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Locked Chromophore Analogs Reveal That Photoactive Yellow Protein Regulates Biofilm Formation in the Deep Sea Bacterium *Idiomarina loihiensis*

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Abstract: *Idiomarina loihiensis* is a heterotrophic deep sea bacterium with no known photobiology. We show that light suppresses biofilm formation in this organism. The genome of *I. loihiensis* encodes a single photoreceptor protein: a homologue of photoactive yellow protein (PYP), a blue light receptor with photochemistry based on trans to cis isomerization of its *p*-coumaric acid (*p*CA) chromophore. The addition of trans-locked *p*CA to *I. loihiensis* increases biofilm formation, whereas cis-locked *p*CA decreases it. This demonstrates that the PYP homologue regulates biofilm formation in *I. loihiensis*, revealing an unexpected functional versatility in the PYP family of photoreceptors. These results imply that *I. loihiensis* thrives not only in the deep sea but also near the water surface and provide an example of genome-based discovery of photophysiological responses. The use of locked *p*CA analogs is a novel and generally applicable pharmacochemical tool to study the in vivo role of PYPs irrespective of genetic accessibility. Heterologously produced PYP from *I. loihiensis* (II PYP) absorbs maximally at 446 nm and has a *p*CA pK_a of 3.4. Photoexcitation triggers the formation of a pB signaling state that decays with a time constant of 0.3 s. FTIR difference signals at 1726 and 1497 cm^{-1} reveal that active-site proton transfer during the photocycle is conserved in II PYP. It has been proposed that a correlation exists between the lifetime of a photoreceptor signaling state and the time scale of the biological response that it regulates. The data presented here provide an example of a protein with a rapid photocycle that regulates a slow biological response.

1. Introduction

Living organisms use a limited number of types of photoreceptor proteins to obtain information about their ambient light climate. These photoreceptor proteins can be grouped into different families, on the basis of their structure and light-absorbing cofactor.[1] Until very recently, such photoreceptor proteins had been found exclusively in phototrophic microorganisms. However, genome sequencing projects have uncovered many examples of photoreceptor proteins in chemotrophic microorganisms.[2,3] Bioinformatics analyses showed that members from most known photoreceptor protein families are present in chemotrophic Bacteria. Most of these have not been characterized regarding their in vivo function or in vitro biochemical and photophysical characteristics. The widespread occurrence of as of yet unstudied photosensory proteins revealed by genome projects indicates that many additional unanticipated photobiological responses in chemotrophic bacteria remain to be discovered.

One example of a chemotrophic bacterium with a photoreceptor encoded in its genome is the deep-sea bacterium *Idiomarina loihiensis*. This organism belongs to the group of γ-proteobacteria and was isolated at 1 km depth in the Hawaiian ocean.[4] The genome of *I. loihiensis* contains a cluster of 32 genes that encode proteins involved in the production and secretion of polysaccharides.[5] This prompted us to study biofilm formation in *I. loihiensis*. In parallel, we examined the genome of *I. loihiensis* for the presence of photoreceptor proteins from all five established photoreceptor families that can be unambiguously recognized based on their sequence,[1] and a single photoreceptor gene was identified.[2] This gene encodes a protein that shows clear homology to photoactive yellow protein (PYP).[2,6] This blue-light photoreceptor protein was first found in *Halorhodospira halophila*,[7,8] and its physicochemical proper-

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ties have been well characterized.[9−11] Studies on the properties of 6 additional PYPs have been initiated (see ref 6 for a review). This work has led to a detailed understanding of PYP photochemistry. Light activation of the initial pG state of PYP (λ_{max} 446 nm) involves the photoisomerization of the vinyl bond of its *p*-coumaric acid (*p*CA) chromophore from trans to cis, resulting in the short-lived, red-shifted pR state. Subsequent protonation of the *p*CA by Glu46[12,13] causes formation of the pB′ state, and subsequently leads to structural changes in the protein that result in the formation of the blue-shifted pB state (λ_{max} 355 nm). The pB state is the presumed signaling state of PYP. The protein then thermally recovers to its pG groundstate on a time scale that ranges from 1 ms for the PYP from *Rhodobacter sphaeroides*,[14] to ∼0.5 s for the PYP from *H. halophila* (Hh PYP),[8,15] to ∼1 h for the PYP from *Salinibacter ruber*.[6,16]

Examination of the *I. loihiensis* genome[5] indicates that its *pyp* gene is located in the immediate vicinity of a gene encoding a diguanylate cyclase. Since c-di-GMP, a recently discovered bacterial signaling molecule, is a central regulator for the synthesis of the exopolysaccharides that are involved in biofilm formation,[17−20] we examined the role of light in regulating biofilm formation in *I. loihiensis*. This revealed that photoactivation of PYP suppresses biofilm formation in this organism.

2. Experimental Section

Culturing and Attachment of *I. loihiensis*. *I. loihiensis* strain L2-TR(T) was cultured in Marine Broth (Difco) at 30 °C with gentle shaking. Biofilm assays were performed at 20 °C, in a well-thermostatted room, using a method adapted from ref 21. An overnight culture of *I. loihiensis* was diluted ten times and incubated in 96-well flat-bottom PVC microtiter plates (Costar 3595). One plate was covered with black cloth, another with transparent plastic foil. Plates were illuminated with a white light bulb (50 W, Philips) at ∼80 cm distance, resulting in a light intensity of ∼80 microeinsteins m^{-2} s^{-1}. Care was taken to minimize effects of illumination on other physical parameters in the experimental setup, such as temperature and humidity. To this end, the experiment was also carried out in an incubator/shaker, thermostatted at 20 °C. The incubator was not rotating and illumination was carried out from the outside, through the glass shield in the lid of the incubator. To ensure that the position in the 96-wells plate did not alter the behavior of the cells, the addition of *p*CA and its analogs was randomized over the plate, and plates were incubated as described above. After 24 h, nonattached cells were removed by washing the plates three times with demi water.

Attached cells were stained for 60 min with 1% crystal violet. Nonbound crystal violet was removed by washing three times with demi water. Crystal violet was resolublized in 80% ethanol/20% acetone, and cell attachment was quantified by measuring the absorbance at 595 nm in a plate reader (Spectramax plus, Molecular devices). To test the effect of *p*CA and its analogs on cell attachment, concentrated solutions of the chromophores (*p*CA, trans-locked, and cis-locked) in DMF were added to the cultures in the wells to a final concentration of 1 mM. The final concentration and solvent used for adding these three chromophores to *I. loihiensis* cell cultures were identical. The values for biofilm formation in the dark and light, and in the dark in the presence of *p*CA and the two *p*CA analogs as determined by crystal violet binding were averaged over 6 wells in a 96 wells microtiter plate, and the reproducibility of the resulting values was confirmed in two independent experiments. Attachment of the cells to the wall of the wells prevented exact quantitation of growth by OD600 measurements. Visual inspection of the 96-well plates indicated that there is only limited growth during overnight incubation in the wells. Addition of chromophores did not noticeably affect cell growth.

Synthesis of pCA Analogs. The *cis*-locked chromophore analog 2-(4-Hydroxy-phenyl)-cyclopent-1-enecarboxylic acid was synthesized from ethyl-2-oxocyclopentane carboxylate as described in the Supporting Information. The identity and purity of the intermediate and final products was determined using FTIR and NMR spectroscopy and mass spectrometry.

Cloning of the Il pyp Gene. On the basis of the genome sequence of *I. loihiensis*,[5] the oligonucleotides 5′-AGGTAACAC-CATGGAGATTGTTCAATT-3′ and 5′-TGAGTCTGGATCCT-TATAGTCGCTTAACGAATA-3′ were used as the sense primer and antisense primer, respectively, for PCR amplification of the *pyp* gene from this organism, thereby introducing *Nco*I and *BamH*I restriction sites. Purified *I. liohiensis* genomic DNA was used as the template in the PCR reaction. The amplified gene was cloned into the pET-16b vector (Novagen) using the *Nco*I and *BamH*I restriction sites. The nucleotide sequence of the cloned Il PYP was confirmed by DNA sequence analysis (BigDye-terminated reaction analyzed on ABI Model 3730 DNA Analyzer, Recombinant DNA and Protein Core Facility at Oklahoma State University). Plasmid DNA was transformed into *E. coli* BL21 (DE3) for apoPYP overproduction.

Overproduction and Purification of Hh PYP and Il PYP. PYP from *H. halophila* was produced and isolated as described in[22] as hexa-histidine tagged apoprotein in *Escherichia coli*. The apoprotein was reconstituted with the 1,1-carbonyldiimidazole derivative of the respective chromophore (i.e., 4-hydroxy-cinnamic acid (*p*CA), 7-hydroxy-coumarin-3-carboxylic acid (trans-locked; Molecular Probes, Invitrogen), 2-(4-Hydroxy-phenyl)-cyclopent-1-enecarboxylic acid (cis-locked), see Figure 1C) as described in.[23] Hh PYP was used in 10 mM Tris-HCl, pH 8.0 without removal of the N-terminal hexahistidine tag.

Overproduction of Il apoPYP was induced by the addition of 1 mM IPTG, extracted from *E. coli* BL21 (DE3) using 8 M urea and reconstituted with *p*-hydroxycinnamic anhydride (Sigma-Aldrich)[24] following the procedure described form the PYP from *H. halophila*.[25] The apoPYP from *I. loihiensis* readily reconstituted upon the addition of the *p*CA anhydride, yielding a bright yellow protein

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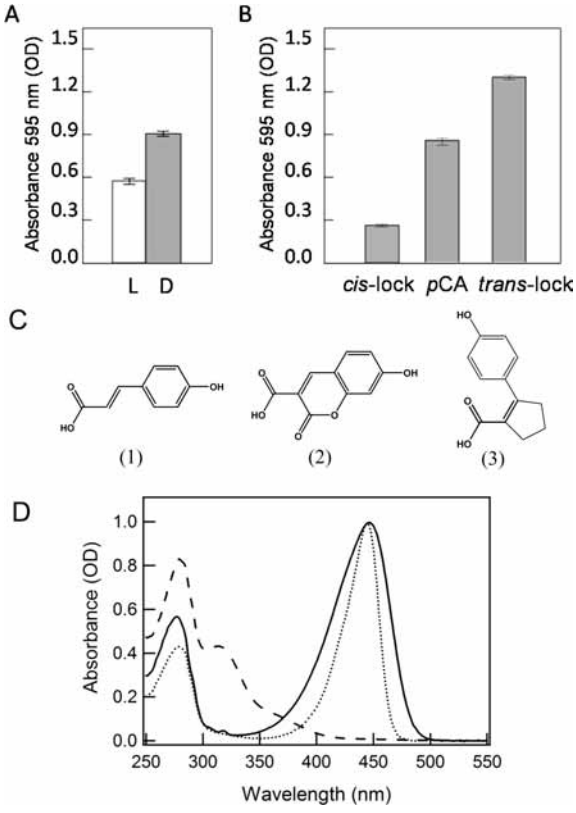

Figure 1. Regulation of biofilm formation in *I. loihiensis* by light and *p*-coumaric acid analogs. Error bars indicate standard deviations. (A) Biofilm formation in growing *I. loihiensis* cultures was measured in the light (open bars) and in the dark (gray bars), revealing that light suppresses biofilm formation. The absorbance of crystal violet at 595 nm is shown, providing a measure for the amount of cells attached to the PVC microtiter plate well. (B) Effect of the addition of *p*CA, cis-locked *p*CA, and trans-locked *p*CA on biofilm formation in *I. loihiensis* cultures grown in the dark. The error in panels (A) and (B) were calculated for values of 6 wells in a 96-well microtiter plate. (C) *p*CA chromophore analogs used to lock PYP into its pG and pB states. The structures of the chromophore used in this study are shown: 4-hydroxy-cinnamic acid (*p*CA, 1), (2) 7-hydroxy-coumarin-3-carboxylic acid (trans-locked, 2), and 2-(4-hydroxy-phenyl)-cyclopent-1-enecarboxylic acid (cis-locked, 3). (D) UV/vis absorbance spectra of Hh PYP containing *p*CA (solid), trans-locked *p*CA (dotted), and cis-locked *p*CA (dashed).

with an absorbance maximum at 446 nm. The reconstituted holo-Il PYP was purified by anion-exchange chromatography using DEAE-sepharose CL-6B (Pharmacia) and sodium chloride as the eluent. The purified protein had a purity index (ratio of absorbance at 280 and 446 nm) of 0.48. Possible proteolytic damage of the purified protein was examined by mass spectrometry (Applied Biosystems Voyager-DE Pro MALDI-TOF MS, Recombinant DNA and Protein Core Facility at Oklahoma State University). This yielded a mass of 14,121 Da, indistinguishable within the error of the instrument from the theoretical mass of 14 131 Da.

UV−Vis Absorbance Spectroscopy. UV−visible absorption spectra were measured at room temperature using an HP-8453 (Hewlett-Packard) diode array spectrophotometer and a monochromator-based Cary300Bio (Varian) spectrophotometer. The relative value of the molar extinction coefficient of the Il PYP was determined based on the effect of denaturing the protein in 10 mM Tris-HCl pH 7.5 by the addition of 2% SDS on its absorbance spectrum.[26] The extinction coefficient of Il PYP was derived by comparing the amplitude of the absorbance band at 345 nm of the denatured Il PYP to that of native and SDS-denatured wild type *H. halophila* PYP, which has a known extinction coefficient at 446 nm (45 500 M^{-1} cm^{-1}).[27] A 150 W halogen quartz light source (Cuda) with a broadband blue filter (band-pass filter 59855 Oriel, CT) was used to initiate the photocycle of Il PYP in 10 mM Tris-HCl pH 7.5. After approximately 15 s of illumination the actinic light was shuttered, and the thermal recovery of the dark state was measured with a time resolution of 100 ms. The photocycle recovery kinetics at 446 and 355 nm was biexponential. A mixed buffer (Glycine, Succinate, MES and MOPS, 100 mM each) was used for pH titration measurement in the pH range from 2.0 to 12.0. The pH was adjusted using 1 N NaOH or HCl. The acid titration was carried out in a darkened room using the minimal intensity of red light needed for handling of the samples and reading of the pH values. The pH dependence of the sample absorbance at its absorption maximum (446 nm) was described using the Henderson−Hasselbalch equation.

FTIR Spectroscopy. Il PYP and Hh PYP samples for FTIR spectroscopy were prepared at 8 mM in 50 mM phosphate buffer in D_2O at pH* 6.6 as described.[12] For each sample, 2.7 μL PYP was sandwiched between two CaF_2 windows (15 mm diameter and 2 mm thickness) with a 12 μm spacer. Steady state and time-resolved infrared measurements were measured using a Bruker IFS 66v FTIR spectrometer with a liquid nitrogen-cooled Mercury Cadmium Telluride (MCT) detector. Water vapor in the optical path was removed by vacuum, except for the sample compartment, which was purging with dry nitrogen gas (2.2 L/min). Sample temperature was controlled at 300 K using a water circulator (Neslab, RTE- 111) in all the FTIR measurements. FTIR absorption spectra of steady state PYP samples were measured in the spectral range of 4000−850 cm^{-1} at 2 cm^{-1} spectral resolution and 40 kHz scanning velocity, and averaged over 175 scans, using CaF_2 windows as a reference. The second derivative of the absorbance was calculated using the Bruker OPUS software based on Savitzky-Golay algorithm with 13 smoothing points.

For time-resolved FTIR spectroscopy, the photocycle of PYP was triggered with blue laser pulses (475 nm, 4 ns pulse duration, and ~3 mJ per pulse) from a Quantel, Brilliant laser with an Opotek OPO. The repetition rate of the pump laser was set at 0.125 Hz, for Hh PYP and Il PYP. Light-induced infrared absorption changes of PYP were measured in rapid-scan FTIR mode at 4.5 cm^{-1} spectral resolution with 200 kHz scanning velocity, and averaged over 256 repetitions. A quadruple splitting technique was employed so that the time resolution for the first spectrum was improved from 46 ms (without splitting) to 8 ms. For proper synchronization between the scanning mirror of the Bruker FTIR system and the laser flashes, a triggering system was designed and utilized where the FTIR system served as the master clock, and two delay generators (SRS, DG535) that were externally triggered by the FTIR system (coupled to the scanning mirror), were used to trigger the laser flash lamps (~10 Hz), the laser Q-switch, and rapid-scan FTIR data acquisition.

A macro program was designed and employed to compute the time-resolved infrared difference spectra, $\Delta A(t,\nu) = C_{pB}(t)\cdot d\cdot[\varepsilon_{pB}(\nu) - \varepsilon_{pG}(\nu)]$, using the infrared absorption of the pG state (preflash spectrum) as the baseline. The peak-to-peak signal of the first infrared difference spectrum of Il PYP was 10 mOD in the Amide I region, with a noise level of 0.03 mOD (3×10^{-5} OD).

Bioinformatics Analysis. The genome of *I. loihiensis* was examined for the presence of *pcl* and *tal*, and for genes encoding photoreceptor proteins using NCBI genomic BLAST tools at: http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. Domain architecture was examined using SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) databases.

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3. Results

Light-Regulated Cell Attachment. During the culturing *I. loihiensis* in glass flasks or tubes, cells were observed by eye to stick together in aggregates or attach to the glass culturing tube in a ring at the air−liquid interface. These aggregates were several millimeters in size and appeared after overnight culturing. In PVC 96-well microtiter plates, cells tended to stick more to the wall of the well, instead of to each other. To examine if light regulates this biofilm formation process, we determined the effects of white light on the attachment of *I. loihiensis* to 96-well microtiter plates. Bacterial cell attachment to the PVC was quantified using a crystal violet staining method.[21] This revealed that the presence of light reproducibly suppresses attachment of *I. loihiensis* cells to the PVC surface by ~35% (Figure 1A).

Since the only photoreceptor protein identified in the genome of *I. loihiensis* is a PYP homologue,[2] we designed an approach to study the role of this photoreceptor in the light regulation of biofilm formation. Because no genetic system is available for *I. loihiensis*, we utilized available knowledge on the effects of locked *p*CA analogues on PYP photochemistry (Figure 1C).[28,29] To block PYP photoexcitation we used 7-hydroxy-coumarin-3-carboxylic acid as a trans-locked *p*CA to essentially lock the protein in its pG dark state. The remaining photochemistry of PYP reconstituted with this *p*CA analog[28] occurs with very low quantum efficiency. The cis-locked *p*CA analog 2-(4-hydroxyphenyl)-cyclopent-1-enecarboxylic acid would be expected to permanently activate PYP by locking it in its pB state.[30] Both chromophores readily attach to Hh PYP. As expected, trans-locked *p*CA results in an absorbance spectrum resembling the pG state (λ_{max} 443 nm), while cis-locked *p*CA resembles the absorbance spectrum of the pB state (Figure 1D). PYP reconstituted with cis-locked *p*CA exhibits a characteristic peak at ~350 nm, but in addition displays a peak at 310 nm. The free form of this chromophore has absorption maxima at 267, 283, and 340 nm at pH 3, 7, and 10, respectively (data not shown). The 310 nm peak may arise from a combination of increased scattering and the n-to-π transition of the carbonyl group of the chromophore. The increased scattering of the solution suggests partial aggregation of PYP reconstituted with cis-locked *p*CA.

Incubation of *I. loihiensis* with these two *p*CA analogs significantly affected biofilm formation in the dark. The addition of trans-locked *p*CA resulted in a 45% increase in cell attachment, whereas *cis*-locked *p*CA resulted in a 75% reduction of attachment, when compared to the addition of *p*CA or no chromophore addition (Figure 1B). In the presence of both chromophore derivatives the cells no longer exhibited the light-induced suppression of biofilm formation. The addition of exogenous *p*CA to the cells did not alter the photoresponse (compare Figure 1A in the dark and 1B in the presence of *p*CA). This result shows that *I. loihiensis* cells synthesize *p*CA, and that the addition of the small amount of DMF used as solvent for the chromophores to the cell culture does not affect biofilm formation. These data provide strong evidence that PYP is the photoreceptor for the light regulation of biofilm formation in *I. loihiensis*. We therefore decided to examine the spectroscopic properties of Il PYP.

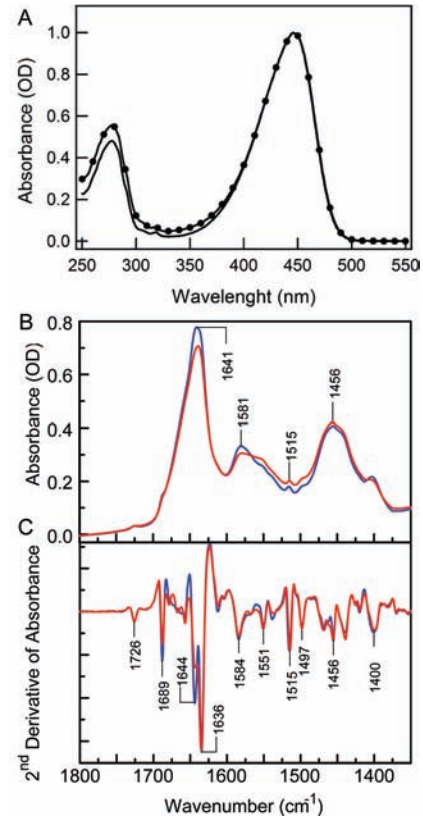

Figure 2. Comparison of the spectroscopic properties of the pG dark state of the PYPs from *Idiomarina loihiensis* and *Halorhodospira halophila*. (A) UV−vis absorbance spectra of Il PYP (dotted line) and Hh PYP (solid line). FTIR spectroscopic comparison of the structure of the pG dark state of Il PYP (red) and Hh PYP (blue) based on infrared absorbance spectra (B) and second derivative spectra (C) in D_2O at pH* 6.6.

Spectroscopy of Il PYP. The UV/vis absorbance spectrum of Il PYP is essentially identical to that of Hh PYP (Figure 2A). The extinction coefficient of the characteristic 446 nm absorbance band was determined to be 43 350 M^{-1} cm^{-1}, very similar to the value of 45 500 for *H. halophila* PYP.[27]

The structure of the pG state of Il PYP was compared to that of the PYP from *H. halophila* using FTIR absorption and second derivative spectroscopy (Figure 2B, C). The FTIR absorbance spectrum of a protein in the 1800−1350 cm^{-1} region contains contributions from the protein backbone and from all polar and charged side chains. The peak positions of these overlapping bands are better resolved in the second derivative of the FTIR spectrum.[31] Thus, the second derivative of the FTIR absorbance of a protein provides a sensitive "fingerprint" for the structure of a protein.[31,32] The second derivative FTIR spectra of the pG states of the PYPs from *I. loihiensis* and *H. halophila* are highly similar, demonstrating that they share a very similar three-dimensional structure. This is in line with the 75% sequence identity shared by these two PYPs.

The pK_a of the *p*CA chromophore in Il PYP was determined by acid titration using UV/vis absorbance spectroscopy. At pH values below 4 the native pG state was converted to a pB_{dark}

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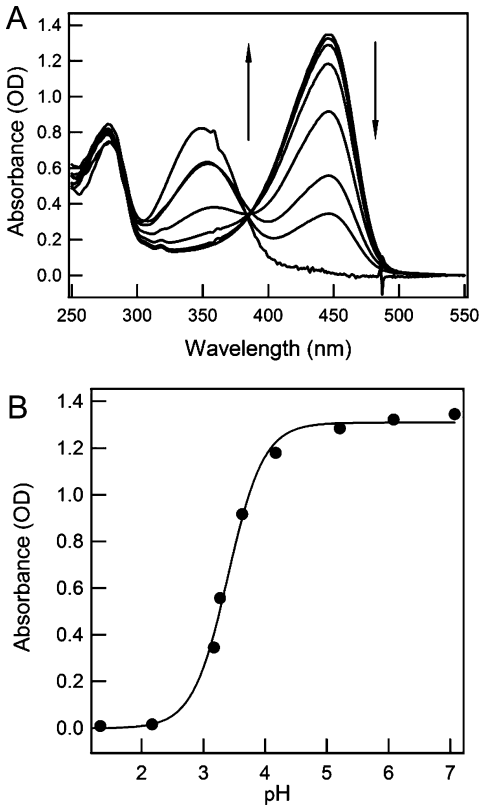

Figure 3. Low pH titration of the PYP from *I. loihiensis*. (A) UV−vis absorbance spectra of Il PYP as a function of pH. Arrows indicate the direction of the change in the amplitudes of the bands at 446 and 350 nm at increasingly lower pH values. (B) pH dependence of the absorbance at 446 nm as a function pH, fit to the Henderson−Hasselbalch equation.

species[7,33] with a protonated *p*CA chromophore and an absorbance maximum at 352 nm (Figure 3A). The transition was described by a single pK_a value of 3.4, with an *n* value of 1.3 (Figure 3B). In Hh PYP this transition occurs with a pK_a of 2.8.[7,33]

The photochemical activity of the protein was examined by exposing it to blue light at pH 7.5. This revealed the formation of a pB photocycle intermediate with an absorbance maximum at 350 nm (Figure 4). The pB state decayed to the initial pG state in a biphasic process with time constants of 0.3 s (79%) and 5 s (21%) (Figure 4). Thus, the *pyp* gene in the *I. loihiensis* genome encodes a genuine, photochemically active PYP. The kinetics of pB decay in the PYP from *I. loihiensis* is very similar to that of the PYP from *H. halophila*.[8,15]

The molecular events that occur upon pB formation in Il PYP were examined using rapid-scan FTIR difference spectroscopy in D_2O (Figure 5A). The pB − pG FTIR difference spectrum for Il PYP is quite similar to that of Hh PYP (Figure 5B). Thus, the published assignments of FTIR signals in Hh PYP[12,13] can be applied to Il PYP. The negative signal at 1726 cm^{-1} is caused by the deprotonation of Glu46, and matches the signals at 1497 and 1515 cm^{-1} caused by *p*CA protonation. The signal at 1726 cm^{-1} also shows that hydrogen bonding between the side chain of Glu46 and the phenolic oxygen of the *p*CA in the pG state is essentially identical in the two PYPs. The main differences in the pB − pG difference spectrum for Il PYP are a ∼20% reduction in the amide I difference signals at 1624 cm^{-1}, and a

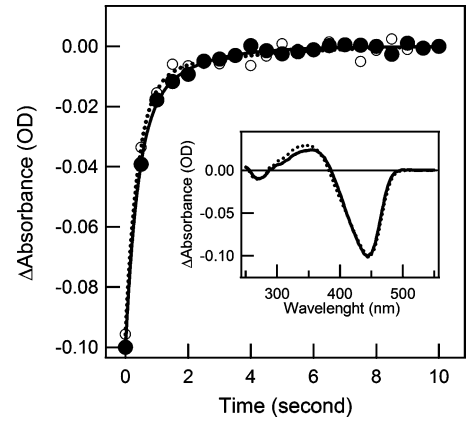

Figure 4. Comparison of the last photocycle step in the PYPs from *I. loihiensis* and *H. halophila* at pH 7.5. Kinetics of pB decay in Il PYP (solid line and closed circles) and Hh PYP (dotted line and open circles) detected at 446 nm. In both PYPs the decay is biexponential. The time constants for Il PYP were 230 ms (80%) and 5.7 s (20%), and for Hh PYP 380 ms (75%) and 1.6 s (25%). The inset depicts the light-dark UV/vis difference spectra of Il PYP (solid) and Hh PYP (dotted) calculated using the absorbance spectra a $t = 0$ s and $t = 10$ s.

reduction in the unassigned side chain signal at 1590 cm^{-1}. This indicates that the light-induced conformational changes in Il PYP are similar but somewhat smaller compared to those in Hh PYP.

To complement the detection of the kinetics of pB decay in Il PYP by visible absorbance spectroscopy (Figure 4), we extracted the time dependence of the amplitude between the negative amide I difference signal at 1642 cm^{-1} and the positive amide I peak at 1624 cm^{-1} (Figure 5C). This revealed a biphasic decay with a major fast phase of 0.30 s and a minor slow phase of 3.6 s, very similar to the values obtained by visible absorbance spectroscopy. The kinetics of the amide I signals of Il PYP during pB decay were compared to those obtained for Hh PYP, confirming that the kinetics of the last photocycle step in these two proteins is very similar (Figure 5C).

Identification of TAL and pCL in *I. loihiensis.* The *in vivo* functioning of PYP requires the biosynthesis of its *p*CA chromophore and covalent attachment to apoPYP. In *Rhodobacter capsulatus* this is achieved by the activity of two proteins: tyrosine ammonia lyase (TAL) and *p*-coumaryl:CoA ligase (*p*CL).[34] TAL converts tyrosine into pCA and pCL activates the *p*CA by attachment to coenzyme A. The coumaryl CoA then reacts with apoPYP, resulting in the formation of a thioester bond between *p*CA and the conserved Cys side chain at the active site of PYP. Since the *I. loihiensis* genome encodes a functional *pyp* gene, we examined the presence of possible *tal* and *pcl* genes in its genome.

The *I. loihiensis* genome encodes two proteins with significant sequence similarity to the known TAL from *Rb. capsulatus* (Supplemental Figure 1, Supporting Information). The product of gene IL135 shares 31% sequence identity and 46% sequence identity with the TAL from *Rb. capsulatus*; for gene IL2450 these number are 28 and 43%. The two TAL candidates in *I. loihiensis* are fairly divergent: they share 28% sequence identity and 49% similarity. In *H. halophila* the *pyp* gene is present in an apparent three-gene operon, together with a *tal* and a *pcl* gene.[22] The TAL from *H. halophila* is 32% identical to product

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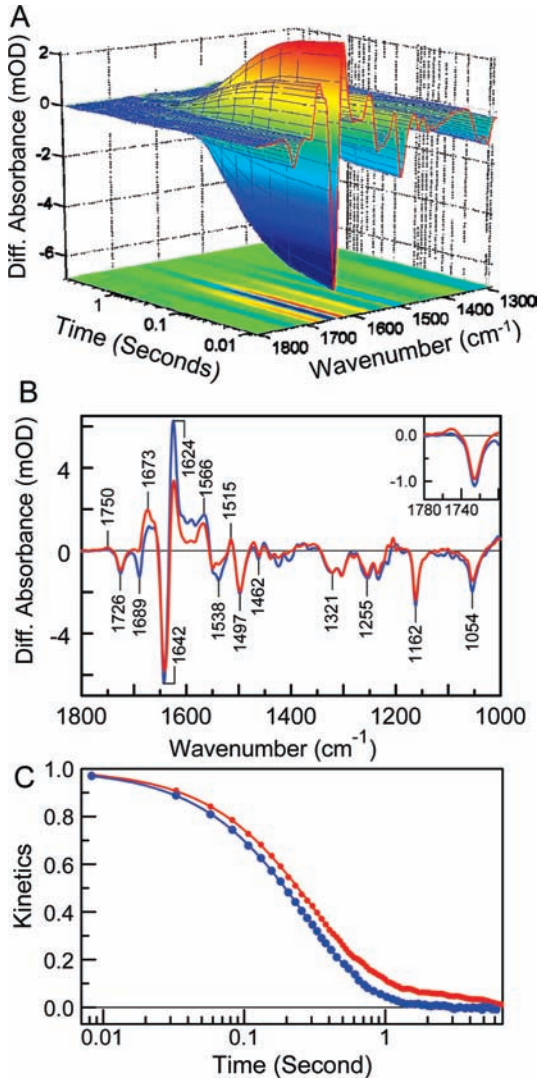

Figure 5. Time-resolved FTIR difference spectroscopy of the PYP from *I. loihiensis*. (A) Three-dimensional plot of the time-resolved rapid-scan FTIR difference absorbance signals during the photocycle of Il PYP in D_2O at pH* 6.6. (B) Light-induced FTIR difference spectra between the long-lived pB intermediate and the initial pG state for the PYPs from *I. loihiensis* (red) and *H. halophila* (blue). In the inset the difference spectra in the spectral region 1780−1760 cm^{-1} are enlarged. (C) Kinetics of the last step in the PYP photocycle of Il PYP (red) and Hh PYP (blue) monitored by the peak to peak difference between the amide I signals at 1642 and 1624 cm^{-1}. The solid lines are a biexponential fits of the data, with time constants of 0.30 s (89%) and 4 s (11%) for Il PYP, and 0.26 s (92%) and 0.8 s (8%) for Hh PYP.

of gene IL2450 and 26% identical to product of gene IL135. These four proteins all are approximately 500 amino acids in length.

A highly conserved Ala-Ser-Gly fragment of ammonia lyases undergoes spontaneous cyclization to form the 4-methylene-imidazole-5-one cofactor of the enzyme.[35] This ASG sequence is conserved in the two TAL candidates in *I. loihiensis*. The crystal structure of the TAL from *Rb. sphaeroides*[36] has revealed 9 residues that form the substrate binding pocket. Of these 9 residues 8 are conserved (Tyr60, Phe66, His89, Leu153, Tyr300, Arg303, Asn435, and Gln436) in the two *I. loihiensis* proteins. His89 is of particular importance, because hydrogen bonds with the phenolic oxygen of the Tyr substrate, and mutagenesis has shown that this residue is critical for its substrate specificity.[36] In view of this high level of conservation of functionally critical residues, the productes of *I. loihiensis* genes IL135 and IL2450 are very likely to encode functional TALs. Both of these genes are not in the immediate vicinity of the *pyp* gene (*I. loihiensis* gene IL2385). This is in contrast to the situation in *H. halophila*,[22] and in *Rb. capsulatus*,[37] where *tal* is in the located very close to the *pyp* gene.

A single gene with high sequence similarity to the *pcl* genes from *Rb. capsulatus* and *H. halophila* is present in the *I. loihiensis* genome (Supplemental Figure 2, Supporting Information). This gene (IL2385) encodes a protein with ∼28% sequence identity and ∼43% sequence similarity to the known *p*CLs from *Rb. sphaeroides* and *H. halophila*;[22,34] all three proteins are ∼400 residues in length. The crystal structure of the 4-chlorobenzoate:CoA ligase from *Alcaligines sp. AL2007* has been reported.[38] This structure revealed functionally important residues in the substrate binding pocket. Comparison with the protein encoded by gene IL2385 reveals that key residues in the substrate binding pocket are conserved: His204, Ile303, and Gly305 (numbering for 4-chlorobenzoate:CoA ligase). In CoA ligases for small acyl substrates Gly305 is substituted with Trp, supporting the conclusion that the *I. loihiensis* enzyme binds the larger *p*-coumaryl substrate. Finally, Val205 in the 4-chlorobenzoate:CoA ligase is substituted with Ile in the CoA ligase in *I. loihiensis*. In plant *p*-coumaryl:CoA ligases this position is important for substrate specificity, and contains Ile,[39] as does the *I. loihiensis* enzyme. Interestingly, gene IL2385 in *I. loihiensis* is located immediately downstream of the *pyp* gene in an apparent operon (Figure 6A). This is strong independent support for its functional assignment as a *p*CL.

4. Discussion

Functional Diversity in the PYP Family. Hh PYP and Il PYP share 75% sequence identity. This level of sequence similarity ensures that the two proteins have very similar three-dimensional structures. However, this sequence-based conclusion does not transfer to their functional properties. For example, the absorbance spectra of the red and green pigments in human color vision differ by 30 nm, while these proteins share 96% sequence identity.[40] Similarly, the absorbance peaks of the green and blue variants of proteorhodopsin differ by 35 nm, but their amino acid sequences are >78% identical.[41] The lifetime of the pB state in the PYP family varies from milliseconds to hours, and single point mutations can greatly alter pB decay kinetics in Hh PYP.[6] The results reported here demonstrate that the properties of Hh PYP and Il PYP are highly similar despite their very different biological functions.

In *Rhodocista centenaria*, a PYP-phytochrome hybrid containing a histidine kinase domain regulates the activity of

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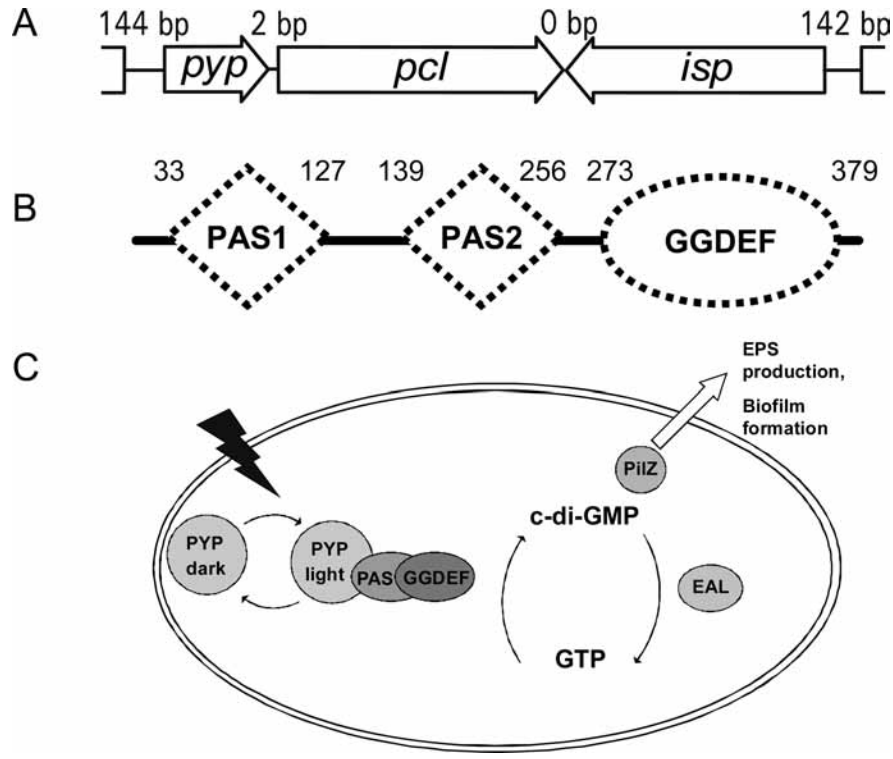

Figure 6. Model for the regulation of biofilm formation in *I. loihiensis*. (A) Genetic context of the *Il pyp* gene. Genes are represented to scale as arrows: *pyp* is photoactive yellow protein (125 residues, IL2385), *pcl* is *p*-coumaryl:CoA ligase (422 residues, IL2386), *isp* is intracellular signaling protein, see panel B (392 residues, IL2387). The length (number of basepairs) of the intergenic regions is also indicated. (B) Domain architecture of the intracellular signaling protein (IL2387) immediately downstream of the *pcl* gene. The numbers of the residues at the start and end of each domain are indicated. (C) Model for light-regulated biofilm formation in *I. loihiensis*. The proposed effect of PYP photoexcitation on the synthesis of c-di-GMP is indicated, together with the resulting physiological changes that result in biofilm formation.

chalcone synthase, which is involved in the biosynthesis of photoprotective pigments.[42] The PYP from *H. halophila* functions as the photoreceptor for negative phototaxis.[43] The biological function of the PYP from *Thermochromatium tepidum* is not known, but it is part of a larger protein that also contains a diguanylate cyclase domain.[44] The data reported here reveal that the biological function of the PYP from *I. loihiensis* is to photoregulate biofilm formation. This indicates a high level of functional versatility in the PYP family. The difference in function between Il PYP and Hh PYP is in striking contrast with the high level of conservation in both amino acid sequence and a range of functional properties: the *p*CA absorbance maximum and pK_a, pB lifetime, and photocycle-associated active site proton transfer and conformational changes. These data suggest a recent divergence in the biological function of these two PYPs.

The diversity of output signals generated by PYP photoexcitation (phototaxis, pigment synthesis, and biofilm formation) appears to be a general theme in photobiology. Also in the case of LOV,[45] BLUF,[46,47] and phytochromes[48] great diversity if seen for the output domains connected to the photosensors and in the biological responses that they trigger.

Approach to Study *in vivo* PYP Function. For 9 of the 12 bacteria known to contain PYP,[6] no genetic tools are available. This presents an important barrier to studies on the *in vivo* function of PYP, and has precluded genetic confirmation of the function of Hh PYP in negative phototaxis. Here we develop and apply the use of locked *p*CA analogs to examine the involvement of PYP in light-regulated biofilm formation in *I. loihiensis*. This provides a generally applicable tool for studies on the *in vivo* role of PYP, even in organisms that are not genetically accessible. Thus, while the biological function of the highly studied PYP from *H. halophila* as the photoreceptor for negative phototaxis still has not been conclusively determined using a genetic approach, the results reported here provide compelling biochemical evidence for the biological function of the PYP in *I. loihiensis*.

Since PYP is the only photoreceptor known to contain *p*CA as a cofactor, the detection of opposing biological effects upon the addition of trans-locked and cis-locked *p*CA provides strong evidence for a role of this photoreceptor in the photoresponse studied. Coumaric acids are known to pass the microbial cell envelope relatively easily.[49] Presumably, after passing the cell membrane, the *p*CA analog will be incorporated into PYP by *p*-coumaryl:CoA ligase (pCL),[34] depending on the substrate specificity of the *p*CL. *De novo* biosynthesis of PYP and PYP turnover will thus result in the incorporation of *p*CA analogs into PYP. The difference between cells exposed to trans-locked

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and *cis*-locked *p*CA would be expected to reveal the maximal physiological response triggered by PYP. This presents a generally applicable chemical approach to examine the involvement of PYP in a photoresponse.

The degree of biofilm formation by *I. loihiensis* in the presence of trans-locked *p*CA would be expected to be identical to that for cultures grown in absolute darkness. However, the experiments (Figure 1) showed a somewhat lower degree of biofilm formation for cultures grown in darkness. Two possible explanations for this observation are (i) that small amounts of light reached the cultures during the course of the experiments, or (ii) that a small amount of dark noise occurs in PYP reconstituted with native *p*CA so that even in the absence of light a basal level of signal is generated. The current data do not allow these two effects to be distinguished.

Correlating the Time Scales of Receptor Activation and Biological Response. The photocycling rates of photosensory proteins cover a wide range of time scales, from milliseconds to hours. In the PYP family the lifetime of the pB state varies from minutes and hours for the PYPs from *Rhodocista centenaria*[42] and *Salinibacter ruber*[6,16] to hundreds of milliseconds for the PYP from *H. halophila*.[8,15] The photocycles for the PYPs from *Rb. sphaeroides* and *Rb. capsulatus* are even faster.[14,37,50]

Interestingly, the lifetime of the signaling states[51] in a photocycle appears to match the typical time scale of the biological response that they trigger.[52,53] A correspondence between the lifetime of an activated signaling component and the time scale of the biological response that it triggers has also been reported for two-component regulatory systems. In this case the lifetime of phosphorylated response regulators varies according to the duration of the response.[54,55] Hh PYP and the archaeal sensory rhodopsins both exhibit a photocycling time of ~0.5 s and both trigger phototaxis responses, which occur on a subsecond to second time scale.[43,56] The slower photocycling time of the PYP from *R. centenaria* (~50 s) corresponds to its function in the regulation of gene expression.[42] A similar correspondence is seen in BLUF domain proteins.[46] The BLUF domain protein AppA functions as an antirepressor, and exhibits a photocycle of 15 min.[57] In contrast, the BLUF protein PixD is a photoreceptor for phototaxis in *Synechocystis*, with a photocycle time of ~10 s.[58,59] The LOV-based phototropins in plants,[60] which trigger phototropism, chloroplast movement, and stomatal opening, also exhibit slow photocycling times (from minutes to hours).

Here, we present an example of a photosensory protein that does not follow this trend. Il PYP exhibits a fast photocycling time but triggers a slow biological response: biofilm formation. Strikingly, despite the highly distinct biological functions of the PYPs from *I. loihiensis* and *H. halophila*, their photocycling times are essentially identical. Thus, the overall kinetics of a photocycle cannot be directly used to infer information regarding the time scale of the biological response that it triggers. This result also has implications for modeling of signaling pathways in the living cell based on kinetic information for the individual signal transduction components.

Biofilm formation in *I. loihiensis* exposed to trans-locked versus cis-locked *p*CA differs by a factor ~5, while incubation in the dark versus light results in a difference by a factor ~1.6. The smaller effect of light exposure is in line with the fairly short lifetime of the pB state of Il PYP. The difference between the two locked *p*CA analogs provides an estimate of the maximal amplitude of this physiological response. Apparently, the light intensities used in the experiments reported here do not trigger this maximal response. In view of the pB lifetime of Il PYP, this light intensity would be expected to yield a photostationary state in which 5−10% of the PYP molecules in the cell are in the pB state, which may yield a submaximal response. This analysis suggests that Il PYP functions as a photoreceptor for relatively high light intensities, providing an explanation for the apparent mismatch between the photocycling time of Il PYP and the time scale of the biological response that it triggers.

Light Regulation of Biofilm Formation. Biofilm formation is a complex process. Two key external stimuli known to regulate biofilm formation are nutrient availability and quorum sensing.[61,62] Here we report a novel external stimulus that affects biofilm formation: light.

Recently it was reported that cell attachment in *Caulobacter crescentus* is regulated by blue light via the LOV-histidine kinase photoreceptor protein LovK.[63] Key differences with the results reported here are that (i) attachment of *C. crescentus* occurs not via expolysaccharides but through a specialized stalk, (ii) in *I. loihiensis* a *p*CA-based PYP and not a flavin-based LOV photoreceptor regulates cell attachment, (iii) blue light inhibits biofilm formation in *I. loihiensis* but promotes cell attachment in *C. crescentus*, and (iv) the photocycling time of the LovK is on the hours time scale, while that of Il PYP occurs on the subsecond time scale. For LovK it has been proposed that light provides information on the position of the organism in the water column.[63] A similar biological role is plausible for light-regulated biofilm formation by PYP in *I. loihiensis*.

A number of features in the *I. loihiensis* genome suggest a model for the signal transduction chain in PYP-directed light regulation of biofilm formation. The Il *pyp* gene is present in an apparent operon with a downstream gene encoding a *p*CL (Figure 6A). The two-gene *pyp-pcl* operon is immediately adjacent to a gene encoding a multidomain signaling protein that is transcribed convergently with the *pcl* gene and overlaps with it by three base pairs. This signaling protein consists of two N-terminal PAS domains and a C-terminal GGDEF domain

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(Figure 6B). The latter domain presumably exhibits diguanylate cyclase activity, which catalyzes the conversion of GTP into c-di-GMP.[17−19] Recently c-di-GMP has emerged as a central regulator for biofilm formation.[20] The *I. loihiensis* genome encodes 32 genes encoding enzymes involved in exopolysaccharide synthesis and transport, 34 proteins containing an GGDEF domain, and 22 proteins containing an EAL domain.[5] The EAL domain is involved in the breakdown of c-di-GMP through its phosphodiesterase activity.[64] In addition, the *I. loihiensis* genome contains 3 proteins that have been annotated to contain a PilZ domain, which function as a receptor for c-di-GMP.[20] These observations suggest the possibility that Il PYP regulates the diguanylate cyclase activity of the downstream GGDEF protein (Figure 6C). A functional link between PYP and c-di-GMP is confirmed by the PYP from *Thermochromatium tepidum*, which contains a diguanylate cyclase domain.[44]

Genome-Based Identification of Photobiology in Chemotrophs. *I. loihiensis* was identified in samples taken 35 km off the coast of Hawai'i at 1300 m below the water surface near a hydrothermal vent and is closely related to an uncultivated organism found at 11 000 m depth in the Mariana Trench.[4] Thus, *I. loihiensis* is regarded as a deep sea bacterium. At such depths no sun light penetrates, and organisms in this environment live in complete darkness. *I. loihiensis* is an aerobic heterotroph capable of amino acid catabolism[5] and has not been reported to exhibit photobiological responses.

The results reported here that *I. loihiensis* contains a functional PYP and exhibits light-induced suppression of biofilm formation are strong evidence that this organism also lives in a part of the ocean's water column where its biology is influenced by light. The inhibition of biofilm formation by light suggests that planktonic *I. loihiensis* cells are favored in the light, while biofilms form at greater depths. The metabolism of *I. loihiensis* is centered around amino acid catabolism, and it has been proposed that the organism grows on the proteinaceous particles that are present near deep sea hydrothermal vents.[5] *I. loihiensis* is likely to form biofilms on such particles and may be dispersed when such particles flow to the ocean's surface.

5. Conclusions

I. loihiensis can form biofilms, and this process is inhibited by light. This light regulation of cell physiology is unexpected for a chemotrophic deep sea bacterium, and indicates that the organism also occurs in illuminated areas of the water column, close to the ocean surface. Genome sequencing projects have revealed photoreceptors in many chemotrophic bacteria. Thus it is likely that many novel light responses remain to be discovered in these organisms.

A novel approach to study *the in vivo* function of PYP was developed based on the use of trans-locked and cis-locked derivatives of the *p*CA chromophore in PYP, which results in constitutively inactive and constitutively active PYP derivatives, respectively. This approach was used to demonstrate that PYP is the photoreceptor for light-induced suppression of biofilm formation in *I. loihiensis*. The approach is generally applicable, and is particularly valuable for studies on organisms for which no genetic tools are available.

The genome of *I. loihiensis* encodes two enzymes that are likely to have TAL activity, and in addition it contains a *pcl* gene that forms an apparent operon with the *pyp* gene. This indicates that *I. loihiensis* contains the two enzymes for *p*CA synthesis and activation that allow holoPYP production.

The identification of Il PYP as the photoreceptor for biofilm regulation in *I. loihiensis* broadens the range of biological processes regulated by PYP. Such diversity in output signals is an emerging theme from studies on different types of photoreceptors.

Heterologously overproduced Il PYP is a yellow and photoactive protein. Its visible absorbance maximum, *p*CA pK_a, active site proton transfer, and pB lifetime are very similar to the well-studied PYP from *H. halophila*, despite the very different biological function of Hh PYP. The genome of *I. loihiensis* encodes proteins with clear homology to *p*CL (immediately downstream of the *pyp* gene) and TAL for the synthesis and activation of the *p*CA chromophore of PYP.

The fast kinetics of pB decay in Il PYP do not match the slow biological function that it triggers, providing a counterexample for a proposed correlation between the time scales for the photocycle of a photoreceptor and the biological response that it causes.

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Supporting Information Available: Synthesis method for locked chromophores; TAL multiple sequence alignment; *p*CL multiple sequence alignment. This material is available free of charge via the Internet at http://pubs.acs.org.

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