Photochemistry and Photobiology, 2015, 91: 1012–1020

Invited Review

Photoreceptors in Chemotrophic Prokaryotes: The Case of *Acinetobacter* spp. Revisited†

Clara B. Nudel*[1] and Klaas J. Hellingwerf[2]

[1]Nanobiotec Institute, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Ciudad de Buenos Aires, Argentina
[2]Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

Received 2 June 2015, accepted 8 June 2015, DOI: 10.1111/php.12491

ABSTRACT

A comprehensive description of blue light using flavin (BLUF) photosensory proteins, including preferred domain architectures and the molecular mechanism of their light activation and signal generation, among chemotrophic prokaryotes is presented. Light-regulated physiological responses in *Acinetobacter* spp. from environmental and clinically relevant strains are discussed. The twitching motility response in *A. baylyi* sp. *ADP1* and the joint involvement of three of the four putative BLUF-domain-containing proteins in this response, in this species, is presented as an example of remarkable photoreceptor redundancy.

INTRODUCTION

When the genome sequencing revolution revealed the protein complement that can be synthesized by any of a large range of living organisms, it became clear that photosensory receptor proteins are also present extensively among the chemotrophic organisms (1). This was to a certain extent surprising because prior to that development it was generally assumed that such functionality would exclusively be found in phototrophic organisms, *e.g.* to regulate expression of the photosynthesis machinery and/or to protect cells from harmful exposure to short-wavelength radiation, with a few notable exceptions like the human visual rhodopsins (2). But now that we know that they exist, it is important to characterize their function, *e.g.* because their presence may cause irreproducibility in the outcome of experiments in which the light climate was not stringently controlled. Reversely, their presence, like the YtvA protein from *Bacillus subtilis*, may open up entirely new opportunities for basic research of the mechanism of signal transduction and signal integration in bacteria (3,4). A basic understanding of their functionality furthermore may help in designing preventive strategies against organisms that turn out to be pathogenic and/or virulent.

Also in members of several of the species in the genus *Acinetobacter*, genes encoding such photosensory receptors have been identified and to a certain extent characterized. Strikingly, the diversity of these photoreceptors in this genus is limited to members of the BLUF family of proteins, but among these significant diversity has emerged, just like for several other signal transduction proteins (5). Here we will review the work carried out on the characterization of photoreceptors and photosensing in the various species of the genus *Acinetobacter*.

Photoreceptors: families, primary photochemistry and domain architecture

Photoreceptor proteins present in phototrophic prokaryotes generally have a role in regulating gene expression for energy conservation and/or in phototactic responses. Recently, however, the finding that photoreceptors involved in a wide array of physiological responses are also present among chemotrophic organisms from various taxonomic groups, has challenged this view. For example, it has been described that in chemotrophs they may have a role in cell signaling processes, pigment synthesis, nucleotide metabolism, adhesion, tactic migration and pathogenesis, thereby integrating other environmental signals such as temperature, redox state and salt stress in some of the responses (6–9). To date we can conclude that all known photoreceptor proteins are to a very high degree conserved between phototrophic and chemotrophic organisms. They belong to six main well-described families, based on the structure of their light-absorbing chromophore: (bacterio)rhodopsins, phytochromes, xanthopsins, cryptochromes/photolyases, phototropins and flavoproteins with BLUF or LOV domains, covering the visible to near infrared range of the spectrum of electromagnetic radiation, between 380 and 750 nm (Fig. 1) (10). Beyond these six, a few less well-characterized families, with only one or a few representatives, have more recently been added, based on the structure and photochemistry of their light sensitive chromophore. Examples are: the orange carotenoid protein (OCP) present in cyanobacteria and probably in Mycobacterium (11) and the UV photoreceptor protein UVR8, so far only described in plants (12), the red-light (stress) receptor RsbP from *B. subtilis* (13) and methylcobalamin containing photosensors, like CarH from *Myxococcus xanthus* (14).

With respect to the specificity of the wavelength of maximal absorption, most rhodopsins are red-yellow light photoreceptors, while some have shifted their maximum to the blue-green part of the visible spectrum; most phytochromes sense in the red and

*Corresponding author email: cnudel@ffyb.uba.ar (Clara B. Nudel)
†Most of the material discussed in this article was presented during the 16th International Congress on Photobiology held in Cordoba, Argentina, in September (8th – 12th), 2014.

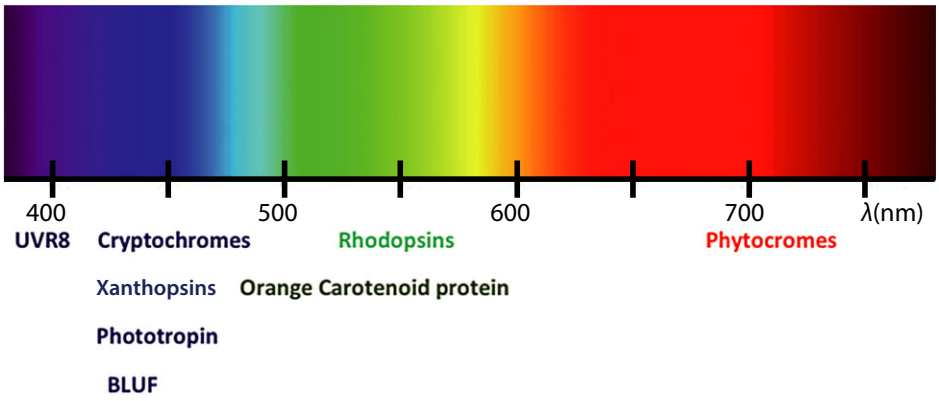

Figure 1. Families of photoreceptory proteins and the wavelength of absorption of their respective chromophores.

far-red zone, but also for these one encounters some blue-green light responding representatives; and xanthopsins, cryptochromes, phototropins and the flavin-containing photoreceptor proteins with a BLUF or a LOV domain, are blue light receptors. OCP absorbs blue-green light and UVR8 is a UV-B photoreceptor (Fig. 1).

Strikingly, only a relatively small number of chromophore structures provide color to photoreceptor proteins. The phytochromes, rhodopsins, xanthopsins and the (orange) carotenoid protein contain (a) linear tetrapyrrole, retinal, coumaric acid and a carotenoid molecule, respectively, all carrying a reactive double bond that can be used to initiate the *trans-cis* isomerization reaction after photon absorption, that is typical for these families. Cryptochromes, phototropins, containing one or more LOV domains, and BLUF-domain-containing proteins, make use of flavins (mostly FMN or FAD), which, upon photoactivation, promote the transfer of an electron or hydrogen atom, to either form an adduct, or initiate an isomerization (*e.g.* a side-chain flip; see further below) in an amino acid side chain of the surrounding apoprotein. The UVR8 photoreceptor is a-typical, as just as in the fluorescent proteins like GFP, its chromophore is formed by a group of highly reactive tryptophan residues, which upon photoactivation form (*via* Trp233) a radical ion pair Trp285(+)–Trp233(−), which disrupts the salt-bridge stabilized UVR8 dimers, and initiates signaling (12).

Another distinct feature of the photoreceptor proteins is the site of binding of their chromophore, which, in most cases, is easily recognizable as a subdomain, with a few highly conserved amino acids in each type of photosensory protein. Typical domains for blue light-absorbing chromophores are the LOV (light, oxygen voltage) domain for FMN, the BLUF (blue light using flavin) domain for FAD, the PYP domain for *p*-coumaric acid and the Cry-DASH domain for FAD plus pterin (MTHF). Note that cryptochromes usually harbor two binding domains for their chromophores, with the flavin as the catalytic one. Typical chromophore-binding domains for red-light-specific sensory proteins, mostly accommodating a bilin, include the PHY (from: phytochrome) and the GAF (c**G**MP-specific phosphodiesterases, **a**denylyl cyclases, **F**hlA) domain.

Photosensory domains are arranged in well-defined architectures; usually one or more of them are part of a multidomain protein. Prototypic arrangements are extensively described in the literature for LOV-domain-containing proteins (15) as well as for phytochrome- and BLUF-domain-containing proteins (16,17). In the latter, the output domains identified so far include SCHICH, EAL, cyclase, HTH, GGDEF, PAS or combinations thereof. Nevertheless, BLUF-domain-enclosed proteins also show a high frequency of "single" or "small" BLUF proteins with no presently known output domains.

Blue light photoreceptors in phototrophic and chemotrophic prokaryotes

It is now generally accepted that photosynthetic as well as non-photosynthetic (*i.e.* chemotrophic) prokaryotes contain photosensory proteins that enable them to integrate light signals into environment-sensing networks. Among the bacterial photoreceptors, the bilin-containing phytochromes appear to be the most abundant and, at the same time, the most variegate family, with genes in approximately 17% of all sequenced bacterial genomes, a wide range of wavelength sensitivity, and displaying either a monochromic or a photochromic response. The next most abundant photoreceptor classes are represented by the flavin-based blue light photoreceptors containing a BLUF or a LOV domain, which each account for approximately 13% of the total. The cryptochromes, rhodopsins and the PAS-domain-based Yellow Proteins are less widely distributed; the total number of photoreceptors belonging to the cryptochrome family is difficult to estimate because of their sequence similarity to photolyases (18).

For both BLUF and LOV domains, it is possible to identify 10 to 12 highly conserved amino acids that can identify all family members of the respective families in a whole/multiple genome search. A similarly strict set of conserved amino acids could not be defined for the family of bilin-binding GAF domains (17). Particularly in the BLUF family, most of the residues that are conserved among all members of the BLUF family serve in cofactor binding, and probably in stabilization of the signaling state. A search using the 10 most conserved amino acids (Tyr-9, Ser-11, Asn-33, Gln-51, Leu-63, Phe-64, Ile-67, Asp-70, Arg-72 and His-73) of the blue light photoreceptor AppA from *Rhodobacter spheroides* in fully sequenced bacterial genomes (2143 at the time of this analysis, March 2013) revealed that around 1/5 (431 strains) harbor genes with a recognizable flavin-binding (BLUF) domain. Surprisingly, most of these strains were chemotrophic (383) and only 42 were phototrophic bacteria (Fig. 2).

Losi *et al.* used a different approach, identifying sequence logos in an alignment including *ca.* 2000 protein sequences from eukaryotic and prokaryotic organisms, for each of the two photosensing domains, LOV and BLUF, present in the proteins, in

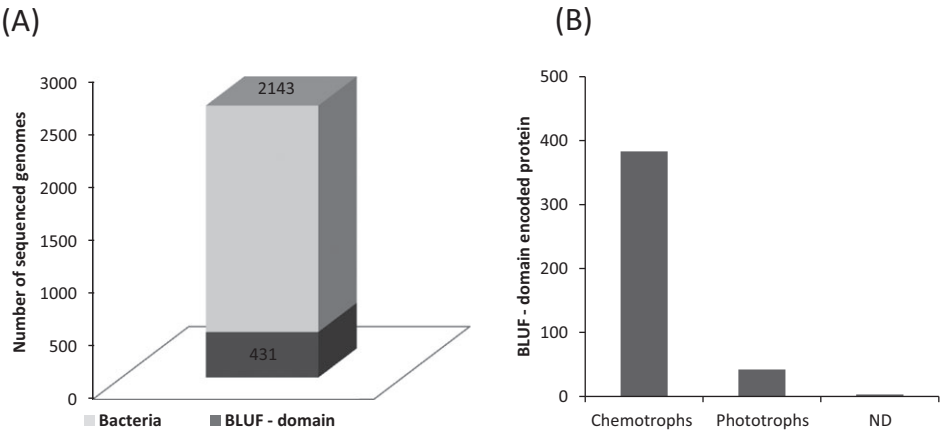

Figure 2. (A) Abundance of BLUF-domain-containing proteins in sequenced bacteria genomes. (B) Distribution of putative proteins with flavin-binding (BLUF) domains in chemotrophs, phototrophs, and non-determined (ND) bacteria.

which besides the essential amino acids interacting with the chromophore, other residues that showed high conservation were included (19). With this new algorithm they identified for the first time 167 archaeal LOV proteins in 86 strains (of 82 species). The search reported also 1705 bacterial BLUF proteins in 1282 strains (in 453 species; many strains were from *E. coli*, *Acinetobacter* sps. and *Klebsiella pneumoniae*) and 1390 LOV domains in 1031 bacterial strains (658 species), which shows that this search revealed many more candidates than previously anticipated. Moreover, in 162 species/strains LOV- and BLUF-domain-containing proteins were both present, hereby expanding the range of putative networks with multiple photosensory proteins functioning in individual cells, a characteristic presently only described for *R. sphaeroides* (19) and *Pseudomonas syringae* (this latter species carries actually three photosensory protein-encoding genes predicted to encode a blue light-sensing LOV histidine kinase (LOV-HK) and two red/far-red light-sensing bacteriophytochromes, BphP1 and BphP) (20).

In spite of the high frequency of occurrence of (putative) photoreceptor sequences in bacterial genomes, the physiological relevance of light sensing has been demonstrated only in a small group of chemotrophic species. Photoresponsive chemotrophs identified at present are *P. aeruginosa* (21)*, B. subtilis* (4), *E. coli* (22), *B. abortus* (23), *S. aurantiaca* (24), *R. leguminosarum* (25), *A. baumanii* (26), *A. tumefasciens* (27) *and C. crescentus* (28), all inhabiting very diverse ecological niches and displaying different lifestyles.

Photoreceptors in the genus *Acinetobacter*

Acinetobacter spp. belong to the Moraxellaceae family of Gamma-proteobacteria. The order (*Pseudomonadales)* is shared by bacteria in which not only single photoreceptor-encoding genes of the bacteriophytochrome type have been identified (*i.e. P. aeruginosa*) but also multiple blue and red light photosensory protein-encoding genes, as in the bean pathogen *P. syringae*, mentioned above. So far, in the *Acinetobacter* genus only BLUF-domain-encoding genes of photoreceptor proteins have been identified through genome mining. The first report on this issue already spotted one putative BLUF-domain-containing protein in *A. baumanii's* genome, whereas the genome of *A. baylyi* sp. *ADP1* was shown to encode four putative photoreceptor proteins of the BLUF type (1). It is noticeable among nonphototrophic species that a certain variation in the number of photoreceptors that they contain occurs, even between closely related genera. Among the enteric bacteria, for instance, all currently sequenced species of *E. coli* contain a single BLUF-domain photoreceptor, whereas organisms from the genera *Salmonella* and *Yersinia* have none, and the *Klebsiella* species have two (18). These numbers are nevertheless modest compared to the redundancy encountered in *Acinetobacter* sp. *ADP1*, so far an uncommon phenomenon among chemotrophic bacteria. Such a distribution, nevertheless, is in line with what has been observed for other signal transduction systems before: the transfer of genes between species and genera generally is a bottleneck in the spreading of genes, but once established in a particular species, a particular system may rapidly diversify through gene duplication mechanisms, as it has been observed with kinases and response regulators of two-component regulatory systems (29).

The analysis of the architecture of BLUF-domain-containing proteins in the genomes of 160 fully sequenced nonphototrophic bacteria confirmed the predominance of the single domain architecture for most of the BLUF-domain-containing proteins ("short BLUF"), including 31 *Acinetobacter* strains. The analysis also revealed the BLUF-EAL arrangement as the second most frequent, and confirmed the relative abundance of multiple photoreceptor proteins in some of the strains (Table 1).

An update on the list of *Acinetobacter* strains harboring putative BLUF-domain-encoding genes includes presently many more organisms of the *A. baumanii* group (that have been sequenced), but also other species, namely *A. oleivorans, A. nosocomialis, A. calcoaceticus, A. soli, A. pitti, A. venetianus, A. radioresistens, A. gyllenbergii, A. beijerinckii, A. guillouiae A. bereziniae, A. parvus, A. haemolyticus, A. ursinggii, A. tandoii, A. lwoffii, A. indicus, A. harbinensis, A. nectaris, A. gerneri, A. johnsonii and A. schindleri.* A total of 198 putative BLUF-domain-encoding genes in 119 fully sequenced *Acinetobacter* genomes were identified as for March 2015 (B. C. Nudel, unpublished).

Additionally, the number of BLUF-domain-encoding gene/s found in specific species of the genus is notably diverse; while a single gene is present in all fully sequenced *A. baumanii* genomes (24 strains), a more mixed composition, with single and multiple genes of the "single-BLUF" architecture are present in other species of the genus, including *A. radioresistens, A. calcoaceticus, A. pitti, A. oleivorans and A. beijerinckii*.

Table 1. Architecture of proteins containing (a) BLUF domain(s) in fully sequenced bacterial genomes and their relative abundance.

	Number of BLUF proteins in same species (frequency)						
Architecture	1	2	3	4	5	6	7
Short BLUF (112 bacteria)	73 (65.2%)	26 (23.2%)	6 (5.4%)	3 (2.7%)	2 (1.8%)	1 (0.9%)	1 (0.9%)
BLUF-EAL (58 bacteria)	37 (63.8%)	19 (32.8%)	2 (3.4%)	–	–	–	–

Molecular basis of photoactivation and signal generation in BLUF domains

The spatial molecular structure of BLUF domains (30) was resolved both with X-ray crystallography (31) and solution NMR (32) studies. The ~125 amino acids of these domains adopt an α/β-sandwich fold with a $\beta\alpha\beta\beta\alpha\beta\beta$ topology, such that a flavin (presumably mostly FAD; 33) is clamped between the two α-helices, while its isoalloxazine ring is resting on the five-stranded β-sheet. A wide range of BLUF domains has been studied at the molecular level, but most detailed information is available for these domains from AppA (34,35) and PixD (36). These will be discussed in the following, together with BlsA, the only photoreceptor protein encoded in the genome of *Acinetobacter baumannii* (26).

Upon photoexcitation of a BLUF domain with a blue photon (*e.g.* of 450 nm) an electronically excited state is formed with a quantum yield of ~0.25 (37), as a first step in a photocycle in which next, within a nanosecond, a stable ground state intermediate forms with a slightly (10–15 nm) red-shifted absorption spectrum. This red-shifted intermediate presumably is the signaling state of the protein. It relaxes to the dark state of the protein with a time constant characteristic for each BLUF domain, which may vary from minutes to hours (38). This very modest spectral red shift of the signaling state of BLUF domains makes it also relevant to consider the biological function of "branching" reactions, initiated by absorption of a second photon, that can be observed in many photosensory receptors (39,40), which effectively accelerate the dark-state recovery. Strikingly, such a branching reaction is not observable in the BLUF domain of AppA (41), which may be related to the importance of a long lifetime of its signaling state. Significantly, absence of branching activity is also observed in YtvA, a slow-recovering LOV domain that activates the general stress response in *B. subtilis* (42). Prior to photoactivation, the flavin chromophore of BLUF domains, like those in LOV domains, have to be in the fully oxidized form, which absorb maximally at 450 nm. Particularly for the BLUF domains, this could imply yet another mode of regulation, because the midpoint potential of these latter photosensory receptors is not far below the midpoint potential of key physiological electron donors (~ −260 mV; see (43) and significantly higher than those of LOV domains (which have their midpoint potential at around – 300 mV).

Consensus has not been reached yet as to how is the red-shifted intermediate exactly formed. For the BLUF domain of AppA it has been proposed (44) that its formation is facilitated by a coupled, reversible, electron-plus-proton transfer from a nearby tyrosine residue (Y21) to the isoalloxazine ring system of the flavin. This reversible "H" transfer allows a key glutamine residue from the β-5 strand of the β-sheet of the protein to "flip" (*i.e.* rotate 180 degrees); the correct positioning of a hydrophobic tryptophan side chain (W104 in AppA) is key to the success of these primary processes (42). This then leads to a change in the secondary structure of this β strand, because of a general weakening of the hydrogen bonds in the β-sheet. In the PixD protein this then eventually leads to dissociation of PixE from PixD and initiation of the phototaxis process (38). The alternative view holds that the key glutamine residue (*i.e.* Q63 in AppA-BLUF) changes its hydrogen-bonding interaction not through a flip, but through keto-enol tautomerization, without involvement of reversible electron or hydrogen transfer to the flavin ring system (45). Possibly both mechanisms are operative in different BLUF domains, *i.e.* the tautomerization in AppA and the transient electron/hydrogen transfer in PixD (46). In both mechanisms, nevertheless, the change in hydrogen-bonding interactions of the key glutamine residue is the primary, structural, signal-generating process (47). This transition also forms the basis of the similarity between BLUF and LOV domains (48), in spite of their very different primary photochemistry, which in LOV domains is based on covalent adduct formation between the ring system of FMN and a nearby cysteine side chain (47).

The signal transfer in BLUF-domain-containing proteins may therefore be as follows: Light absorption by the flavin chromophore results in hydrogen bond rearrangements between the flavin and the conserved residues lining the chromophore-binding pocket, and leads to a conformational change involving the β5 strand of the β-sheet. This induces further structural changes in the C-terminal, mostly α-helical part of the protein, which in turn affects the interaction with a cognate downstream signal transduction partner protein (38). The C-terminal α-helices in BLUF proteins are known to be able to adopt two different conformations (Fig. 3): They can lie either parallel (*e.g.* for BlrP1) or perpendicular to the β-sheet (*e.g.* in PixD). Biochemical analysis has shown that PixD interacts with signaling partner PixE only in the dark, whereas PapB interacts with its partner protein PapA under dark and light conditions. This indicates that the interactions of PapB and PixD with their corresponding partner protein differ. Significantly, also the light-induced NMR signals are different for the BLUF domains of AppA and BlrP1: Photoactivation broadens peaks in the AppA spectrum (32), whereas it shifts them in the BlrP1 spectrum (49). These characteristics may be related to different phototransduction mechanisms for the two proteins.

Using a chimeric protein consisting of the N-terminal core region of the BLUF domain of PixD, and the C-terminal α-helices of PapB, Ren *et al.* (50) could show that specificity for interaction with the downstream signaling partner resides in the (short) C-terminal domain of the protein. These results suggest that the C-terminal domain acts as an intermediary by receiving the light-induced signal detected by the flavin moiety of the BLUF domain and transmitting it to a downstream partner (protein). The different orientations of the α-helices in the C-terminal domain may reflect different light-induced signal transduction mechanisms to specific downstream components.

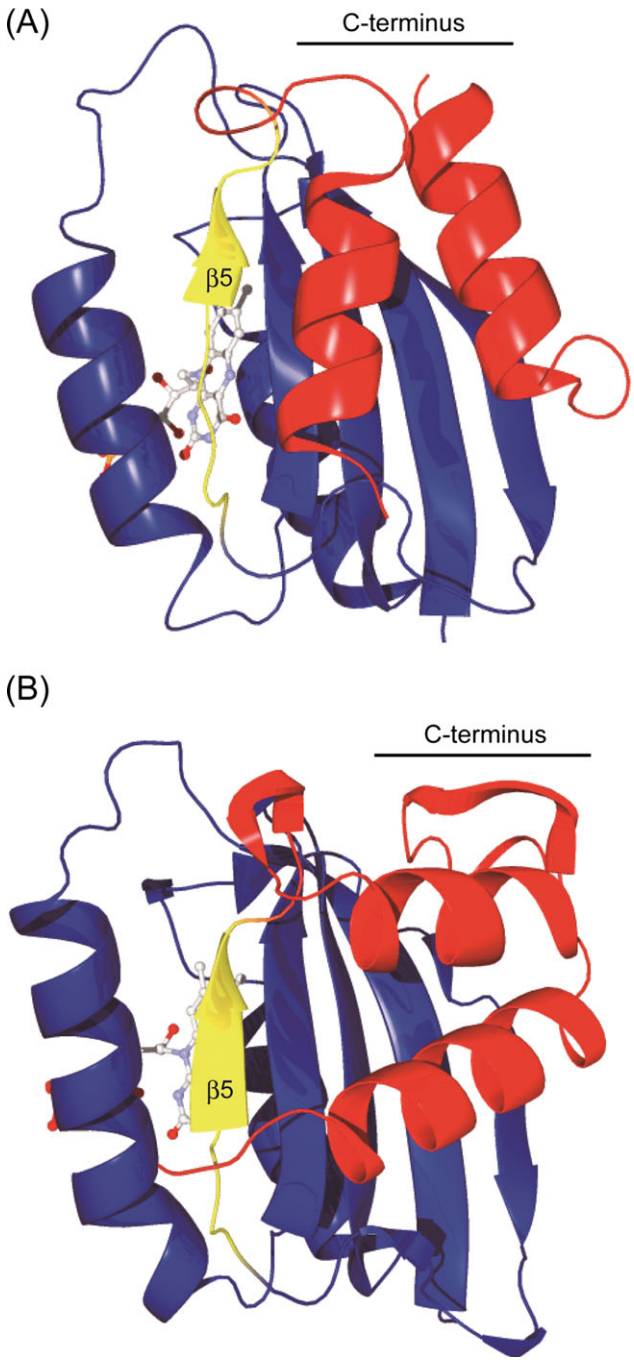

Figure 3. Ribbon diagrams of the BLUF-domain crystal structures from BlrP1 and PixD. (A) BlrP1 (PDB code 3GFZ). (B) PixD (PDB code 2HFN). The BLUF-domain core, the C-terminal α-helices and the β5 strand are colored blue, red and yellow, respectively. The β5 strand is responsible for signal transfer coordination between the flavin-binding pocket and the C-terminal α-helices. Reproduced with permission (38).

Also between BlsA and AppA the structural dynamics in the BLUF domain at the slower timescales (*i.e.* those relevant for structural dynamics in proteins) differ in at least one key aspect: The altered H-bonding around the glutamine that occurs upon photoactivation of the FMN chromophore strengthens rather than weakens the H-bonding in the BlsA β-sheet. This was revealed (51) by the change of sign of the β-strand marker mode in static IR-difference spectroscopy studies of these two proteins. This suggests that also the BlsA photoreceptor functions such that its activation by blue light leads to formation of a complex with a downstream target (protein) rather than that it would lead to complex dissociation.

Light-regulated physiological responses in *Acinetobacter* sps

During the past three decades the genus of the Gram-negative coccobacillus *Acinetobacter* has turned from a genus of questionable pathogenicity to a genus including a collection of species considered infectious agents of importance to hospitals worldwide (52). Whereas the spp. *baumanii, calcoaceticus, lwoffi* are the most commonly reported in the clinical literature, *Acinetobacter baylyi* sp. *ADP1* is considered a nonpathogenic strain, common in soil and ubiquitous in nature. It is metabolically very versatile and shows an interesting system for natural transformation. Although the genus has been identified by its lack of motility ("akinesis") due to the absence of motility-providing flagella, it uses polar thick fimbriae for surface migration through twitching motility (53,54).

Interestingly, we observed that migration through twitching motility in *Acinetobacter* sp. *ADP1* was modulated (*i.e.* inhibited) by illumination with visible light, but the molecular basis of this effect was not addressed at that time (55). A subsequent study extended this observation to a set of clinically relevant *Acinetobacter* strains; notably, all members of the so-called *A. calcoaceticus–A. baumannii* complex (including *A. baumannii* RUH134 and RUH875, *A. nosocomialis* RUH0503, *A. pittii* RUH0509 and *A. calcoaceticus* RUH0584) displayed blue light inhibition of twitching motility, whereas the strains *A. Iwoffi* RUH0045, *A. haemolyticus* RUH0044 and *A. junii* RUH0204 did not respond to blue light in assays of this characteristic (56).

The single *bluf*-domain-encoding gene, and the corresponding protein, from *A. baumanii* ATCC 17978 were studied by Mussi *et al.* (26). In this important contribution it was reported that in this strain, both, twitching motility and biofilm formation, were dependent on the expression of the gene A1S_2225, which encodes an 18.6-kDa protein carrying an N-terminal BLUF domain that lacks (a) detectable output domain(s) (*i.e.* a "short BLUF" or "single BLUF" domain). Moreover, the abovementioned phenotypes were observed only when bacterial cells were incubated in darkness, whereas the killing of *Candida albicans* filaments was enhanced when cocultured with bacteria in the light. Spectral analyses of purified recombinant protein (BlsA) showed its ability to sense light by an illumination-induced 10 nm red shift of its main absorption band, typical of BLUF proteins (26) (see above for a detailed description of the mechanistic aspects of the photoactivation of BLUF domains, including the BlsA photoreceptor).

The study on the influence of blue light and temperature (24°C *vs* 37°C) regulation of motility and biofilm formation was recently expanded to a large set of *Acinetobacter* strains harboring putative *bluf* genes in their genome (57). In spite of the broad gene distribution, the phenotype of light inhibition of twitching motility was only observed in a handful of isolates of the species *A. baylyi, A. calcoaceticus, A. nosocomialis, A. oleivorans, A. pittii* and *A. tjernbergiae*. The results on light regulation of biofilm formation were more diverse, in part due to the inherent differences in the type of biofilm formed by the strains (wall attached *vs* sedimented). It was reported that at least one strain of *A. baylyi, A. bereziniae, A. calcoaceticus,*

A. gerneri and A. rudis formed large amounts of biofilm on tubes incubated stagnantly under blue light for 4 days, while the levels of biofilms formed in the dark were significantly lower or negligible. Some strains of *A. beijerinckii, A. brissouii, A. guillouiae, A. johnsonii, A. lwoffii, A. nosocomialis, A. pittii, A. ursingii and A. venetianus* also showed photoregulation of biofilm formation, but the number of wall-attached biofilms was lower than in the aforementioned strains (57). So far we were unable to detect a statistically significant light driven response in the amount of biofilm formation in *Acinetobacter* sp. *ADP1*, both at 24°C and at 30°C (58). The question whether or not light regulated additional physiological responses, *e.g.* the transformation frequency and the influence of iron concentration on twitching motility and siderophore synthesis, are still under investigation (J. C. Arents and M. Bitrian unpublished results).

On the redundancy of the BLUF-domains in *Acinetobacter* sp. *ADP1*

We constructed single, double and triple knockouts of all four genes predicted in the genome of *Acinetobacter* sp. *ADP1* as encoding a blue-light-sensing using flavin (BLUF)-domain-containing protein, and measured the twitching motility response in the dark and under moderate blue light intensity at 24°C. The results obtained showed that individual knockout mutants of the genes ACIAD1499, ACIAD2125 and ACIAD2129, but not ACIAD2110, fully abolished the light dependency of the twitching motility response (Fig. 4). Double and triple knockouts confirmed the same phenotype as the corresponding single knockouts (not shown). From these results it was concluded that ACIAD2110, although expressed both in light and in darkness, as shown by RT-PCR experiments, was not related (or at most very minimally) to the twitching motility response.

Quantitative analysis of the transcript levels of the three genes involved in the light-dependent twitching response with Q-PCR showed a decreased expression in the light, with dark/light ratios of 1.65 ± 0.28, 1.79 ± 0.21 and 2.69 ± 0.39, for the genes ACIAD2125, ACIAD2129 and ACIAD1499, respectively. Moreover, a neighbor-joining tree of BLUF-domain sequences, built with members of the class of *Gammaproteobacteria,* grouped ACIAD2110 separately from the other three *bluf*-encoding genes (ACIAD1499, ACIAD2125 and ACIAD2129) and their relatedness with the *A. baumanii* sequences was supported by a bootstrap value of 66% (Figure S1, Supporting information). The same groups were described in the alignment published by Golic *et al.* (57) but instead they suggested an active role in the blue light inhibition of twitching motility response for ACIAD2110, supported by the fact that all four *A. baylyi* sp. *ADP1* BLUF-photoreceptors functionally replaced *in vivo* the *A. baumannii* 17978 BlsA protein, albeit with lower efficiency for ACIAD2110. This discrepancy with respect to the precise role of ACIAD2110 in the overall twitching motility response in *A. baylyi* sp. *ADP1* still needs to be further resolved**.**

Complementation of the single knockout strains (ACIAD1499KO, ACIAD2125KO and ACIAD2129KO), or the triple knockout on the same three genes (B++M3), with any of the three active BLUF-domain-encoding genes, fully restored the inhibition of twitching motility by blue light that is also observed in the wild type strain (Table 2). Notably, only ACIAD2110 did not (58). We conclude therefore that *A. baylyi* sp. *ADP1 s*hows a high degree of redundancy in the genes that encode BLUF-containing

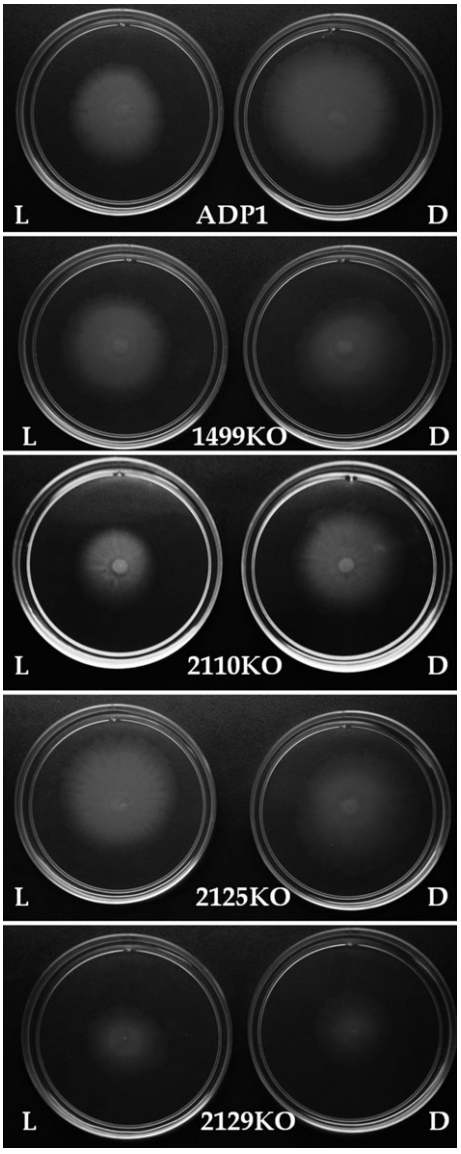

Figure 4. Effect of blue light on twitching motility in *A. baylyi* ADP1 and four single *bluf* knockout mutants, showing that, except KO2110, all knockouts lost the light dependent response. Cultures growing exponentially (OD_{600} = 0.3) were inoculated with 3 μL of *A. baylyi* ADP1, 1499KO, 2110KO, 2125KO or 2129KO in soft agar plates and incubated in darkness (D) or under blue light (L) at 24°C for 15 h. Reproduced with permission (58).

photoreceptors for the motility response. Additionally, as all plasmid-complemented strains, expressing any of the BLUF proteins irrespective of the specific set of deleted photoreceptors, restored the light-sensitivity phenotype compared to the wild type ($P << 0.001$), it is our working hypothesis that the three genes ACIAD1499, ACIAD2125 and ACIAD2129, are jointly required to inhibit twitching motility under moderate blue light illumination in the wild-type strain (58). This redundancy, however, does not extend to ACIAD2110.

A transcriptomic analysis in dark and light conditions, with cells grown for 8 h in soft agar plates at 28°C, revealed that the genes ACIAD0969, ACIAD2048, ACIAD2325 and ACIAD1053 (likely involved in iron transport/storage), ACIAD1205 (identified as a stress-response DNA-binding protein and ferritin-like

Table 2. Effect of blue light and darkness on twitching motility of *A. baylyi* ADP1, a triple *bluf*-knockout mutant (B++M3), and (B++M3) complemented with each of the same three *bluf* genes (ACIAD2125, ACIAD2129 or ACIAD1499).

	Diameter of Twitching (cm) ± SD	
Strain	Blue Light (L)	Darkness (D)
A. baylyi ADP1	3.80 ± 0.179	5.90 ± 0.135
B++M3 (triple knockout of ACIAD2125, ACIAD2129 and ACIAD1499)	5.07 ± 0.293	5.20 ± 0.132
B++M3 complemented with ACIAD1499	1.00 ± 0.354	4.07 ± 0.115
B++M3 complemented with ACIAD2125	0.93 ± 0.076	3.68 ± 0.126
B++M3 complemented with ACIAD2129	1.43 ± 0.104	4.63 ± 0.153

gene) and ACIAD0507 (TonB coding gene) were differentially transcribed under blue light illumination and in darkness (59).

CONCLUSIONS

The wide distribution of BLUF-domains containing proteins within the genus *Acinetobacter* does not appear to follow the signal transduction rule that was derived for the two components regulatory systems: slow dispersal by lateral gene transfer, followed by a much more rapid diversification *via* gene duplication, as the BLUF domain in this genus appear to be monophyletic (57,59). On the other hand, the multiplicity of BLUF domains in one particular species presumably did primarily derive from gene duplication. But also on this rule there may be exceptions, like the ACIAD2110 gene, which might have been acquired through lateral gene transfer. Regarding physiological function, however, only the first glimpses of the very complex issue of assigning a function to all the identified BLUF domains, have been obtained, which for some aspects are even still rather contradictory (see above).

Only three of the four BLUF-domain-encoding genes present in the genome of the nonpathogenic environmental strain *A. baylyi* sp. *ADP1* were jointly necessary to make up for the blue light inhibition of twitching motility observed in the wild type strain at low temperature (24°C). Moreover, any of the genes (ACIAD1499, ACIAD2125 or ACIAD2129) if overexpressed *via* plasmid-based expression, can fully restore the response in all knockout strains, including double and triple knockouts mutants. The fourth BLUF-domain-encoding gene, ACIAD2110, is phylogenetically linked to the *A. baumanii* group and failed to contribute significantly to the motility response in *A. baylyi* sp. *ADP1*, although it is transcriptionally expressed under dark and light condition. Preliminary transcriptome data analysis suggests an involvement of iron traffic/storage-related genes in light–dark responses.

Acknowledgements—The authors are thankful to Mariana Bitrian and Jos Arents for sharing unpublished results. This work has been supported partially by UBACYT B095 (University of Buenos Aires), PICT 2008-1155 (ANPCYT) and PIP1937 (CONICET).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree of BLUF domains (pfam04940) of *Gammaproteobacteria* obtained by the neighbor-joining method.

REFERENCES

1. van der Horst, M. A., J. Key and K. J. Hellingwerf (2007) Photosensing in chemotrophic, non-phototrophic bacteria: Let there be light sensing too. *Trends Microbiol.* **15**, 554–562.
2. Hurley, J. B., M. Spencer and G. A. Niemi (1998) Rhodopsin phosphorylation and its role in photoreceptor function. *Vision. Res.* **38**, 1341–1352.
3. Gaidenko, T. A., T. J. Kim, A. L. Weigel, M. S. Brody and C. W. Price (2006) The blue-light receptor YtvA acts in the environmental stress signaling pathway of *Bacillus subtilis*. *J. Bacteriol.* **188**, 6387–6395.
4. Avila-Perez, M., K. J. Hellingwerf and R. Kort (2006) Blue light activates the sigmaB-dependent stress response of *Bacillus subtilis* via YtvA. *J. Bacteriol.* **188**, 6411–6414.
5. Losi, A. and W. Gärtner (2012) The evolution of flavin-binding photoreceptors: An ancient chromophore serving trendy blue-light sensors. *Annu. Rev. Plant Biol.* **63**, 49–72.
6. Elıas-Arnanz, M., S. Padmanabhan and F. J. Murillo (2011) Light-dependent gene regulation in nonphototrophic bacteria. *Curr. Opin. Microbiol.* **32**, 128–135.
7. Purcell, E. B. and S. Crosson (2008) Photoregulation in prokaryotes. *Curr. Opin. Microbiol.* **11**, 168–178.
8. Idnurm, A. and S. Crosson (2009) The photobiology of microbial pathogenesis. *PLoS Pathog.* **5**(11), e1000470.
9. Takano, H., S. Obitsu, T. Beppu and K. Ueda (2005) Light-induced carotenogenesis in *Streptomyces coelicolor* A3(2): Identification of an extracytoplasmic function sigma factor that directs photodependent transcription of the carotenoid biosynthesis gene cluster. *J. Bacteriol.* **187**, 1825–1832.
10. van der Horst, M. A. and K. J. Hellingwerf (2004) Photoreceptor proteins, star actors of modern times: A review of the functional dynamics in the structure of representative members of six different photoreceptor families. *Acc. Chem. Res.* **37**, 13–20.
11. Batra, P. (1971) Mechanism of light induced carotenoid synthesis in non photosynthetic plants. In *Photophysiology: In Current Topics in Photobiology and Photochemistry* (Edited by A. C. Giese), pp. 47–74. Academic Press, New York.
12. Voityuk, A. A., A. Rudolph, A. Marcus and M.-E. Michel-Beyerle (2014) On the mechanism of photoinduced dimer dissociation in the plant UVR8 photoreceptor. *PNAS* **111**, 5219–5224.
13. Ávila-Pérez, M., J. B. van der Steen, R. Kort and K. J. Hellingwerf (2010) Red light activates the σB-mediated general stress response of *Bacillus subtilis* via the energy branch of the upstream signaling cascade. *J. Bacteriol.* **192**, 755–762.
14. Pérez-Marín, M. C., S. Padmanabhan, M. C. Polanco, F. J. Murillo and M. Elías-Arnanz (2008) Vitamin B12 partners the CarH repressor to downregulate a photoinducible promoter in *Myxococcus xanthus*. *Mol. Microbiol.* **67**, 804–819.
15. Herrou, J. and S. Crosson (2011) Function, structure, and mechanism in bacterial photosensory LOV proteins. *Nat. Rev. Microbiol.* **9**, 713–723.
16. Losi, A. and W. Gärtner (2008) Bacterial bilin- and flavin-binding photoreceptors. *Photochem. Photobiol. Sci.* **7**, 1168–1178.
17. Mandalari, C., A. Losi and W. Gaertner (2013) Distance-tree analysis, distribution and co-presence of bilin-and flavin-binding prokaryotic photoreceptors for visible light. *Photochem. Photobiol. Sci.* **12**, 1144–1157.
18. Gomelsky, M. and W. D. Hoff (2011) Light helps bacteria make important lifestyle decisions. *Trends Microbiol.* **19**, 441–448.
19. Losi, A., C. Mandalari and W. Gärtner (2014) From plant infectivity to growth patterns: The role of blue-light sensing in the prokaryotic world. *Plants* **3**, 70–94.
20. Wu, L., R. S. McGrane and G. A. Beattie (2013) Light regulation of swarming motility in *Pseudomonas syringae* integrates signaling pathways mediated by a bacteriophytochrome and a LOV protein. *MBio* **4**(3), e00334-13.

21. Barkovits, K., B. Schubert, S. Heine, M. Scheer and N. Frankenberg-Dinkel (2011) Function of the bacteriophytochrome BphP in the RpoS/Las quorum-sensing network of *Pseudomonas aeruginosa*. *Microbiology* **157**, 1651–1664.
22. Tschowri, N., S. Busse and R. Hengge (2009) The BLUF-EAL protein YcgF acts as a direct anti-repressor in a blue-light response of *Escherichia coli*. *Genes Dev.* **23**, 522–534.
23. Swartz, T. E., T. S. Tseng, M. A. Frederickson, G. Paris, D. J. Comerci, G. Rajashekara, J. G. Kim, M. B. Mudgett, G. A. Splitter, R. A. Ugalde, F. A. Goldbaum, W. R. Briggs and R. A. Bogomolni (2007) Blue-light-activated histidine kinases: Two-component sensors in bacteria. *Science* **317**, 1090–1093.
24. Qualls, G. T., K. Stephens and D. White (1978) Light-stimulated morphogenesis in the fruiting myxobacterium *Stigmatella aurantiaca*. *Science* **201**, 444–445.
25. Bonomi, H. R., D. M. Posadas, G. Paris, M. C. Carrica, M. Frederickson, L. I. Pietrasanta, R. A. Bogomolni, A. Zorreguieta and F. A. Goldbaum (2012) Light regulates attachment, exopolysaccharide production, and nodulation in *Rhizobium leguminosarum* through a LOV-histidine kinase photoreceptor. *PNAS* **109**, 12135–12140.
26. Mussi, M. A., J. A. Gaddy, M. Cabruja, B. A. Arivett, A. M. Viale, R. Rasia and L. A. Actis (2010) The opportunistic human pathogen *Acinetobacter baumannii* senses and responds to light. *J. Bacteriol.* **192**, 6336–6345.
27. Oberpichler, I., R. Rosen, A. Rasouly, M. Vugman, E. Z. Ron and T. Lamparter (2008) Light affects motility and infectivity of *Agrobacterium tumefaciens*. *Environ. Microbiol.* **10**, 2020–2029.
28. Purcell, E. B., D. Siegal-Gaskins, D. C. Rawling, A. Fiebig and S. Crosson (2007) A photosensory two-component system regulates bacterial cell attachment. *Proc. Natl Acad. Sci. USA* **104**, 18241–18246.
29. Capra, E. J. and M. T. Laub (2012) Evolution of two-component signal transduction systems. *Annu. Rev. Microbiol.* **66**, 325–347.
30. Gomelsky, M. and G. Klug (2002) BLUF: A novel FAD-binding domain involved in sensory transduction in microorganisms. *Trends Biochem. Sci.* **27**, 497–500.
31. Anderson, S., V. Dragnea, S. Masuda, J. Ybe, K. Moffat and C. Bauer (2005) Structure of a novel photoreceptor, the BLUF domain of AppA from *Rhodobacter sphaeroides*. *Biochemistry* **44**, 7998–8005.
32. Grinstead, J. S., S. T. Hsu, W. Laan, A. M. Bonvin, K. J. Hellingwerf, R. Boelens and R. Kaptein (2006) The solution structure of the AppA BLUF domain: Insight into the mechanism of light-induced signaling. *ChemBioChem* **7**, 187–193.
33. Laan, W., M. Gauden, S. Yeremenko, R. van Grondelle, J. T. Kennis and K. J. Hellingwerf (2006) On the mechanism of activation of the BLUF domain of AppA. *Biochemistry* **45**, 51–60.
34. Masuda, S. and C. E. Bauer (2002) AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in *Rhodobacter sphaeroides*. *Cell* **110**, 613–623.
35. Braatsch, S., M. Gomelsky, S. Kuphal and G. Klug (2002) A single flavoprotein, AppA, integrates both redox and light signals in *Rhodobacter sphaeroides*. *Mol. Microbiol.* **45**, 827–836.
36. Okajima, K., S. Yoshihara, Y. Fukushima, X. Geng, M. Katayama, S. Higashi, M. Watanabe, S. Sato, S. Tabata, Y. Shibata, S. Itoh and M. Ikeuchi (2005) Biochemical and functional characterization of BLUF-type flavin-binding proteins of two species of cyanobacteria. *J. Biochem.* **137**, 741–750.
37. Gauden, M., S. Yeremenko, W. Laan, I. H. van Stokkum, J. A. Ihalainen, R. van Grondelle, K. J. Hellingwerf and J. T. Kennis (2005) Photocycle of the flavin-binding photoreceptor AppA, a bacterial transcriptional antirepressor of photosynthesis genes. *Biochemistry* **44**, 3653–3662.
38. Masuda, S. (2013) Light detection and signal transduction in the BLUF photoreceptors. *Plant Cell Physiol.* **54**, 171–179.
39. Spudich, J. L. and R. A. Bogomolni (1984) Mechanism of colour discrimination by a bacterial sensory rhodopsin. *Nature* **312**, 509–513.
40. Miller, A., H. Leigeber, W. D. Hoff and K. J. Hellingwerf (1993) A light-dependent branching-reaction in the photocycle of the yellow protein from *Ectothiorhodospira halophila.* Biochimica et Biophysica Acta (BBA). *Bioenergetics* **1141**, 190–196.
41. Toh, K. C., I. H. van Stokkum, J. Hendriks, M. T. Alexandre, J. C. Arents, M. Avila Perez, R. van Grondelle, K. J. Hellingwerf and J. T. Kennis (2008) On the signaling mechanism and the absence of photoreversibility in the AppA BLUF domain. *Biophys. J.* **95**, 312–321.
42. Song, S. H., D. Madsen, J. B. van der Steen, R. Pullman, L. H. Freer, K. J. Hellingwerf and D. S. Larsen (2013) Primary photochemistry of the dark- and light-adapted states of the YtvA protein from *Bacillus subtilis*. *Biochemistry* **52**, 7951–7963.
43. Arents, J. C., M. Avila Perez, J. Hendriks and K. J. Hellingwerf (2011) On the midpoint potential of the FAD chromophore in a BLUF-domain containing photoreceptor protein. *FEBS Lett.* **585**, 167–172.
44. Gauden, M., J. S. Grinstead, W. Laan, I. H. van Stokkum, M. Avila-Perez, K. C. Toh, R. Boelens, R. Kaptein, R. van Grondelle, K. J. Hellingwerf and J. T. Kennis (2007) On the role of aromatic side chains in the photoactivation of BLUF domains. *Biochemistry* **46**, 7405–7415.
45. Domratcheva, T., B. L. Grigorenko, I. Schlichting and A. V. Nemukhin (2008) Molecular models predict light-induced glutamine tautomerization in BLUF photoreceptors. *Biophys. J.* **94**, 3872–3879.
46. Laptenok, S. P., A. Lukacs, R. Brust, A. Haigney, A. Gil, M. Towrie, G. M. Greetham, P. J. Tonge and S. R. Meech (2015) Electron transfer quenching in light adapted and mutant forms of the AppA BLUF domain. *Faraday Discuss.* **177**, 293–311.
47. Losi, A. (2007) Flavin-based Blue-Light photosensors: A photobiophysics update. *Photochem. Photobiol.* **83**, 1283–1300.
48. Okajima, K., Y. Fukushima, H. Suzuki, A. Kita, Y. Ochiai, M. Katayama, Y. Shibata, K. Miki, T. Noguchi, S. Itoh and M. Ikeuchi (2006) Fate determination of the Flavin photoreceptions in the cyanobacterial blue light receptor TePixD (Tll0078). *J. Mol. Biol.* **363**, 10–18.
49. Wu, Q. and K. H. Gardner (2009) Structure and insight into blue light-induced changes in the BlrP1 BLUF domain. *Biochemistry* **48**, 2620–2629.
50. Ren, S., M. Sawada, K. Hasegawa, Y. Hayakawa, H. Ohta and S. Masuda (2012) A PixD—PapB chimeric protein reveals the function of the BLUF domain C-terminal α-helices for light signal transduction. *Plant Cell Physiol.* **53**, 1638–1647.
51. Brust, R., A. Haigney, A. Lukacs, A. Gil, S. Hossain, K. Addison, C. T. Lai, M. Towrie, G. M. Greetham, I. P. Clark, B. Illarionov, A. Bacher, R. R. Kim, M. Fischer, C. Simmerling, S. R. Meech and P. J. Tonge (2014) Ultrafast structural dynamics of BlsA, a photoreceptor from the pathogenic bacterium *Acinetobacter baumannii*. *J. Phys. Chem. Lett.* **5**, 220–224.
52. Muñoz-Price, L. S. and R. A. Weinstein (2008) Acinetobacter infection. *N. Engl. J. Med.* **358**, 1271–1281.
53. Harshey, R. M. (2003) Bacterial motility on a surface: Many ways to a common goal. *Annu. Rev. Microbiol.* **57**, 249–273.
54. Mattick, J. S. (2002) Type iv pili and twitching motility. *Annu. Rev. Microbiol.* **56**, 289–314.
55. Hoff, W. D., M. A. der van Horst, C. B. Nudel and K. J. Hellingwerf (2009) Prokaryotic phototaxis. In *Chemotaxis: Methods and Protocols* (Edited by T. Jin and D. Hereld), pp. 25–50. Humana Press, New York.
56. Bitrian, M., C. M. Solari, R. H. González and C. B. Nudel (2012) Identification of virulence markers in clinically relevant strains of *Acinetobacter* genospecies. *Int. Microbiol.* **15**, 79–88.
57. Golic, A., M. Vaneechoutte, A. Nemec, A. M. Viale, L. A. Actis and M. A. Mussi (2013) Staring at the cold sun: Blue light regulation is distributed within the genus *Acinetobacter*. *PLoS ONE* **8**, e55059.
58. Bitrian, M. (2013) Estudio funcional de genes de densidad poblacional (luxR/luxI) y de dominios fotorreceptores sospechados como reguladores de respuesta de virulencia en *Acinetobacter*. Ph.D. thesis, Universidad de Buenos Aires.
59. Bitrian, M., R. H. González, G. Paris, K. J. Hellingwerf and C. B. Nudel (2013) Blue-light dependent inhibition of twitching motility in *Acinetobacter baylyi ADP1*: Additive involvement of three BLUF domain-containing proteins. *Microbiology* **159**, 1828–1841.

AUTHOR BIOGRAPHIES

Clara B. Nudel obtained PhD in Industrial Biochemistry at the University of Buenos Aires in 1988, followed by a 1 year postdoctoral training in Molecular Microbial Physiology at the University of Amsterdam. Since 1991, Dr. Nudel is Professor in Biotechnology and Industrial Microbiology in the Faculty of Pharmacy and Biochemistry and currently chairs the research institute Nanobiotec (UBA-CONICET). Main research topics in the group are the investigation of novel mechanisms of microbial interactions with the environment and their transfer into biotech applications.

Klaas J. Hellingwerf received his master degree in Chemistry from the University of Groningen (1975) and his PhD in Biochemistry from the University of Amsterdam (1979) on the topic of: "Reconstitution of bacteriorhodopsin in liposomes." As a staff member of the University of Groningen he studied anoxygenic photosynthesis in Rhodobacter sphaeroides in relation to membrane transport. Since 1988 he holds the chair in General Microbiology at the University of Amsterdam and supervises the Molecular Microbial Physiology research group of the Swammerdam Institute for Life Sciences on the subject of molecular regulation mechanisms in bacterial signal transduction and systems and synthetic biology of prokaryotic chemotrophs and phototrophs.