation of *Rb. sphaeroides* proteins, with the approximate molecular mass of MCPs, occurs on the time scale of tens of minutes, after a long period of starvation, followed by the addition of complete medium. This phenomenon was not observed after addition or removal of individual attractants (Armitage & Schmitt, 1997). So far, all *Rb. sphaeroides* tactic responses show adaptation on relatively long time scales, taking 10 seconds up to 60 minutes (Gauden & Armitage, 1995; Packer & Armitage, 1994). This seems also to be true for the blue-light motility response, described in the previous section of this thesis. Interestingly, the motility response towards a step up in blue-light is very similar to the response that *Rb. sphaeroides* cells show after a step down in photosynthetic light. The primary photosensor for this response, with full adaptation after 40 s, is most probably the photosynthetic apparatus (Grishanin, Gauden & Armitage, 1997).

This paper describes light-induced changes in the release of volatile methylated compounds from the photosynthetic eubacterium *Rb. sphaeroides*. These studies provide evidence supporting the mechanism of adaptation of the motility responses observed in this bacterium to a step down in photosynthetic light (Grishanin et al., 1997) and to a step up in blue-light (chapter 3.1). Especially, the identification of methylation-dependent adaptation pathway in blue-light sensing is of great importance, because it suggests the existence of a methyl-accepting protein as a downstream partner of the blue-light induced phototransduction pathway.

**Materials and Methods**

*R. sphaeroides* RK1 cells were cultured in medium described by Sistrom (1962) at 28 °C in the presence of 0.1 mM methionine to enhance methionine uptake. Culturing was carried out under anaerobic conditions in the light in 20 ml screw cap tubes, under semi-anaerobic conditions in the dark in 1/6 filled 300 ml erlenmeyers at relatively low rotation.
Motility and adaptation responses

speed and under aerobic conditions in the dark in 1/25 filled 500 ml erlenmeyers at high rotation speed. The flow assay, developed by Ke­hry and collaborators for measurement of stimulus-induced changes in methylesterase activity of intact E. coli cells (Kehry, Doak & Dahlquist, 1984), was carried out with a 2 ml cell-suspension (OD\text{600} \sim 0.8). Cells were washed three times in Sistrom medium without methionine and incubated under (semi-)anaerobic or aerobic conditions for 40 min in the presence of 200 \mu L-[methyl-\text{\textsuperscript{3}H}] methio­nine with a specific activity of 75 Ci/mmol and a concentration of 1 mCi/ml (DuPont) in a total volume of 2 ml (final concentration is 1.3 \mu M labeled methionine). After labeling, cells were washed once in Sistrom medium with 0.1 mM methionine and pumped onto a 0.2 \mu m syringe filter (Nalgene). Cells were equili­brated for 10 min in a continuous flow of Sis­trom medium of 28\textdegree C, supplied with a rate of 1 ml/min. Every 30 seconds, a fraction of 0.5 ml was collected in a 1.5 ml Eppendorf tube. Photostimuli were obtained by the use of a tungsten or xenon lamp, heat filters and broad band interference filters. All light intensities were adjusted to 2.0 \times 10^5 \text{ergs cm}^{-2} \text{s}^{-1}, except for light of 400 (+/- 20) nm, which maximal intensity was 5.0 \times 10^3 \text{ergs cm}^{-2} \text{s}^{-1}. Light intensity measurements were carried out with a Kettering Radiant Power Meter (Scientific Instruments). The photostimuli had a negligible effect on the temperature at the position of the immobilized cells in the filter. Eppendorf tubes containing the collected samples were carefully transferred to vials with scintillation fluid and incubated for 24 hours at room temperature in the dark, to allow transfer of labeled methanol \textsuperscript{3}H-decay was measured in the scin­tillation counter for 1 min per vial.

Results and discussion

Light-induced release of volatile tritiated compounds was analyzed using the flow assay, with intact Rb. sphaeroides cells immobilized on a filter. The nature of the labeled volatile compound has not been identified, but we assume that it is \textsuperscript{3}H-methanol, because this is the only compound detected so far in this type of experiment carried out with eubacteria. The transient increase in methanol production, indicated in figures 1A and 1B, is most probably the result of a cellular response to a step-up in blue-light intensity, inducing a methylation-dependent adaptation reaction for negative phototaxis. A blue-light repellent motility response, i.e. an increase in the stop frequency, has been observed in anaerobically cultured Rb. sphaeroides cells (chapter 3.1). Under semi­anaerobic conditions, Rb. sphaeroides cells show an increased release of methanol groups as well (figure 1B), be it less pronounced, while under aerobic conditions this increase is not visible (figure 1C). It should be noted that under semi-anaerobic and aerobic conditions in the dark the incorporation of radioactive label is less efficient, leading to lower turn­over of labeled methyl groups, and lower

![Figure 1. Release of \textsuperscript{3}H-labeled methanol by R. sphaeroides cells as a function of time. Each dot indicates the counts per minute for a 0.5 ml fraction, collected during a period of 30 seconds. The arrow indicates the start of a step-up in blue-light (450 nm). A cells grown under anaerobic conditions in the light B cells grown under semi-anaerobic conditions in the dark C cells grown under aerobic conditions in the dark.](image-url)
light-induced signals. The results in figure 1 match nicely the previously observed blue-light motility response, which also is observed in cells grown anaerobically in the light and is less pronounced in cells grown under semi-anaerobic conditions in the dark. Apparently, the blue-light motility response, as well as the blue-light induced release of methanol, are observed exclusively in cells grown under low oxygen tension. This may indicate that the genes involved in these blue-light responses are controlled by oxygen rather than light, like the genes encoding the photosynthetic pigments.

In a previous study, methanol release in *Rhodobacter sphaeroides* WS8 was not observed in response to the addition or removal of a combination of the attractants serine and succinate (Sockett, Armitage & Evans, 1987). This could be explained by the lower specific activity and amount of added $^3$H-methionine label: 15 Ci/mmol and 30 $\mu$Ci in the experiment by Sockett *et al.* and 75 Ci/mmol and 200 $\mu$Ci used in the experiment described here. In *Escherichia coli*, where an attractant stimulus decreases methylesterase activity and recovers over the course of several minutes to prestimulus rate and a negative stimulus produces a transient increase in methylesterase activity. The sustained decrease and the transient increase of methanol release indicated in figure 2 can be rationalized by the assumption that activated CheB-P has a relatively short lifetime, causing a transient response, while inhibition of CheA kinase activity, increasing the pool of stable, unphosporylated CheB molecules has a more sustained effect.

The opposite effects of blue and infrared photostimuli on methanol production in intact
Motility and adaptation responses

*Rhodobacter sphaeroides* cells urged us to investigate the wavelength dependence of this response. Accordingly, we used a set of broad band interference filters, with transmission spectra as indicated in figure 3. The data in figure 4 show that the wavelength dependence of the increase in $^3$H-methanol release matches the absorbance spectrum of the photosynthetic pigment for wavelengths of 500 nm and longer. We hypothesize that adaptation of the phototactic response towards a decrease in photosynthetic light, reported to be mediated by the photosynthetic pigments as the primary photosensor, involves a methylation-dependent system. This would then be the first true signaling response mediated by a photosynthetic pigment. The mechanism of signal transfer from the photosynthetic pigment to a methyl-accepting transducer protein is still unknown. One could speculate about different mechanisms for this signal transfer: (i) reduced quinone could be directly sensed by a membrane-spanning protein, (ii) the phosphorylation potential could be sensed by a cytoplasmic protein or (iii) the membrane potential ($\Delta \Psi$) could be sensed by a membrane-spanning protein; in this case one could hypothesize that the conformation of transmembrane-spanning $\alpha$-helices, containing a dipole, could be changed upon changes in $\Delta \Psi$, triggering a signal for sensory output (see figure 5). One of the presumed methyl-accepting transducer-like proteins, TlpB, could be a possible candidate for one of the sensing mechanisms described above, since it is specifically produced in *Rhodobacter sphaeroides* under anaerobic growth conditions (Armitage & Schmitt, 1997), under which methanol release responses are most pronounced.

An inverted methanol release response is observed for blue light (400 and 450 nm). It remains to be determined whether a separate photoreceptor is responsible for this latter response. Recently, the gene encoding the blue-light photosensor photoactive yellow protein (*pyp*) has been identified in *Rhodobacter sphaeroides* (chapter 2). It has been shown however, that this gene is not mediating this blue-light induced methanol release response (chapter 3.1; figure 5). Interestingly, a recent transposon mutagenesis study on mutants defective in phototactic colony migration in the photosynthetic eubacterium *Rhodospirillum centenum* showed that signals for positive and negative phototactic responses probably both involve light-driven photosynthetic electron transport (Jiang *et al.*, 1998).

Acknowledgement

Remco Kort is very thankful to Bastianella Perazzona for expert assistance with the methylation assay.

![Figure 4. Wavelength dependence of light-induced release of $^3$H-labeled methanol by *Rhodobacter sphaeroides* cells. The values were calculated by differences in counts per minute between the last fraction before the stimulus and the first fraction during the stimulus. A indicates the difference in methanol release before and after a light step up; B shows the difference in methanol release before and after a light step down. For blue light (450 nm), and infrared light, values of 3 independent experiments were averaged. It should be noted that the light intensity used for 400 nm-light is 4 times lower than those used for all the other wavelengths. For comparison, the absorption spectrum of *Rhodobacter sphaeroides* cells, cultured under anaerobic conditions in the light is shown in B.](attachment:figure4.png)
Figure 5. Model for light-induced signaling in *Rb. sphaeroides*. The photocycle of the blue-light photosensor photoactive yellow protein (PYP) is indicated with isomerization and protonation states of the 4-hydroxy cinnamic acid chromophore. The ground state (pG), the red-shifted intermediate (pR) and the blue-shifted intermediate (pB) and their absorbance maxima are indicated in the figure. The latter intermediate is the presumed signaling state and interacts with a so far unidentified downstream partner. The *pyp* gene from *Rb. sphaeroides* has been cloned (chapter 2.2) and inactivated (chapter 3.1), but its physiological role has not been elucidated. The data presented in the figure are based on those available for PYP from the purple sulfur bacterium *E. halophile*; for a review see Hellingwerf, Hoff & Crielaard (1996). For an extensive description of the multiple sensory pathways in *Rb. sphaeroides* see chapter 1.2. Abbreviations: R, CheR; B, CheB; Y1, CheY1; Y2, CheY2; Y3, CheY3; A1, CheA1; A2, CheA2; W1, CheW1; W2, CheW2; M, FliM; N, FliN; RC, reaction center; QH, reduced quinone; BC1, BC1 complex. The dashed arrow indicates a putative interaction of the reduced quinone with a presumed redox sensor.
References


