Staring at the Cold Sun: Blue Light Regulation Is Distributed within the Genus *Acinetobacter*

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Abstract

We previously showed that the opportunist nosocomial pathogen *Acinetobacter baumannii* is able to sense and respond to light via BlsA, a BLUF (Blue-Light-sensing Using FAD)-domain photoreceptor protein. Here, we extend our previous studies showing that light regulation is not restricted to *A. baumannii*, but rather widespread within the genus *Acinetobacter*. First, we found that blue light modulates motility and biofilm formation in many species of the genus, including members of the *Acinetobacter calcoaceticus-A. baumannii* complex. In many of these species blue light acts as a key factor guiding the decision between motility or sessility at 24°C, whereas in *A. baumannii*, light inhibits both motility and biofilm formation. We also show that light regulation of motility occurred not only at 24°C but also at 37°C in non-*A. baumannii* species, contrasting the situation of *A. baumannii* which only shows photoregulation at 24°C. Second, we show that *Acinetobacter baylyi* (strain ADP1) BLUF-photoreceptors can functionally replace in vivo the *A. baumannii* 17978 BlsA protein and that the pathways leading to biofilm formation are inversely regulated at 24°C between these two microorganisms. Finally, we found the presence of predicted genes coding BLUF-containing proteins in all *Acinetobacter* sequenced genomes, even though the copy number is variable among them. Phylogenetic analysis suggests a common origin for all BLUF domains present in members of this genus, and could distinguish well-differentiated clusters that group together BLUF homologs from different species, a situation particularly clear for members of the ACP complex. Despite a role played by these BLUF domain-containing proteins in the photoregulation observed in the members of the genus *Acinetobacter* is a likely scenario given our findings in *A. baumannii* and *A. baylyi*, further research will contribute to confirm this possibility.

Introduction

The members of the genus *Acinetobacter* are strictly aerobic, oxidase negative, ubiquitous Gram-negative coccobacilli that are frequently found in the environment but also in the hospital setting, where some particular groups of the genus have been associated with outbreaks of nosocomial infections [1]. Currently, the genus comprises 27 species with valid names ([www.bacterio.ict.fr/a/acinetobacter.html](http://www.bacterio.ict.fr/a/acinetobacter.html)) and several putative species with provisional designations including nine genomic species delineated ([cict.fr/a/acinetobacter.html](http://cict.fr/a/acinetobacter.html)) and several putative species with provisional designations including nine genomic species delineated. In many of these species blue light acts as a key factor guiding the decision between motility or sessility at 24°C, whereas in *A. baumannii*, light inhibits both motility and biofilm formation. We also show that light regulation of motility occurred not only at 24°C but also at 37°C in non-*A. baumannii* species, contrasting the situation of *A. baumannii* which only shows photoregulation at 24°C. Second, we show that *Acinetobacter baylyi* (strain ADP1) BLUF-photoreceptors can functionally replace in vivo the *A. baumannii* 17978 BlsA protein and that the pathways leading to biofilm formation are inversely regulated at 24°C between these two microorganisms. Finally, we found the presence of predicted genes coding BLUF-containing proteins in all *Acinetobacter* sequenced genomes, even though the copy number is variable among them. Phylogenetic analysis suggests a common origin for all BLUF domains present in members of this genus, and could distinguish well-differentiated clusters that group together BLUF homologs from different species, a situation particularly clear for members of the ACP complex. Despite a role played by these BLUF domain-containing proteins in the photoregulation observed in the members of the genus *Acinetobacter* is a likely scenario given our findings in *A. baumannii* and *A. baylyi*, further research will contribute to confirm this possibility.

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A. baumannii photoregulation was later confirmed by both biophysical and genetic approaches, and therefore it was designated BlsA for blue light sensing A [5]. Interestingly, light regulation was lost at 37°C, a temperature compatible with warm-blooded hosts [5]. This temperature-dependency prompted speculations on the role of light sensing in the lifestyle of A. baumannii. Our current hypothesis postulates that light sensing might play a role during the pathogens environmental life, modulating its behavior outside the human body.

Many questions arise from our previous work, such as whether blue light regulation is widely distributed within the genus Acinetobacter. In such a case, relevant questions would be whether environmental species show light-mediated responses and temperature dependence similar to that described above in A. baumannii. Alternatively, blue light regulation could be restricted only to a subgroup in which A. baumannii is contained, suggesting that acquisition of light sensing genes occurred by recent horizontal gene transfer events.

Therefore, in this work we evaluated whether other species within the genus Acinetobacter also share the light-dependent responses described in A. baumannii, in particular, by studying photoregulation of motility and biofilm formation. We also provide insights into the evolution of BLUF-domains encoded within Acinetobacter genomes, shown to mediate light regulation in A. baumannii, by inferring and analyzing their phylogenetic relationships. This knowledge may contribute to our current understanding of the role, importance and evolution of light sensing and regulation in these bacterial species.

Materials and Methods

Bacterial Strains

The A. baumannii and E. coli strains, as well as the plasmids constructed and/or used in this work are listed in Table 1. The Acinetobacter strains used in this study, which include representatives of 25 validated named species, and their origin (if known) are listed in Table 2. A. indicus and A. ohivorans are effectively but not validly published names for single strains (CCM 7832 and DR1, respectively), and therefore are mentioned between apostrophes. Many of these strains have been reported in literature before while others, selected from our collection, have been identified at the species level by amplified ribosomal DNA restriction analysis (ARDRA) [9]. All this information is also indicated in Table 2.

Cell Motility Experiments and Biofilm Assays

Cell motility was tested on “swimming agarose” (Tryptone 1%, NaCl 0.5%, agarose 0.3%; 5) or LB agarose (Peptone 1%, NaCl 1%, yeast extract 0.5%, agarose 0.3%) plates incubated in the presence or absence of light. “Swimming agarose” is an inherited name for plates of this composition, but it should be noted that members of the genus Acinetobacter do not perform flagella-mediated swimming as they do not produce this type of cell appendage [10]. Biofilm assays were carried out in glass tubes as described before [5]. All assays were performed at least in triplicate for both light and dark conditions.

Plates or biofilm tubes were incubated at 24°C or 37°C in the dark or under light emitted by LED (light-emitting diode) arrays with an intensity of 6 to 10 μmol photons/m2/s, determined using a Li-250A Light Meter (LI-COR). Each array was built using three-LED module strips (containing three LEDs each) emitting blue, green, or red light with emission peaks centered at 462 nm, 514 nm, and 636 nm, respectively, as determined using a LI-COR LI-1800 spectroradiometer [5]. It should be noted that temperature of both the liquid or agar medium under the illumination conditions used in these experiments did not vary significantly from that measured under dark conditions or in the incubation chamber.

For quantification of biofilms, duplicate cultures for each sample at each condition were used. One tube was sonicated immediately for 3 s with a thin probe and the OD900 of the culture was determined to estimate total cell biomass. The supernatant of the other tube was aspirated and rinsed thoroughly with distilled water. The cells attached to the tube walls were visualized and quantified by staining with crystal violet and solubilization with ethanol–acetone as described in ref. [11]. The OD900/OD600 ratio was used to normalize the amount of biofilm formed to the total cell content of each sample tested, to avoid variations due to differences in bacterial growth under different experimental conditions. Error bars show standard error of the mean for 3 different biological replicates (n = 3).

General DNA Procedures

Genomic and plasmid DNA were isolated as described before [4], and DNA restriction and cloning experiments were carried out using standard protocols [12]. DNA sequences were determined at the DNA Sequencing Facility of the University of Maine, Orono, ME, USA; or at Macrogen (Korea).

Construction of Complementation Plasmids

DNA fragments of 836, 890, 942 and 772 bp, corresponding to A. baileyi ACIAD1499, ACIAD2110, ACIAD2129, and ACIAD2125 predicted BLUF-domain-containing genes and cognate promoters were amplified by PCR using a A. baileyi strain ADP1 total DNA and primers 1499F (5’-ggatcccttacaataaga-3’) and 1499R (5’-ggatcccttacaacaggttaa-3’), 2110F (5’-ggatcccttataaggtccttaa-3’) and 2110R (5’-ggatctttactttgctat-3’), 2125F (5’-ggatcccttacaagttataa-3’) and 2125R (5’-ggatcccttataaggtccttaa-3’), all of which were sequenced with BamHI restriction sites (indicated in italics in the primer sequences). The corresponding amplicons were first cloned into pGEM-T Easy (Promega) and then subcloned as BamHI fragments into the cognate site of pWH1286 [13]. Proper constructions of the complementing plasmids were verified by automated DNA sequencing. Plasmid DNA was electroporated into A. baumannii ATCC 17978 blsA mutant (17978.0R) as described before [5].

Amplification and Sequencing of BLUF-coding Genes

The presence of BLUF domain-coding genes in strains A. calcoaceticus ACI 412, A. nosocomialis ACI 32 and A. pittii ACI 988, was analyzed by colony PCR using sequence information derived from the genome-sequenced strains A. calcoaceticus PHEA-2, A. nosocomialis RUH 2624 and A. pittii SH 024 to design specific primers that amplify the cognate BLUF genes: A. calcoaceticus ADY82057, primers 82057F 5’-ggatcccttacaataaga-3’ and 82057R 5’-ggatcccttacaacaggttaa-3’, A. nosocomialis EEW98085, primers 98085F 5’-ggatcccttataaggtccttaa-3’ and 98085R 5’-ggatcccttataaggtccttaa-3’, A. nosocomialis EEWX0046, primers 46F 5’-ggatcccttataaggtccttaa-3’ and 46R 5’-ggatcccttataaggtccttaa-3’. To amplify A. baumannii BlsA close homologs present in these strains (ADY82317, EEX01065 and EFX06339), we used primers EhsA/R/5’ (5’-ggaactgcca-taagacctgcta-3’) and EhsA/F/6’ (5’-ggaactgcca-taagacctgcta-3’) previously described in ref. [5]. The
amplified fragments were purified by gel extraction (GFX, Amersham) and sequenced by Macrogen (Korea).

Disc Diffusion Antibiotic Susceptibility Test

Mueller Hinton (MH) agar plates were inoculated with a culture of each tested strain, which were previously adjusted to 0.5 McFarland standard turbidity using fresh culture medium or saline solution, according to the recommendations of the National Committee for Clinical Laboratory Standards [14]. Antimicrobial commercial discs (BBL, Cockeysville, MD, USA) containing 10 mg of ampicillin, 30 mg of amikacyn, 30 mg of cefepim, 30 mg of cefotaxime, 30 mg of cefoxitin, 30 mg of cephalotin, 30 mg of chloramphenicol, 5 mg of ciprofloxacin, 10 mg of gentamycin, 10 mg of meropenem, 100 mg of piperacillin or 5 mg of rifampicin were placed on the surface of plates, which were latter incubated overnight at 24°C or darkness. The assays were performed in triplicate.

Sequence Analyses

Protein sequences were retrieved from databases at NCBI (http://www.ncbi.nlm.nih.gov) and Pfam [15], and were aligned using ClustalW version 1.7 [16]. To exclude the high sequence variability often found after BLUF domains which -in some cases- supposes diversity of the accompanying effector domains [17], only the region corresponding to the BLUF domain (comprising 93–96 amino acid residues, depending on the organism) was extracted from the complete sequences and used for the alignments. Gaps were removed from the alignments using BioEdit version 7.05.3 [18].

Phylogenetic Inferences

Phylogenetic relationships were inferred from amino acid sequence alignments using the programs provided in the PHYLIP package, version 3.69 [19] (http://evolution.genetics.washington.edu/phylip.html). The maximum-likelihood method (PROTML) was used for the construction of the BLUF-domain phylogenetic tree. In all cases, confidence levels were calculated from 1,000 bootstrap resamplings (SEQBOOT) of alignments used for phylogenetic inferences by both neighbor-joining method using a Dayhoff PAM distance matrix (PROTDIST) and the parsimony (PROTPARS) method, also included in the PHYLIP software package [19].

Results

Motility is also Regulated by Blue Light in non-A. baumannii Members of the Genus Acinetobacter

Given that light regulates motility in A. baumannii [5], we analyzed whether other species of this genus are also able to move and respond to light at 24°C. We found that A. baylyi, A. calcoaceticus, A. nosocomialis, A. oleovorans, A. pittii and A. tjernbergiae...
Table 2. Blue light and temperature (24°C vs. 37°C) regulation of motility and biofilm formation by *Acinetobacter* strains studied in this work.

| Species/Strain | Origina | Reference | BLUF-containing genesb | Motility 24°C | 37°C | Biofilm formationc |
|----------------|---------|-----------|------------------------|--------------|-----|-------------------|
|                |         |           |                        | L | D | L | D | 24°C | L | D |
| *A. baumannii* |         |           |                        |   |   |   |   |      |   |   |
| ATCC 17978T (**) cerebrospinal fluid [27] | 1 | | | | | | | | | |
| 17978.ORrp | | | | | | | | | |
| 17978.ORC1899 | | | | | | | | | |
| 17978.ORC2110 | | | | | | | | | |
| 17978.ORC2125 | | | | | | | | | |
| 17978.ORC2129 | | | | | | | | | |
| 17978.ORC229A | | | | | | | | | |
| ATCC 19606T | | | | | | | | | |
| Ab244 | | | | | | | | | |
| *A. baylyi* | soil | [28] | 4 | ± | + | ± | + | ± |
| *A. beijerinckii* | | | | | | | | | |
| CCUG 56139 | air sacculitisa (horse) [21] | | | | | | | | |
| NIPH 838T | wound | [21] | | | | | | | |
| NIPH1065 | toe-web | [21] | | | | | | | |
| *A. bereziniae* | soil | (1) | | | | | | | |
| ACI 449 | | | | | | | | | |
| ACI 552 | unknown | (1) | | | | | | | |
| LMG9988T | wound | [29] | | | | | | | |
| *A. bouvettii* | | | | | | | | | |
| DSM 14964T | activated sludge plants [30] | | | | | | | | |
| *A. brisouii* | peat | [31] | | | | | | | |
| CCUG 61636T | | | | | | | | | |
| *A. calcoaceticus* | soil | (1) | 2 (1) | | | | | | |
| ACI 412 | | | | | | | | | |
| ACI 27 | soil | (1) | | | | | | | |
| PHEA-2 (**) | waste water [32] | 2 | | | | | | | |
| ACI 23 | sputum | (1) | | | | | | | |
| LMG 1046T | soil | [27] | | | | | | | |
| *A. gerneri* | | | | | | | | | |
| DSM 14967T | activated sludge plants [30] | | | | | | | | |
| *A. guillouiae* | | | | | | | | | |
| ACI 46 | urine | (1) | | | | | | | |
| ACI 47 | wound | (1) | | | | | | | |
| LMG1003T | wound | [29] | | | | | | | |
| CUGG 50621 | unknown | | | | | | | | |
| *A. gyllenbergii* | | | | | | | | | |
| NIPH 975 | tracheal exudate | [21] | | | | | | | |
| NIPH 822 | wound | [21] | | | | | | | |
| NIPH 230/CCUG56138 | vagina | [21] | | | | | | | |
| NIPH 2150T | urine | [21] | | | | | | | |
| *A. haemolyticus* | | | | | | | | | |
| ACI 25 | air | (1) | | | | | | | |
| ACI 31 | pus | (1) | | | | | | | |
| ACI 927 | unknown | (1) | | | | | | | |
| Species/Strain | Origin | Reference | BLUF-containing genes | Motility | Biofilm formation |
|---------------|--------|-----------|----------------------|----------|------------------|
|               |        |           | 24°C | 37°C | 24°C |
|               |        |           | L | D | L | D | L | D |
| ACI 928       | unknown | (')       | – | – | ND | ND | – | – |
| CCM 2358T     | sputum  | [27]      | – | – | ND | ND | – | – |
| 'A. indicus'  |        |           |   |   |   |   |   |   |
| CCM 7832      | soil    | [33]      | – | – | ND | ND | – | – |
| A. johnsonii  |        |           |   |   |   |   |   |   |
| LMG 999T      | duodenum | [27]     | – | – | ND | ND | ± | ± |
| ACI 166       | unknown | (')       | – | – | ND | ND | – | – |
| ACI 197       | unknown | (')       | – | – | ND | ND | – | – |
| SH046/CCUG 57820(**) | perineum | [34] | 1 | – | – | ND | ND | – | – |
| A. junii      |        |           |   |   |   |   |   |   |
| LMG 998T      | urine   | [27]      | – | – | ND | ND | ± | ± |
| DSM 14968T(***)| activated sludge plants | [30] | – | – | ND | ND | – | – |
| ACI 191       | unknown | (')       | – | – | ND | ND | – | – |
| ACI 282       | unknown | (')       | – | – | ND | ND | – | – |
| A. lwofii     |        |           |   |   |   |   |   |   |
| ACI 26        | blood   | (')       | – | – | ND | ND | – | – |
| LMG 985       | gangrenous lesion | [27] | – | – | ND | ND | ± | ± |
| LMG 1029T     | unknown | [27]      | – | – | ND | ND | ± | ± |
| ACI 172       | unknown | (')       | – | – | ND | ND | ± | ± |
| ACI 174       | unknown | (')       | – | – | ND | ND | – | – |
| SH145/CCUG 57819(**) | hand | [34] | 2 | – | – | ND | ND | ± | ± |
| A. nosocomialis |        |           |   |   |   |   |   |   |
| ACI 32        | urine   | (')       | – | + | – | + | ± | ± |
| ACI 57        | skin front | (')     | – | – | ND | ND | ±(*) | ±(*) |
| ACI 911       | unknown | (')       | – | – | ND | ND | + | + |
| LMG 10619T    | sputum  | [2]       | – | + | – | + | ± | ± |
| RUH 2624 (= CCUG 57817)(**) | Forehead skin | [2] | 3 | – | – | ND | ND | + | ± |
| 'A. oleivorans' |        |           |   |   |   |   |   |   |
| DR1 (**)     | rice paddy | [35] | 2 | – | + | – | + | + | + |
| A. parvus     |        |           |   |   |   |   |   |   |
| NIPH 384T     | ear     | [36]      | – | – | ND | ND | – | – |
| NIPH 399      | eye     | [36]      | – | – | ND | ND | – | – |
| A. pittii     |        |           |   |   |   |   |   |   |
| ACI 37        | wound   | (')       | – | – | ND | ND | + | + |
| ACI 38        | cerebrospinal fluid | (')     | – | – | ND | ND | ±(*) | ±(*) |
| LMG1035T      | cerebrospinal fluid | [2] | – | – | ND | ND | ±(*) | ±(*) |
| ACI 988       | unknown | (')       | 2(·) | – | + | ± | ± | ± |
| SH024/CCUG 57818 (**) | axilla | [2] | 2 | – | – | ND | ND | + | ± |
| A. radioresistens |        |           |   |   |   |   |   |   |
| ACI 49        | urine   | (')       | – | – | ND | ND | – | – |
| ATCC 43998T   | cotton tampon | [37] | – | – | – | – | – | – |
| ACI 62        | hospital pillow | (') | – | – | ND | ND | ±(*) | ±(*) |
| ACI 183       | unknown | (')       | – | – | ND | ND | ±(*) | ±(*) |
| SH164/CCUG 57822(**) | forehead | [34] | 4 | – | – | ND | ND | – | – |
| SK8 (**)     | unknown |           | 6 |   |   |   |   |   |
| A. rudis      |        |           |   |   |   |   |   |   |
| CCUG 57889T   | raw milk | [38] | – | – | ND | ND | + | – |
showed light-dependent regulation of motility, at least in one from three to five strains assayed (see Figure 1 and Table 2). Just as described for *A. baumannii* [5], motility in these bacteria was inhibited under blue light, while in the dark they spread all over the surface of the plate (Figure 1). In the case of *A. baylyi* and *A. pittii*, blue light inhibition was not absolute and bacteria were still able to move, even though to a much lesser extent than in the dark (Figure 1). It is interesting to note that the appearance of *A. pittii* is different from that observed for other species such as *A. baumannii* or *A. calcoaceticus*. *A. pittii* did not move homogeneously from the inoculation point, but rather formed independent striations irradiating from the center. We did not observe motility in any of the *A. beijerinckii*, *A. berezinae*, *A. boucettii*, *A. brissouii*, *A. gerrei*, *A. guillouae*, *A. gyllenbergii*, *A. haemolyticus*, *A. indicus*, *A. johnsonii*, *A. junii*, *A. kuifii*, *A. parvus*, *A. radioresistens*, *A. rudis*, *A. schindlerii*, *A. tandoi*, *A. towneri*, *A. ursingii*, and *A. venetianus* strains analyzed, even though as much as five different strains were tested for several species (Table 2). In our previous study we showed that light regulation of motility in *A. baumannii* occurred at 24°C but not at temperatures associated with warm-blooded hosts such as 37°C [5]. We thus evaluated next whether any of the strains that showed photo-regulation at 24°C also exhibited light regulation at 37°C and found that, in contrast to *A. baumannii*, all of them did respond to light at a higher incubation temperature (Figure 1 and Table 2). It should be noted that similar results were obtained when all strains were tested using LB agarose 0.3% instead of “swimming agarose” under similar experimental conditions.

**Table 2.** Cont.

| Species/Strain | Origin* | Reference | BLUF-containing genes† | Motility | Biofilm formation‡ |
|----------------|---------|-----------|------------------------|----------|--------------------|
|                |         |           |                        | 24°C     | 37°C         | 24°C     |
|                |         |           |                        | L        | D          | L        |
|                |         |           |                        | D        | L          | D        |
| *A. schindleri* |         |           |                        |          |            |          |
| NIPH 883       | urine   | [39]      |                        | –        | –          | ND       |
| NIPH 1034      | urine   | [39]      |                        | –        | –          | ND       |
| ACI 940        | unknown | (1)       |                        | –        | –          | ND       |
| *A. tandoi*    |         |           |                        |          |            |          |
| DSM 14670      | activated sludge plants | [28] | –                      | –        | –          | ND       |
| NIPH 2309      | non-medical environment | ()    | –                      | –        | –          | ND       |
| *A. tjernbergiae* |       |           |                        |          |            |          |
| DSM 14971      | activated sludge plants | [30] | –                      | –        | –          | ND       |
| 7B02           | activated sludge plants | [30] | ±                      | +        | ±          | +        |
| *A. towneri*   |         |           |                        |          |            |          |
| DSM 14962      | activated sludge plants | [30] | –                      | –        | –          | ND       |
| *A. ursingii*  |         |           |                        |          |            |          |
| NIPH 137       | blood   | [39]      | –                      | –        | –          | ND       |
| NIPH 840       | urine   | [39]      | –                      | –        | –          | ND       |
| NIPH 841       | blood   | [39]      | –                      | –        | –          | ND       |
| NIPH 842       | urine   | [39]      | –                      | –        | –          | ND       |
| ACI 941        | unknown | (1)       | –                      | –        | –          | ND       |
| *A. venetianus* |         |           |                        |          |            |          |
| LMG 19082      | taron beach | [40] | –                      | –        | –          | ND       |
| CCUG 60049     | blood of Dermochelyscoriacea[41] (turtle) |       | –                      | –        | –          | ND       |
| T4             | Sea water | [40] | –                      | –        | –          | ND       |

*If not indicated otherwise, strains are of human origin.

†Number of BLUF-containing genes deduced from the available sequenced genomes.

‡All biofilms correspond to wall biofilms unless stated.

§Type strains.

**(1) These strains have been identified by ARDRA.

(1) These strains have been unambiguously identified by rpoB sequencing.

(*) Only pellicle biofilm formation.

(**) Strains whose genomes have been sequenced.

(***) The type strain of *A. gronontii*, a junior synonym of *A. junii*.

(†) Wall and pellicle biofilm formation simultaneously.

(‘) The presence of BLUF-coding genes was determined by amplification using specific primers and posterior sequencing. See Materials and Methods for details.

ND, not determined.

doi:10.1371/journal.pone.0055059.t002

Biofilm Formation is also Regulated by Blue Light in non-*A. baumannii* Members of the Genus Acinetobacter

We further evaluated whether biofilm formation was photo-regulated in different species within the genus by studying their...
Figure 1. Effect of light and temperature on bacterial motility. Cells of different species within the genus Acinetobacter were inoculated on the surface of swimming plates. Plates were inspected and photographed after incubated overnight in darkness (D) or in the presence of blue light (BL), green light (GL) or red light (RL) at 24°C or 37°C. Only some strains displaying photoregulated motility are shown. doi:10.1371/journal.pone.0055059.g001
ability to form biofilms on glass both under blue light and in the dark at 24°C. We found 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 four days, while the levels of biofilms formed in the dark were significantly lower or negligible (Figure 2, Figure S1 and Table 2). 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 (Figure 2, Figure S1 and Table 2), but the levels of wall biofilms were lower than in the aforementioned strains (Figure 2A). It is interesting to note that even though there is photoregulation of biofilm formation in these species (similarly to *A. baumannii*), the amount of wall biofilms formed by non-*A. baumannii* species was much greater under blue light than in darkness, in contrast to *A. baumannii* in which larger levels are observed in the dark [5]. In non-*A. baumannii* species, we observed mainly the presence of wall biofilms only. The presence of pellicles (with no wall biofilm occurring at the same time) was evident only in a few strains of *A. bereziniae*, *A. guillouiae*, *A. nosocomialis*, *A. pittii* and *A. radioresistens*, and no light regulation was detected on them. The effect of light on biofilm formation at 37°C by the *Acinetobacter* strains used in this study could not be evaluated because of wide variations observed in different assays. Such a response is not surprising, as similar situations have been described before for clinical isolates of *A. baumannii* [5].

### A. baylyi: a Case Study

The fact that *A. baylyi* harbors four paralogous genes encoding BLUF-containing putative photoreceptors, designated ACIAD1499, ACIAD2110, ACIAD2125 and ACIAD2129 (Table 2), suggests that light might play a key role in the lifestyle of the bacterium, which could justify the abundance of these genes and their possible functional redundancy. To better characterize the response of this bacterium to light, we compared its ability to move under red or green light with its response under blue light or darkness at 24°C. We observed that green light inhibited motility, even though to a lesser extent than blue light (Figure 2). In contrast, under red light the bacteria behaved as in darkness. Thus, *A. baylyi* is able to sense and respond to green light in a similar way as *A. baumannii* [5], indicating that at least one of the four putative photoreceptors is capable to respond to green light.

We assayed next whether any of the different photoreceptors present in *A. baylyi* were able to rescue the lost photoregulation of motility and biofilm formation at 24°C of the *A. baumannii* ATCC 17978 bldA mutant (17978.OR) [5]. For this purpose, we cloned each of the *A. baylyi* predicted photoreceptor genes under their own promoter control in the shuttle plasmid pWH1266 to generate pWH1499, pWH2110, pWH2125 and pWH2129 (Table 1). Then, we transformed the 17978.OR mutant strain with these constructions, as well as with the empty vector, to generate 17978.ORc1499, 17978.ORc2110, 17978.ORc2125, 17978.ORc2129 and 17978.ORp, respectively. Finally, we analyzed whether these strains showed photoregulation of motility or biofilm formation at 24°C.

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**Figure 2. Effects of light on biofilm formation.**

A. The biofilms formed by cells of the different species within the genus *Acinetobacter* on glass tubes in the presence of blue light (L) or in darkness (D) were recorded after static incubation for 96 h at 24°C by direct visual inspection and staining with crystal violet. Only representative examples are shown. B. Quantification of the biofilms of cognate samples shown in A. Error bars show standard error of the mean for 3 different biological replicates (n = 3). OD$_{580}$/OD$_{600}$, optical density at 580 or 600 nm, respectively. L: light; D: Dark.

doi:10.1371/journal.pone.0055059.g002
Figure 3. Effect of blue light on biofilm and motility mediated by *A. baylyi* photoreceptors in an *A. baumannii* genetic background.

A. Cells of the ATCC 17978.OR *blsA* mutant, transformed with plasmids pWH1499, pWH2110, pWH2125 and pWH2129 or the empty pWH1266 vector, were inoculated on the surface of swimming plates. Plates were inspected and photographed after overnight incubation in darkness (D) or the presence of blue light (L) at 24°C. B. The biofilms formed by ATCC 17978.OR *blsA* mutant, transformed with plasmids pWH1499, pWH2110, pWH2125 and pWH2129 or the empty pWH1266 vector on glass tubes were recorded after static incubation for 96 h at 24°C by direct visual inspection and

C. Graph showing the change in optical density (OD) at 540 nm (OD$_{540}$) of biofilms treated with blue light (L) and darkness (D) compared to the control (D).
Figure 3A shows that 17978.ORG harboring the empty pWH1266 plasmid (17978.ORGp) does not exhibit photoregulation of motility, spreading throughout the plate either under blue light or in the dark at 24°C. In contrast, 17978.ORG1999, 17978.ORG2125, and 17978.ORG2129 showed a tight inhibition of motility under blue light while spreading throughout the plate in the dark. Such response resembles that of the complemented 17978.ORGBLUF strain harboring the native A. baumannii blsA wild-type allele (see Figure 3A) [5]. Therefore, the corresponding A. baylyi putative photoreceptors are able to fully complement the blsA gene. Conversely, the A. baylyi photoreceptor encoded by ACIAD2110 only partially complemented the blsA mutation in ATCC 17978 showing that it is not able to fully restore BlsA functioning in A. baumannii, at least regarding motility (Figure 3A).

We also analyzed the effects of the expression of the different A. baylyi photoreceptors on the ability of the A. baumannii ATCC 17978.ORG cells to form biofilms at 24°C (Figure 3B and C). In this case, all four A. baylyi BLUF-domain containing photoreceptors restored the original phenotype, behaving as the 17978.ORGBLU strain when tested as described before [5].

Blue Light and Resistance to Antibiotics

As many clinically-relevant species of Acinetobacter show an outstanding ability to rapidly evolve resistance to antibiotics, reducing therefore the available therapeutic approaches [4], we speculated whether blue light also modulates resistance to antibiotics. For this purpose, we conducted disc-diffusion antibiotic susceptibility assays both at 24°C as well as 37°C under blue light or in the darkness, using strains A. nosocomialis ACI 32, A. pittii ACI 988, both of which show photoregulation of motility and biofilm formation, and A. haemolyticus ACI 25. Despite various antibiotics belonging to different groups such as ampicillin (β-lactam), cefazidime (β-lactam), cephalotin (β-lactam), chloramphenicol (chloramphenicol), ciprofloxacin (fluoroquinolone), gentamycin (aminoglycoside), imipenem (carbapenem β-lactam), meropenem (carbapenem β-lactam), piperacillin (β-lactam) and rifampicyn (rifamycin) were tested, no significant differences were detected between light and dark conditions at either temperature for these strains (Table S1).

We also evaluated the effect of light on antibiotic resistance in A. baumannii strains, as this species is the most frequently recovered in clinical settings and the one that has been extensively reported to exhibit resistance to multiple antibiotics [4]. We used here strain ATCC 17978 (as it has been reported to respond to light), which is sensitive to most antibiotics; as well as strain AB244 [4], which shows resistance to multiple antibiotics and a slight photoregulation of biofilm formation (Figure S1). Here again, although many antibiotics belonging to different groups were tested, e.g., imipenem, meropenem, cloramphenicol, rifampicin, ampicillin, amoxicillin (aminoglycoside), piperacillin, cefoxitin (β-lactam), cephaparin (β-lactam), cefotaxime (β-lactam), cefepim (β-lactam), and cefazidime (β-lactam), no significant differences were observed that could result from differential resistance mediated by light (Table S1).

BLUF-domain Containing Proteins in non-A. baumannii Members of the Genus Acinetobacter

We showed previously that the BLUF domain-containing protein encoded in the A. baumannii ATCC 17978 genome is an active photoreceptor that modulates different traits such as motility and biofilm formation [5], and also that the four BLUF domain-containing proteins encoded in A. baylyi strain ADP1 are active photoreceptors able to sense light and transduce the signal in the A. baumannii genetic background modulating motility and biofilm formation in this organism. To further extend our knowledge of BLUF domain-containing proteins, we analyzed the presence and phylogenetic relationships of these domains in other members of the genus Acinetobacter, as they could likely play similar roles in other species. For this purpose, we screened the complete (or almost complete) sequenced genomes available in databases of the members of the genus Acinetobacter for the presence of genes coding for BLUF-domain containing proteins, to determine if their presence is distributed in the genus and evaluate their phylogenetic relatedness. Genes coding for BLUF-containing proteins were present in all of the screened genomes, i.e. those of A. baumannii, A. baylyi, A. calcoaceticus, A. johnsonii, A. lwofii, A. nosocomialis, A. oleivorans, A. pittii, A. radioresistens, and Acinetobacter sp. ATCC 27294 (Table 2). It is worth mentioning that based on the Pasteur MLST scheme [20], as well as tnpR sequence comparisons and phenotypic analyses [21], ‘A. oleivorans’ DR1 is highly related to one of the two strains designated ‘Between 1 and 3’, being therefore also a member of the ACB complex [A. Nemec, unpublished data].

As shown in Table 2, the number of predicted BLUF-containing proteins encoded per genome in the above species fluctuates from one to six. Indeed, it is noteworthy that close species such as those comprised within the ACB complex show variability in the number of genes coding for BLUF-domain containing proteins. As seen in Table 2, A. calcoaceticus PHEA-2, A. pittii SH024 and ‘A. oleivorans’ DR1 encode two, while A. baumannii and A. nosocomialis RUH2624 encode one and three BLUF-domain containing proteins, respectively. Besides, A. baylyi ADP1 encodes four putative BLUF-photoreceptors while A. radioresistens SK3 and SH164 encode six and four, respectively. All of the predicted BLUF-proteins found in members of the genus Acinetobacter correspond to the most common bacterial BLUF photoreceptors, small proteins containing a flavin-binding photosensin core lacking a recognizable effector or output domain(s), such as BlsA from A. baumannii [5].

To determine the phylogenetic relationships between the BLUF domains present within the Acinetobacter genus, we retrieved 93 protein sequences corresponding to the BLUF domains of predicted and known blue-light photoreceptors of different members of this genus, and also of organisms belonging to different taxa such as α, β, γ and δ Proteobacteria; and from eukaryotes such as Euglenozoa and Fungi (Figure 4) [22]. In some cases such as those of Euglena and Enterotelia, which contain two BLUF-domains in the same protein molecule, both sequences were included in the analyses. Figure 4 shows the maximum likelihood phylogenetic tree constructed from the alignments of the above sequences, whereby bootstrap values were calculated by the neighbor joining and parsimony methods. The tree clearly illustrates that all the BLUF domains present in members of the genus Acinetobacter are grouped together in a well-supported monophyletic cluster (bootstrap values of 100% and 99% by NJ and parsimony, respectively), suggesting that all of these putative photoreceptor domains share a common origin. The Acinetobacter cluster contains two major branches, B1 and B2 (Figure 4). Each branch contains at least one paralog gene from each species (similar colors in B1 and B2), with the exceptions of A. lwofii...
Blue Light Regulation in Acinetobacter

Presence of BLUF-coding Genes in Strains Showing Photoregulation of Motility and Biofilm Formation

Finally, we analyzed whether strains that presented photoregulation of motility and biofilm formation such as Acinetobacter ACI 412, A. nosocomialis ACI 32 and A. pittii ACI 988 contained BLUF domain-containing genes. For this purpose, we used information derived from the genome-sequenced strains A. calcoaceticus PHEA-2, A. nosocomialis RUH 2624 and A. pittii SH 024 to design specific primers that amplify the cognate BLUF-coding genes (acc. numbers ADY82057 and ADY82317 for A. calcoaceticus; EEW98085, EEX00046 and EEX01065 for A. nosocomialis; and EFF86081 and EFF86339 for A. pittii), and investigated their presence by PCR and nucleotide sequencing. We found the presence of homologs showing 100% identity to ADY82057 and ADY82317, in A. calcoaceticus ACI 412, EEX00046 and EEX01065 in A. nosocomialis ACI 32, and EFF86081 and EFF86339 in A. pittii ACI 988 (Table 2). We were not able to obtain an amplification product in the case of A. nosocomialis EEW98085, despite we assayed different amplification conditions.

Discussion

In this work, we show that light regulation is not restricted to A. baumannii but is rather widespread within the genus Acinetobacter. In fact, we found that blue light effectively regulates motility and biofilm formation at 24°C in many Acinetobacter species, including members of the ACB complex, such as A. calcoaceticus, A. nosocomialis and A. pittii (see Figure 1 and Table 2). Yet, in contrast to A. baumannii, in which the formation of biofilms is inhibited under blue light while stimulated in the dark, the opposite was observed in all the other species where blue light regulation was detected: biofilm formation was inhibited in the dark while stimulated under blue light. In non-A. baumannii species where both biofilm formation and motility were regulated such as A. baylyi, A. calcoaceticus, A. nosocomialis and A. pittii, low biofilm correlated with high motility under dark conditions. This response makes sense since a large amount of data support the notion that motility and biofilm formation can be expected to be mutually exclusive and counter-regulated, i.e. being sticky seems counterproductive for moving around, whereas adhesion and settling down might require reduced activity of the motility machinery [23–25]. Therefore, in these bacteria blue light contributes to the decision between motility and sessility and also may facilitate acclimation to different environments. In our previous work, we showed that blue light regulation in A. baumannii occurred only at low temperature, suggesting that it is important during its life in the environment perhaps allowing bacteria to sense environmental locations outside the human host [5]. However, the results presented here show that many environmental species such as A. baylyi, A. calcoaceticus and A. gernbergiae, as well as the clinically relevant species A. nosocomialis and A. pittii displayed blue light regulation of motility also at 37°C. This differential behavior at 37°C compared to A. baumannii may result from the extra content of BLUF-domain putative photoreceptors encoded in the genomes of these non-A. baumannii species (Figure 4 and Table 2). Indeed, only one photoreceptor is encoded in the A. baumannii genome. The protein present in strain ATCC 17978, BlsA, most probably functions only at 24°C: blsA mRNA levels at 37°C are significantly lower with respect to levels at 24°C [5, and the content of BlsA protein in the cells at 37°C is negligible or null [Mussi et al., unpublished data]. Alternatively, the differential behavior at 37°C between A. baumannii and other Acinetobacter species could result from idiosyncratic differences in expression patterns of photoreceptors and/or pathways and
partners modulating motility functions downstream the photosensing step in these organisms. In any case, *A. baumannii* might have become “blind” to light at 37°C because there is no positive selection to respond to this stimulus in the relative darkness of the warm-blooded host tissues.

The ability of *A. baylyi*’s BLUF-domain containing genes to restore photoregulation at 24°C in the ATCC 17978 *bla* mutant not only confirms that they encode bona fide BLUF photoreceptors, but also that they can transduce the light signal into *A. baumannii* motility and biofilm regulatory cascades, probably by using BlsA partner/s. In this context, *A. baylyi* formed large amounts of biofilms under blue light and almost negligible amounts were produced under dark conditions. It is therefore noteworthy that the opposite situation is observed when *A. baumannii* photoreceptors are expressed in the *A. baumannii* *bla* mutant, restoring in all cases the wild type phenotype corresponding to this species. Therefore, both cascades in *A. baumannii* and *A. baylyi* seem to be inversely affected at 24°C, independently of the origin of the photoreceptor used.

Moreover, we found that BLUF-domain containing genes, shown to be active photoreceptors in *A. baumannii* and *A. baylyi*, are present in all completely sequenced genomes available for members of this genus, and also in strains showing photoregulation of motility and biofilm formation, such as *A. calcoaceticus* ACI 412, *A. nosocomialis* ACI 32 and *A. pittii* ACI 988. The variable number of genes coding for BLUF-domain containing proteins (from one to six) in the different species analyzed in this study suggests that sensing and responding to light might be of differential importance among them, probably reflecting their different lifestyles and the diversity in niches in which they thrive. Phylogenetic analysis suggests a common origin for all BLUF domains within *Acinetobacter* and could distinguish well-differentiated clusters that group together BLUF homologs from different species, a situation particularly clear for members of the ACB complex, which most likely correspond to groups of orthologs. The different clusters may reflect a closer phylogenetic relationship among the species with corresponding proteins may not be active, or downstream partners modulating other cellular processes that remain unidentified in these species. Yet, functional characterization of BLUF domain-containing proteins encoded in species of the genus *Acinetobacter* other than those of *A. baumannii* or *A. baylyi* still needs to be conducted to ascertain their role as photoreceptors involved in light perception in these microorganisms. Regarding other cellular processes affected by light, it is worth mentioning that we could not detect light regulation of resistance to antibiotics in different clinically-relevant species of the genus *Acinetobacter*. Nevertheless, further research would contribute to draw a final conclusion in this sense.

Our understanding of the signal transduction mechanisms and regulatory cascades involved in *A. baumannii* BlsA and its homologs present in other species of the genus *Acinetobacter* is still scarce and currently under study in our laboratory. The final goal is to gain a full comprehension of light regulation in relation to host’s niches and lifestyles, which would perhaps need further understanding of *Acinetobacter* biology.

**Supporting Information**

**Figure S1** Quantification of the biofilms produced by different strains showing photoregulation of biofilm formation within the genus *Acinetobacter*. Error bars show standard error of the mean for 3 different biological replicates (n = 3). OD580/600, optical density at 580 or 600 nm, respectively. (TIF)

**Table S1** Blue light and resistance to antibiotic. Disc diffusion antibiotic susceptibility assay under blue light or in the dark at 24 or 37°C, of some strains of *A. nosocomialis*, *A. pittii* and *A. baumannii* which showed photoregulation of motility and/or biofilm formation. The *A. haemolyticus* strains analyzed in this work did not show photoregulation neither of motility nor biofilm formation, but one strain was included in this study due to the importance of this species in the clinical settings. The diameter of inhibition from three independent experiments (mm +/– SEM of three biological replicates) is indicated. AM, ampicillin; AN, amikacin; FEP, cepolin; CTX, cefotaxime; FOX, cefoxitin; CAZ, ceftazidime; CF, cephalotin; G, chloramphenicol; CIP, ciprofloxacin; IPM, imipenem; GM, gentamycin; MEM, meropenem; PIP, piperacillin; RA, rifampicin. (XLSX)

**Acknowledgments**

We are indebted to R. J. Seviour for providing strains representative of some of the different species within the genus *Acinetobacter*. We also thank Dr. Woojun Park for providing A. sp. DR1 (“A. oleivorans”), and Dr. Harald Seifert for providing genome-sequenced *Acinetobacter* strains. M.A.M. and A.M.V. are career investigators of CONICET.

**Author Contributions**

Conceived and designed the experiments: MAM LAA. Performed the experiments: AG MAM. Analyzed the data: MAM LAA MV AN. Contributed reagents/materials/analysis tools: MAM AMV. Wrote the paper: MAM LAA AMV AN MV.
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