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Blue-light-dependent inhibition of twitching motility in *Acinetobacter baylyi* ADP1: additive involvement of three BLUF-domain-containing proteins

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Twitching motility in *Acinetobacter baylyi* ADP1 is inhibited by moderate intensities of blue light in a temperature-dependent manner (maximally at 20 °C). We analysed the involvement of four predicted blue-light sensing using flavin (BLUF)-domain-containing proteins encoded in the genome of this strain in the twitching motility phenotype. All four genes were expressed both in light and in darkness. A phylogenetic tree showed that one BLUF domain, ACIAD2110, grouped separately from the other three (ACIAD1499, ACIAD2125 and ACIAD2129). Individual knockout mutants of the latter three, but not of ACIAD2110, fully abolished the light dependency of the twitching motility response. Quantitative analysis of transcript level of the three genes 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 ACIAD2125, ACIAD2129 and ACIAD1499, respectively. Double and triple knockouts of ACIAD1499, ACIAD2125 and ACIAD2129 confirmed the same phenotype as the corresponding single knockouts. Complementation of all the single knockouts and the triple knockout mutants with any of the three BLUF-domain-encoding genes fully restored the inhibition of twitching motility by blue light that is observed in the wild-type strain. *A. baylyi* ADP1 therefore shows a high degree of redundancy in the genes that encode BLUF-containing photoreceptors. Moreover, all plasmid-complemented strains, expressing any of the BLUF proteins irrespective of the specific set of deleted photoreceptors, displayed increased light-dependent inhibition of twitching motility, as compared to the wild-type ($P<0.001$). We conclude that the three genes ACIAD1499, ACIAD2125 and ACIAD2129 are jointly required to inhibit twitching motility under moderate blue-light illumination.

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INTRODUCTION

The ability to colonize surfaces with the purpose of growth, development or survival in different environments, is a fundamental property of bacterial cells. Twitching motility is a special kind of surface translocation, driven by cycles of extension, tethering and retraction of type IV pilus (tfp) fibres (Wall & Kaiser, 1999; Merz *et al.*, 2000). Twitching motility was first described by Loutrop as a flagella-independent surface movement in *Acinetobacter calcoaceticus* (Lautrop, 1962). Later, Henrichsen and Blom confirmed the observations and portrayed twitching motility as an intermittent and jerky movement of predominantly single cells, although smaller moving aggregates could also occur (Henrichsen & Blom, 1975). However, the underlying mechanism of this movement was unknown until 1980, when Bradley proposed that retraction of polar pili (later referred to as tfp) was in fact its driving force (Bradley, 1980).

Twitching motility appears to be restricted to a group of Gram-negative bacteria that includes important pathogens of animals, plants, and fungi (Bieber *et al.*, 1998; Dörr *et al.*, 1998; Fullner & Mekalanos, 1999; Hahn, 1997; Liles *et al.*, 1998; Liu *et al.*, 2001; Craig *et al.*, 2004; Collyn *et al.*, 2002). In addition, tfp was shown to be required for a number of functions, namely protein secretion (Li *et al.*, 2007), biofilm formation (Li *et al.*, 2007; O'Toole & Kolter,

Abbreviations: BLUF, blue-light sensing using flavin; CV, crystal violet; HK, histidine kinase; LED, light-emitting diode; tfp, type IV pilus.

1998; Chiang & Burrows, 2003), fruiting body development (Kaiser, 2003), virulence (Shi & Sun, 2002) and many forms of horizontal gene transfer (Dubnau, 1999; Wolfgang *et al.*, 1998).

A great diversity of environmental factors, including media components, regulate twitching motility, but the mechanism of action by which they affect this process is in most cases poorly understood (Rashid & Kornberg, 2000; Terry *et al.*, 1991; Harshey, 2003). This is e.g. the case in *Pseudomonas aeruginosa* where twitching motility is responsive to iron (Patriquin *et al.*, 2008), medium viscosity (Glick *et al.*, 2010), inorganic polyphosphate (Rashid & Kornberg, 2000) and to phosphatidylethanolamine (Kearns *et al.*, 2001), a lipid which has been shown to enhance social gliding motility in *Myxococcus xanthus* (Kearns & Shimkets, 2001). Another environmental signal that has been shown to control motility is light, in both phototrophic and chemotrophic organisms. In the cyanobacterium *Synechocystis* PCC6803, for instance, light and carbon source regulate motility (and phototaxis) via a cluster of genes, where signalling is linked to chromophore-binding photoreceptor domains (Bhaya *et al.*, 2001).

In silico analysis of genomes of non-phototrophic (i.e. chemotrophic) bacteria led to the identification of numerous putative photoreceptors (van der Horst *et al.*, 2007). Strictly focusing in prokaryotes, known photoreceptors that absorb in the blue region of the spectrum include cryptochromes, blue-light sensing using flavin (BLUF)-domain-containing-proteins, LOV-containing proteins (i.e. phototropins), all of them using flavins as chromophores, and the photoactive yellow proteins, using *p*-cumaric acid as the chromophore. So far, researchers have demonstrated the physiological relevance of light in a handful of chemotrophic bacterial species. Blue-light responsive chemotrophs defined at present are *Bacillus subtilis* (Avila-Pérez *et al.*, 2006), *Brucella abortus* (Swartz *et al.*, 2007), *Escherichia coli* (Tschowri *et al.*, 2009), *Caulobacter crescentus* (Purcell *et al.*, 2007), *Stigmatella aurantiaca* (Purcell *et al.*, 2007), *Acinetobacter baumannii* (Mussi *et al.*, 2010) and *Acinetobacter baylyi* ADP1 (Hoff *et al.*, 2009), and recently also *Rhizobium leguminosarum* (Bonomi *et al.*, 2012), each inhabiting niches in which the ability to tightly regulate cell physiology or development at the interface of soil or water with air provides an adaptive advantage to the cell.

A. baylyi ADP1 expresses two types of pili (type I and type IV), displays typical twitching motility on solid surfaces (Henrichsen & Blom, 1975; Gohl *et al.*, 2006) and encodes in its genome putative light-sensing proteins of the BLUF-family type. *In silico* alignments indicate that, in the genus *Acinetobacter*, the majority of putative bacterial BLUF-containing proteins are 'short' proteins composed of a BLUF domain plus 30–70 additional amino acids, unlike e.g. AppA in *Rhodobacter sphaeroides* and YcgF in *E. coli,* which are 'complex' multi-domain proteins (Gomelsky & Klug, 2002).

We previously reported that twitching motility in the environmental strain *A. baylyi* ADP1 was affected by light (Hoff *et al.*, 2009). Molecular studies in the opportunistic pathogen *A. baumannii* ATCC 17978 subsequently identified and characterized a blue-light sensing gene (*blsA*) (Mussi *et al.*, 2010). Interestingly, while *A. baumannii* has a single BLUF-encoding gene, the *A. baylyi* ADP1 genome harbours four sequences predicted to encode BLUF-domain-containing proteins, a fact that, according to bioinformatic data, is not exceptional in the class of *Gammaproteobacteria* (Losi & Gärtner, 2008). The reason(s) for the need for such an abundance of these genes is presently unknown. In this study we addressed this question and conclude that there is considerable redundancy of the BLUF-encoding genes present in the *A. baylyi* ADP1 genome. This conclusion is based on gene knockout experiments and phenotypic analyses of the wild-type, knockout mutants, and complemented strains.

METHODS

Strains, plasmids and culture conditions. *A. baylyi* ADP1 and its isogenic mutants were grown at 30 °C in lysogeny broth (LB) in batch culture at 200 r.p.m. Single *A. baylyi* ADP1 knockout mutants were grown in LB supplemented with 15 μg kanamycin ml^{-1}. Complemented strains were grown in LB with 15 μg tetracycline ml^{-1}. Single, double and triple knockout mutants, constructed by gene plus marker deletion, were grown at 30 °C and 200 r.p.m. without added antibiotics. Strains and plasmids used in this work are listed in Table 1.

Cell motility. Cell motility was assayed as previously described (Mussi *et al.*, 2010). Briefly, Petri plates were prepared with freshly poured medium containing 10 g tryptone l^{-1}, 5 g NaCl l^{-1} and a variable concentration of agarose, adjusted according to the temperature used in the assay. A 0.3 % (w/v) agarose concentration was used for assays incubated at 20 °C or 24 °C during 48 h and 15 h, respectively. For assays performed at 30 °C or 35 °C the plates were prepared with 0.4 % (w/v) agarose and incubated for 15 h and 9 h, respectively. The plates were inoculated on their surface with 3 μl of a bacterial culture, growing exponentially in LB to OD_{600} 0.3, and were incubated in darkness or in red or blue light. The light sources were red- and blue-light emitting diodes (LED), with emission peaks centred at 634 nm and 456 nm, respectively, and an intensity of 5–7 μmol m^{-2} s^{-1}, as determined by a LI-COR LI-1800 spectroradiometer. After the indicated incubation periods, the diameter of colonies expanding through twitching motility was measured. The assays were performed in triplicate plates and the mean ± SD was calculated and plotted. In order to compare assays performed at different temperatures the results were expressed as the ratio between the diameters measured in dark versus light (D/L). To test the hypothesis of increased blue-light dependent motility response of all complemented knockout mutants (n=12) with respect to the wild-type strain (n=4) we performed an ANOVA test on calculated D/L motility ratios.

General DNA procedures. Genomic DNA was isolated with the Fast DNA kit (Bio 101 System) according to the manufacturer's instructions. Plasmid DNA was isolated using commercial kits (Promega). DNA digestions were performed with restriction enzymes as indicated by the supplier (Promega) and fragment size was analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989).

Construction of single mutants of *A. baylyi* ADP1. For single-gene knockout mutants, internal segments of genes ACIAD1499,

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics*	Source or reference
Strains		
Acinetobacter baylyi ADP1		
A. baylyi ADP1	Wild-type	Dr. K. Hellingwerf, University of Amsterdam, The Netherlands
Mutants of *A. baylyi* ADP1		
1499KO	ACIAD1499::nptII, Km^r	This work
2110KO	ACIAD2110::nptII, Km^r	This work
2125KO	ACIAD2125::nptII, Km^r	This work
2129KO	ACIAD2129::nptII, Km^r	de Berardinis *et al.* (2008)
B++M1	ΔACIAD1499	This work
B++M2	ΔACIAD1499/ΔACIAD2125	This work
B++M3	ΔACIAD1499/ΔACIAD2125/ΔACIAD2129	This work
Complemented strains		
1499KO-C1499	1499KO harbouring pWp1499, Km^r Tet^r	This work
1499KO-C2125	1499KO harbouring pWp2125, Km^r Tet^r	This work
1499KO-C2129	1499KO harbouring pWp2129, Km^r Tet^r	This work
2125KO-C1499	2125KO harbouring pWp1499, Km^r Tet^r	This work
2125KO-C2125	2125KO harbouring pWp2125, Km^r Tet^r	This work
2125KO-C2129	2125KO harbouring pWp2129, Km^r Tet^r	This work
2129KO-C1499	2129KO harbouring pWp1499, Km^r Tet^r	This work
2129KO-C2125	2129KO harbouring pWp2125, Km^r Tet^r	This work
2129KO-C2129	2129KO harbouring pWp2129, Km^r Tet^r	This work
B++M3-C1499	B++M3 harbouring pWp1499, Tet^r	This work
B++M3-C2125	B++M3 harbouring pWp2125, Tet^r	This work
B++M3-C2129	B++M3 harbouring pWp2129, Tet^r	This work
Escherichia coli		
DH5α	Used for DNA recombinant methods	Gibco-BRL
Top10	Used for DNA recombinant methods	Invitrogen
Plasmids		
pGEM3zf(+)	PCR cloning vector, Ap^r	Promega
pAMB6K	pGEM3zf(+) *Bam*HI/*Pst*I internal sequence ACIAD1499, Ap^r Km^r	This work
pAMB7K	pGEM3zf(+) *Bam*HI/*Pst*I internal sequence ACIAD2110, Ap^r Km^r	This work
pAMB8K	pGEM3zf(+) *Bam*HI/*Pst*I internal sequence ACIAD2125, Ap^r Km^r	This work
pK19*mobsacB*	Vector for allelic exchange in *C. glutamicum* (pK18 oriVE.c., *sacB*, *lacZa*), Km^r	Schäfer *et al.* (1994)
pK1499	pK19*mobsacB* derivative containing an overlap extension PCR product composed of the up- and downstream regions of ACIAD1499, Km^r	This work
pK2125	pK19*mobsacB* derivative containing an overlap extension PCR product composed of the up- and downstream regions of ACIAD2125, Km^r	This work
pK2129	pK19*mobsacB* derivative containing an overlap extension PCR product composed of the up- and downstream regions of ACIAD2129, Km^r	This work
pWH1266	*E. coli–Acinetobacter* shuttle vector, *Acinetobacter* I*woffi* plasmid cloned into pBR322 *Pvu*II site, Ap^r Tc^r	Hunger *et al.* (1990)
pGp1499	Amplicon harbouring ACIAD1499 and its promoter cloned into pGEM-T Easy, Ap^r	This work
pGp2125	Amplicon harbouring ACIAD2125 and its promoter cloned into pGEM-T Easy, Ap^r	This work
pGp2129	Amplicon harbouring ACIAD2129 and its promoter cloned into pGEM-T Easy, Ap^r	This work
pWp1499	pWH1266 with a wild-type copy of ACIAD1499 with its native promoter cloned into *Pst*I and *Eco*RI sites, Ap^s Tc^r	This work
pWp2125	pWH1266 with a wild-type copy of ACIAD2125 with its native promoter cloned into *Pst*I and *Eco*RI sites, Ap^s Tc^r	This work
pWp2129	pWH1266 with a wild-type copy of ACIAD2129 with its native promoter cloned into *Pst*I and *Eco*RI sites, Ap^s Tc^r	This work

*Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Ap^s, ampicillin sensitive; Tc^r, tetracycline resistance.

ACIAD2110 and ACIAD2125 were amplified by PCR using primers 1499KOPF/PR, 2110KOPF/PR and 2125KOPF/PR, respectively (Table 2). Amplicons were cloned in the *Bam*HI and *Pst*I sites of pGEM-3zf(+) (Promega) and a kanamycin cassette was introduced in the *Sma*I site, resulting in plasmids pAMB6K, pAMB7K and pAMB8K, respectively. *A. baylyi* ADP1 was naturally transformed with 2 μg of each plasmid as previously described (Palmen *et al.*, 1993) and transformants from single cross-over recombination events were selected by plating on LB plates containing 15 μg kanamycin ml^{-1}. To confirm the knockout mutagenesis and plasmid integration into the *A. baylyi* ADP1 genome, PCR amplifications were performed with a designed primer for the SP6 promoter region of pGEM-3zf(+) and primers 1499PR, 2110PR or 2125PR, which bind to the 5′ end of each gene. A single knockout mutant of ACIAD2129 was kindly provided by Genoscope (de Berardinis *et al.*, 2008).

Construction of single, double and triple knockout mutants by gene deletion of *A. baylyi* ADP1. The procedure described by Jones & Williams (2003) was adapted to obtain multiple knockout mutants. Briefly, flanking regions of ACIAD1499, ACIAD2125 and ACIAD2129 were amplified using primers 1499PF_up/1499PR_up, 1499PF_down/1499PR_down; 2125PF_up/2125PR_up, 2125PF_down/2125PR_down;

Table 2. Primers used in this work

Primer	Nucleotide sequence
1499KOPF	5′-GCATCTGCAGATACGTTTTAACTCAAT-3′
1499KOPR	5′-GCATGGATCCATAAGGATTAAATTCGT-3′
2110KOPF	5′-GCATCTGCAGGAATTTAACTCAAATAATC-3′
2110KOPR	5′-GCATGGATCCAGGCAAAGTTGTTTTATC-3′
2125KOPF	5′-GCATCTGCAGAATGACATTACAGGGGT-3′
2125KOPR	5′-GCATGGATCCAGAGTCTGTAGTAAGCA-3′
SP6PF	5′-GCATGGATCCATTTAGGTGACACTATAGAATACT-3′
1499PR	5′-GCATCTGCAGTCAGGATGAATGAGGGTAAGGGTCA-3′
2110PR	5′-GCATCTGCAGCTATAAGAATGGATTAATTCCTCTG-3′
2125PR	5′-GCATCTGCAGCTAATGCATATCAGCTTGCTGAT-3′
1499PF_up	5′-GAGAATTCCACTTGAAGCACTTTATCAAC-3′
1499PR_up	5′-GTTATCAGGATGAATGAGGGATCTAACATAGGCCATAGAAGTTC-3′
1499PF_down	5′-GAACTTCTATGGCCTATGTTAGATCCCTCATTCATCCTGATAAC-3′
1499PR_down	5′-GCACTGCAGGTTTTCATTAAAACTGGCC-3′
2125PF_up	5′-GAGAATTCCCATAACCATTGGTGGGTG-3′
2125PR_up	5′-CAATTCTAATGCATATCAGCTTCTTACTTGCATACATCAAGC-3′
2125PF_down	5′-GCTTGATGTATGCAAGTAAGAAGCTGATATGCATTAGAATTG-3′
2125PR_down	5′-GCACTGCAGATTCAGGCAATATCATTTGAC-3′
2129PF_up	5′-GAGAATTCTATGTACTCACTCAAATAGAG-3′
2129PR_up	5′-GTACTTAAGTTTAAACATTATTTGGTAATTAAAACAATACAAATACTTG-3′
2129PF_down	5′-CAAGTATTTGTATTGTTTTAATTACCAAATAATGTTTAAACTTAAGTAC-3′
2129PR_down	5′-GCACTGCAGAGAATCATAACCAACCAGAGTC-3′
PF1499DEL	5′-ATTCTGCTGCATAAAGTCCAGC-3′
PR1499DEL	5′-ATTCCATTCTTCTCGATCAGC-3′
PF2125DEL	5′-GCTTATCATTATGATTTGCCAGTTG-3′
PR2125DEL	5′-ACCCACATCTTTGTTCTATCGATAC3′
PF2129DEL	5′-ACTTGGCAATTCGGTTATGC-3′
PR2129DEL	5′-CATGAGTATCGTCAATACCAAACG-3′
P1499-PF	5′-GACCTGCAGCACTAATTACGCTCAAACAGTCG-3′
P1499-PR	5′-GCGAATTCTCAGGATGAATGAGGGTAAGG-3′
P2125-PF	5′-GACCTGCAGTTTCATGGTTCTGCATTAAACAG-3′
P2125-PR	5′-CTAAGCTTCATCAATTCTAATGCATATCAGCTTG-3′
P2129-PF	5′-GACCTGCAGCGCAATAAAATCATTCCAGATTAA-3′
P2129-PR	5′-CTAAGCTTTCCACTTTCAAATTAAATATAAAGGAT-3′
RT 1499PF	5′-TGTCAGCAAAACCGCCAAACA-3′
RT 1499PR	5′-CGTATTGCTGTTGTCAAGATTTCCA-3′
RT 2110PF	5′-AACGGGACCTTCTGGAAGAC-3′
RT 2110PR	5′-CGTCTGCATAATACAGGACACC-3′
RT 2125PF	5′-CGAAACCATCGGAAAATCGATCC-3′
RT 2125PR	5′-TGGTGCAAACTTCATGCTCCAA-3′
RT 2129PF	5′-ATGCAAGAATGCTGTATGTGAG-3′
RT 2129PR	5-GTGCACCACAAATAGAATTTG-3′
RecA_PF	5′-CGAATTGCATGGTAATCTTCATT-3′
RecA_PR	5′-CTTGACCAATACGGCGTATATCT-3′

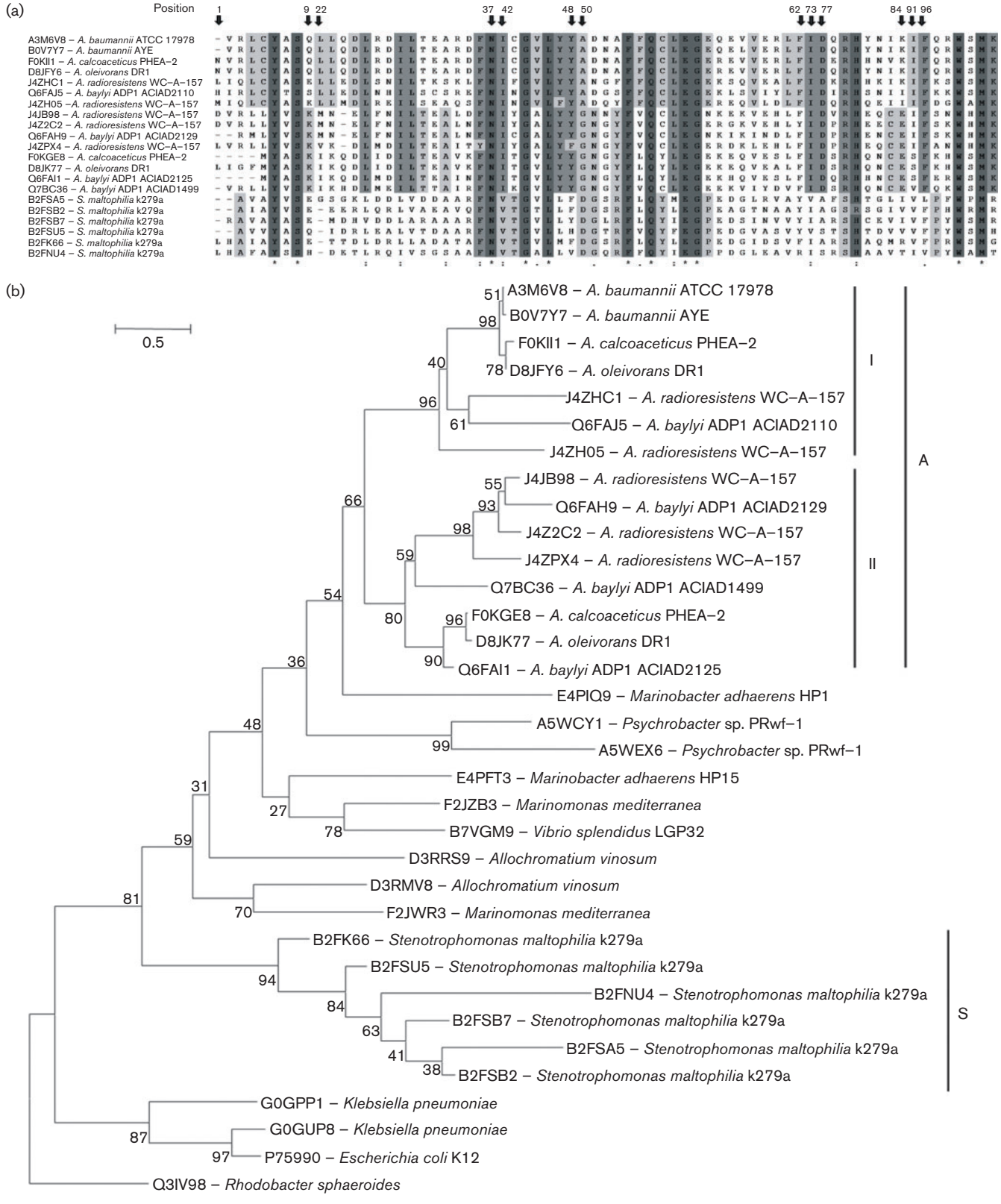

Fig. 1. Phylogenetic analysis of BLUF domains from *Gammaproteobacteria*. (a) Multiple alignment of putative BLUF domains (pfam04940) in members of *Acinetobacter* and *Stenotrophomonas maltophilia* K279a generated by CLUSTAL W and further adjusted manually. Species names are shown with Uniprot accession number. Arrows indicate the position of the alignment, positions 10–21, 38–41, 49, 63–72, 74–76, 85–90 and 92–95 were not informative sites and were deleted for the figure.

Asterisk (*), positions which have a single, fully conserved residue; colon (:), conservation between groups of strongly similar properties – scoring >0.5 in the Gonnet PAM 250 matrix; period (.), conservation between groups of weakly similar properties – scoring ⩽0.5 in the Gonnet PAM 250 matrix. (b) Phylogenetic tree of BLUF domains (pfam04940) of *Gammaproteobacteria* obtained by the Neighbour-joining method. Sequence belonging to *Alphaproteobacteria* (*Rhodobacter sphaeroides*) acts as an outgroup. Species names are shown with Uniprot accession number. Bar indicates the mean number of substitutions per site. Node labels indicate bootstrap values. Group A refers to the *Acinetobacter* sequences, organized in subgroups I and II, according to their most likely origin. Group S refers to the *S. maltophilia* K279a sequences which show a single (monophyletic) origin.

2129PF_up/2129PR_up, 2129PF_down/2129PR_down, respectively (Table 2). For each gene, flanking regions were joined by cross-over PCR using PR-up and PR-down sequences and cloned in *Eco*RI and *Pst*I sites of pK19*mobsacB* (Schäfer *et al.*, 1994) and transformed into *E. coli* DH5α to generate plasmids pK1499, pK2125 and pK2129, respectively (Table 1). *A. baylyi* ADP1 was naturally transformed with 2 μg of pK1499 as previously described (Palmen *et al.*, 1993) and selection was made on LB plates, containing 15 μg kanamycin ml^{-1}. The selected colonies had the plasmid inserted in one of the flanking regions and they were kanamycin-resistant but were not able to grow on LB plates containing 10 % (w/v) sucrose after incubation for 48 h at 16 °C. To select double cross-over mutants, a single colony was grown for 24 h in non-selective LB broth at 30 °C and 200 r.p.m. A dilution of the culture was plated onto LB plates containing 10 % (w/v) sucrose and incubated for 48 h at 16 °C. A sucrose-resistant and kanamycin-sensitive colony was selected in appropriate media and the ACIAD1499 deletion was confirmed by PCR and automated DNA sequencing, using primers PF1499DEL/PR1499DEL which anneal outside the flanking regions (Table 2). The knockout mutant in ACIAD1499 (B++M1, Table 1) was transformed with pK2125 and the selection procedure was repeated in order to obtain the double knockout mutant (B++M2, Table 1). Deletion of ACIAD2125 was then confirmed by PCR and automated DNA sequencing, using primers PF2125DEL/PR2125DEL. To construct the triple knockout mutant (B++M3, Table 1), B++M2 was transformed with pK2129 and selection was performed as described above. Deletion of ACIAD2129 was confirmed by PCR and automated DNA sequencing, using primers PF2129DEL/PR2129DEL. Accordingly, a triple knockout mutant was selected with the three putative BLUF-domain-encoding genes, ACIAD1499, ACIAD2125 and ACIAD2129, deleted.

Construction of complemented strains. Chromosomal fragments harbouring ACIAD1499, ACIAD2125 and ACIAD2129, including their predicted promoter region, were PCR-amplified using *A. baylyi* ADP1 total genomic DNA and primers P1499-PF/P1499-PR, P2125-PF/P2125-PR and P2129-PF/P2129-PR, respectively (Table 2). Each amplicon was cloned into pGEM-T Easy, thus obtaining pGp1499, pGp2125 and pGp2129, respectively, and subsequently subcloned into the *Eco*RI and *Pst*I sites of pWH1266 in *E. coli* Top10, thereby generating plasmids pWp1499, pWp2125 and pWp2129. *A. baylyi* ADP1 single knockouts (1499KO, 2125KO and 2129KO) and B++M3 triple knockout were naturally transformed with 2 μg of pWp1499, pWp2125 or pWp2129 as previously described (Palmen *et al.*, 1993) and tetracycline-resistant colonies were selected. Thus, nine complemented strains in the single knockouts (1499KO-C1499, 1499KO-C2125, 1499KO-C2129, 2125KO-C1499, 2125KO-C2125, 2125KO-C2129, 2129KO-C1499, 2129KO-C2125 and 2129KO-C2129) and three complemented strains in the triple knockout (B++M3-C1499, B++M3-C2125 and B++M3-C2129) were obtained (Table 1).

Protein alignment and phylogenetic analysis. Only *Gammaproteobacteria* with complete genomes reported in the NCBI database were selected for the phylogenetic analysis. BLUF domain (pfam04940) sequences obtained from the UniprotKB database were aligned using CLUSTAL W (Thompson *et al.*, 1994) and neighbour-joining trees were produced with the MEGA5 program (Tamura *et al.*, 2011), using the Jones–Taylor–Thornton model of amino acid substitution with a gamma distribution of 0.8. Confidence in neighbour-joining trees was determined by analysing 1000 bootstrap replicates. Identical tree topologies were obtained when the maximum-likelihood and minimal evolution methods were used.

Biofilm assay. An overnight LB-grown culture of *A. baylyi* ADP1 was diluted 1/100 in fresh LB broth maintained at 30 °C. Two millilitres of this dilution was inoculated into standard 15 ml polypropylene tubes and incubated for 4 days stagnantly at 24 °C or 30 °C, either in darkness or under white light (intensity ~11 μmol m^{-2} s^{-1}). After this period, supernatants were carefully removed with a Pasteur pipette and the tubes were washed five times with PBS, dried in the upside-down position, and stained with 1 % (w/v) crystal violet (CV) for 20 min. The CV-stained cells were solubilized in 2 ml of 96 % ethanol, incubated for 30 min with gentle mixing (80 r.p.m.), after which the OD at 587 nm was measured. To normalize the amount of biofilm formed to the total biomass present in each sample, including pellicles, and attached and suspended cells, additional tubes were included in the assay. For this determination, tubes were centrifuged, supernatants were discarded, and the pellets were resuspended in 2 ml of PBS, sonicated for 10 s at low power with a thin probe, and vortexed for 1 min. The OD at 540 nm of the resuspended cells was then measured and the results were expressed as OD_{587}/OD_{540}. The assay was performed twice in quintuplicate. Results were expressed as mean ± SD.

Transcriptional analysis. Cells from six motility plates, incubated at 24 °C in the dark or under blue LED light, were washed with 2 ml of RNA*later* solution (Life Technologies). Samples from the same incubations, i.e. dark or light, were pooled and processed as a single sample. 'Light' and 'dark' samples were centrifuged at 5000 ***g*** for 2 min at 4 °C and each pellet was subjected to total RNA extraction with Trizol reagent as indicated by the supplier (Invitrogen). Samples were treated with DNase I (Promega) and retro-transcription was performed with M-MLV reverse transcriptase (Promega). Samples without enzyme added were included as negative controls. The cDNA samples from 'dark' and 'light' conditions were used both in a conventional PCR and for qPCR assays. Amplification was performed with primers RT1499PF and RT1499PR, RT2110PF and RT2110PR, RT2125PF and RT2125PR, RT2129PF and RT2129PR (Table 2) corresponding to internal regions of ACIAD1499, ACIAD2110, ACIAD2125 and ACIAD2129, respectively. A conventional PCR was performed after reverse transcription (RT-PCR) under the following conditions: 95 °C for 5 min, 30 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min. qPCR was performed using SYBR green as fluorescence dye in the Mx3005P QPCR System (Strategene) under the following conditions: 95 °C for 5 min; 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s; finally 95 °C for 1 min and 55 °C for 30 s, in order to obtain the melting curve. Three biological samples were analysed in triplicate and expression levels were normalized to the *recA* expression level in each RNA sample. Although results from triplicates of one biological sample are shown, similar results were obtained with the other two biological samples.

RESULTS

Phylogenetic analysis of BLUF domains from *Gammaproteobacteria*

In silico analysis of the five fully sequenced *Acinetobacter* genomes revealed that all species harbour BLUF-coding genes. While ten strains referred to as '*A. baumannii*' carry only one BLUF-coding gene, members of the other four species (*A. baylyi* ADP1, *A. calcoaceticus* PHEA-2, *A. oleivorans* DR1 and *A. radioresistens* WCA157) carry two or more predicted proteins containing this domain. Particularly, *A. baylyi* ADP1 and *A. radioresistens* harbour four and five putative BLUF sequences, respectively. The corresponding hypothetical genes in the *A. baylyi* ADP1 genome were tagged ACIAD1499, ACIAD2110, ACIAD2125 and ACIAD2129 in the NCBI database.

A multiple sequence alignment of putative BLUF domains from six *Acinetobacter* strains and *Stenotrophomonas maltophilia* K279a is shown in Fig. 1(a). These two genera have a pairwise distance average of 74.82 %. The alignment revealed 11 highly conserved amino acids (indicated with asterisks). Seven of them are fully conserved; they are identical to the corresponding residues of the canonical BLUF domain, defined according to pfam04940. The other four amino acids, Leu46, Phe54, Trp99 and Met101, are generally considered as partially conserved residues for this domain family (Gomelsky & Klug, 2002). This analysis suggests that, in spite of the relatively low percentage of identity among the four BLUF-containing protein sequences of *A. baylyi* ADP1 (37–55 %), they all conserve the amino acids involved in the flavin cofactor binding, namely Tyr6, Asn37, Gln56 and Trp99, considered essential in this type of photoreceptor (Fig. 1a).

A phylogenetic tree of putative BLUF domains with members of the class of *Gammaproteobacteria* is shown in Fig. 1(b). As several members of this class have genes coding for 'complex' proteins composed of various domains, we restricted the analysis only to the BLUF domain, consisting of 90–98 amino acids length. The neighbour-joining tree built with these sequences showed that the six BLUF domains encoded in the *S. maltophilia* K279a genome may have been derived from a single (monophyletic) origin (Group S), whereas the BLUF domains encoded in the *Acinetobacter* genomes (Group A) are most likely diverted into two branches, subgroup IA and subgroup IIA, as supported by the bootstrap value of 66 %, thus either pointing to two distinct origins, or to a post-speciation gene-duplication event. Note that the putative *A. baylyi* ADP1 BLUF-domain-encoding sequences display a distribution that differs from the one expected according to the species in which they have been identified, with ACIAD2110 corresponding to subgroup IA, while the other three BLUF-domain-encoding genes, ACIAD2125, ACIAD2129 and ACIAD1499, belong to subgroup IIA. Additionally, the calculated pairwise distance average within Group A and Group S were 47.57 % and 49.99 %, respectively, suggesting that the differences between sequences within each category (A and S) could be due to evolutionary mutation rate. Thus, the retrieval of two or more putative BLUF-domain-encoding genes in a single strain in the same category could be due to gene duplication from a common ancestor, followed by gene mutations. Furthermore, for *A. baumannii*, the additional hypothesis of gene loss should be considered to explain the presence of a single BLUF-domain-encoding gene.

Analysis of the genetic organization in *A. baylyi* ADP1 indicated that the four BLUF-domain-encoding genes harbour, in addition to the BLUF domain pfam04940, a short ~55 aa C-terminal peptide that shows no significant similarity to known domains or motifs from the PFAM database, thus clustering the BLUF domains in this strain among the category of the 'short' BLUF proteins. Interestingly, the analysis of the predicted secondary structure of the four BLUF-containing proteins indicates that their C-terminal tail would consist in all cases of two well-conserved alpha helices.

Blue light inhibits twitching motility in a temperature-dependent manner

We have previously shown in *A. baylyi* ADP1 that surface migration through twitching motility is modulated by illumination with visible light (Hoff *et al.*, 2009). Therefore we analysed twitching motility under red and blue light and determined that motility is specifically inhibited by blue light (Fig. 2a). The assay was carried out at four different temperatures, as shown in Fig. 2(b, c). When the motility assay was performed at 30 °C or 35 °C the migration ratio between darkness and light was close to 1.0, suggesting a modest effect of blue light on motility under these conditions. However, when the assay was performed at 24 °C or 20 °C this ratio increased to 1.5 and 1.6, respectively, which means that the inhibition of motility by blue light was considerably more significant at lower temperatures. All successive experiments were therefore done at 24 °C, as a compromise between rate of twitching-based migration and light sensitivity, which was also the temperature selected for experiments performed in *A. baumannii* (Mussi *et al.*, 2010).

Other phenotypes assayed, including the transformation frequency and the production of catechol type siderophores, were also affected by visible light (data not shown). On the other hand, biofilm formation was not responsive to light, as the amounts measured under blue light ($OD_{587}/OD_{540}=1.11 \pm 0.18$) or darkness ($OD_{587}/OD_{540}=1.27 \pm 0.13$) were not significantly different.

Three BLUF domains are required for blue-light dependent inhibition of twitching motility

Assays of 1499KO, 2110KO, 2125KO and 2129KO single knockout mutants revealed that the phenotype of inhibition of twitching motility by blue light was abolished in three of

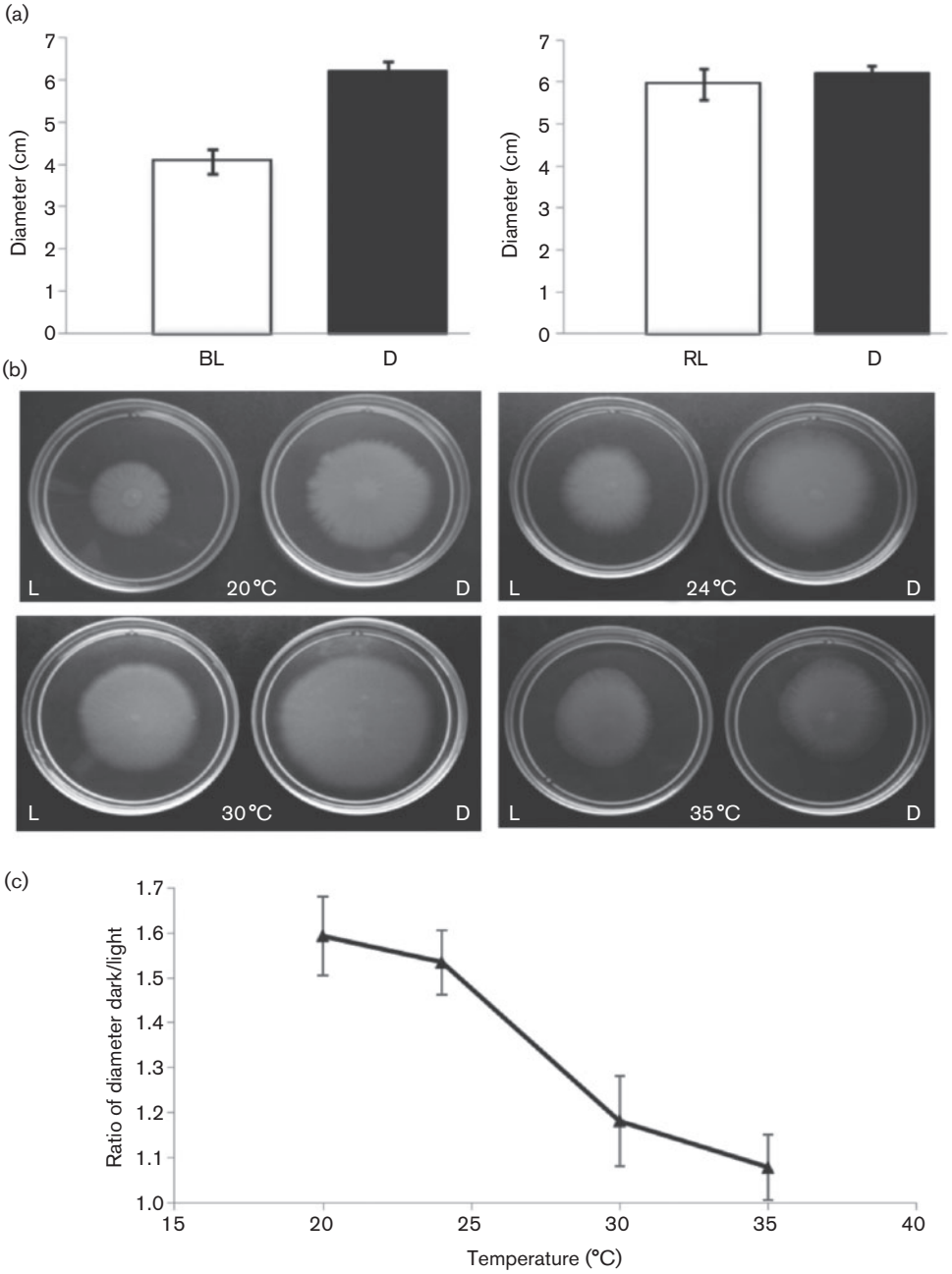

Fig. 2. Effects of light and temperature on *A. baylyi* ADP1 motility. (a) Plate surfaces were inoculated with 3 μl of *A. baylyi* ADP1 cultures growing exponentially (OD_{600}=0.3), incubated in darkness (D) or under blue (BL) or red (RL) light at 24 °C during 15 h and motility diameters were measured. (b) Plate surfaces were inoculated with 3 μl of *A. baylyi* ADP1 cultures growing exponentially (OD_{600}=0.3) and incubated in darkness (D) or under blue light (L) at 20 °C, 24 °C, 30 °C or 35 °C during 48 h, 15 h, 15 h or 9 h, respectively. (c) Ratio of colony diameter in dark versus light at different temperatures. Error bars represent ±SD, n=3.

the strains, while 2110KO remained fully sensitive, just as the wild-type strain (Fig. 3a). From these results we concluded that the BLUF-containing proteins encoded by ACIAD1499, ACIAD2125 and ACIAD2129 were actively and simultaneously involved in the regulation of twitching motility, whereas ACIAD2110 was not. Moreover, a double knockout mutant (B++M2), lacking both ACIAD1499 and ACIAD2125, and a triple knockout mutant (B++M3) lacking all these three genes active in regulation of twitching motility (i.e. ACIAD1499, ACIAD2125 and ACIAD2129), displayed the same phenotype, that is, no inhibition of twitching motility by blue light (Fig. 3b). Note that the triple

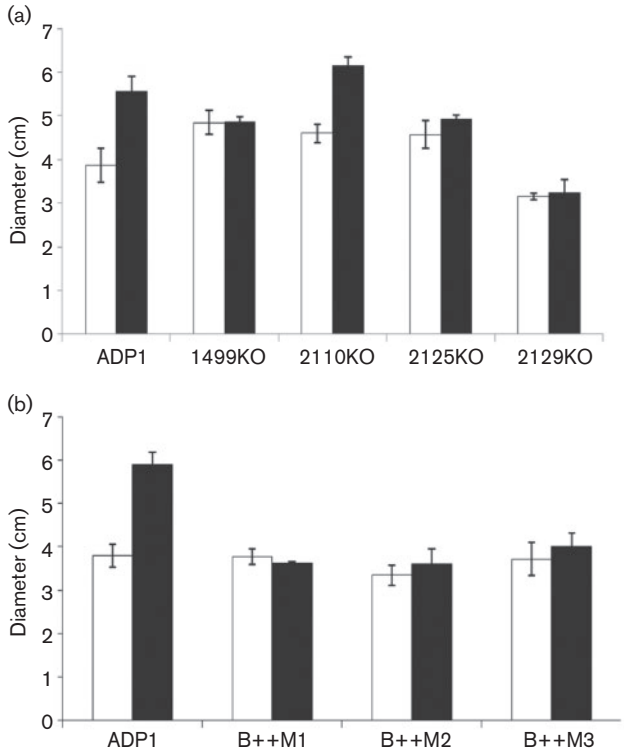

Fig. 3. Effect of blue light on twitching motility in *A. baylyi* ADP1 and knockout *bluf* mutants. Plate surfaces were inoculated with 3 µl cultures growing exponentially (OD_{600}=0.3), incubated in darkness (black bars) or under blue light (white bars) at 24 °C for 15 h and motility diameters were measured (n=3). (a) *A. baylyi* ADP1, 1499KO, 2110KO, 2125KO or 2129KO. (b) *A. baylyi* ADP1, B++M1 (lacking ACIAD1499), B++M2 (lacking ACIAD1499 and ACIAD2125) or B++M3 (lacking ACIAD1499, ACIAD2125 and ACIAD2129).

knockout mutant does contain the ACIAD2110 gene, which provides additional confirmation that the BLUF domain that this gene encodes is not involved in blue-light-mediated inhibition of twitching motility.

In order to solve the way in which the three BLUF-domain-encoding sequences, ACIAD1499, ACIAD1225 and ACIAD2129, are involved in the light-induced regulation of the twitching response, the single knockout mutants were complemented with a plasmid carrying one of the three genes, cloned with its own promoter. As shown in Fig. 4(a–c), all three single knockout mutants, complemented with any of the three plasmids expressing a BLUF-domain-containing protein (pWp1499, pWp2125 or pWp2129) reversed the twitching motility inhibition phenotype to that of the wild-type. These results confirm that all three genes contribute to light inhibition of twitching motility and, more interestingly, that the different proteins can complement (and replace) each other in this property. In addition, all plasmid-complemented strains revealed a significant increase in the light-dependent inhibition of twitching motility, as compared to the wild-type strain by ANOVA test on D/L ratios ($P<0.001$), suggesting that the requirement for the simultaneous expression of the three genes might be related to a low amount of transcript that each of these three genes generates individually (see below). Additional supporting evidence was revealed in the analysis of the complemented strains obtained in the triple knockout mutant (B++M3) host. As shown in Fig. 4(d), complementation of B++M3 with any of the three plasmids, pWp1499, pWp2125 or pWp2129, presumably expressing increased amount of BLUF protein from their endogenous promoter, fully restored the light inhibition of the twitching phenotype, indicating that any single photoreceptor protein is able to restore the light response, if adequately expressed. These results confirmed the redundancy of the three genes, and contribute to the hypothesis that the necessity of the three proteins for inhibition of twitching under blue light in the wild-type strain might be due to a low amount of transcripts and/or protein that each of the BLUF encoded genes individually makes available.

Analysis of expression of putative BLUF-encoding proteins in *A. baylyi* ADP1

Studies on the expression pattern of the four BLUF-domain-encoding genes from *A. baylyi* ADP1 were performed by RT-PCR analysis, on samples of the wild-type strain obtained from motility assays at 24 °C (Fig. 5). We demonstrated that the four genes were expressed in the light and in the dark. Quantitative analysis of transcripts of the three genes involved in blue-light inhibition of twitching motility (ACIAD1499, ACIAD2125 and ACIAD2129, but not ACIAD2110) showed differences in transcript levels between light and dark conditions. For all three genes, expression levels were higher in the dark than in the light, with measured dark/light ratios of 2.69 ± 0.39 for ACIAD1499, 1.79 ± 0.21 for ACIAD2129 and 1.65 ± 0.28 for ACIAD2125 (mean ± SD; n=3). This lower expression level of the genes encoding BLUF-domain-containing proteins under blue light complicates the straightforward interpretation of the molecular mechanism of light inhibition of twitching motility (see Discussion).

DISCUSSION

Genomics analyses

Blue-light sensing photoreceptor proteins of varied domain composition have been described in non-phototrophic organisms. Examples are the YcgF (BLUF-EAL) antirepressor in *E. coli* and the YtvA (LOV-STAS) protein in *B. subtilis*. Also histidine kinases (HK) have been described that contain an N-terminal LOV domain and exhibit increased autophosphorylation activity in the light (Losi & Gärtner, 2008). Such kinases have also been found responsible for processes, i.e. the regulation of cell attachment in *C. crescentus* (Purcell *et al.*, 2007), virulence in the human/animal pathogen *B. abortus* (Swartz *et al.*,

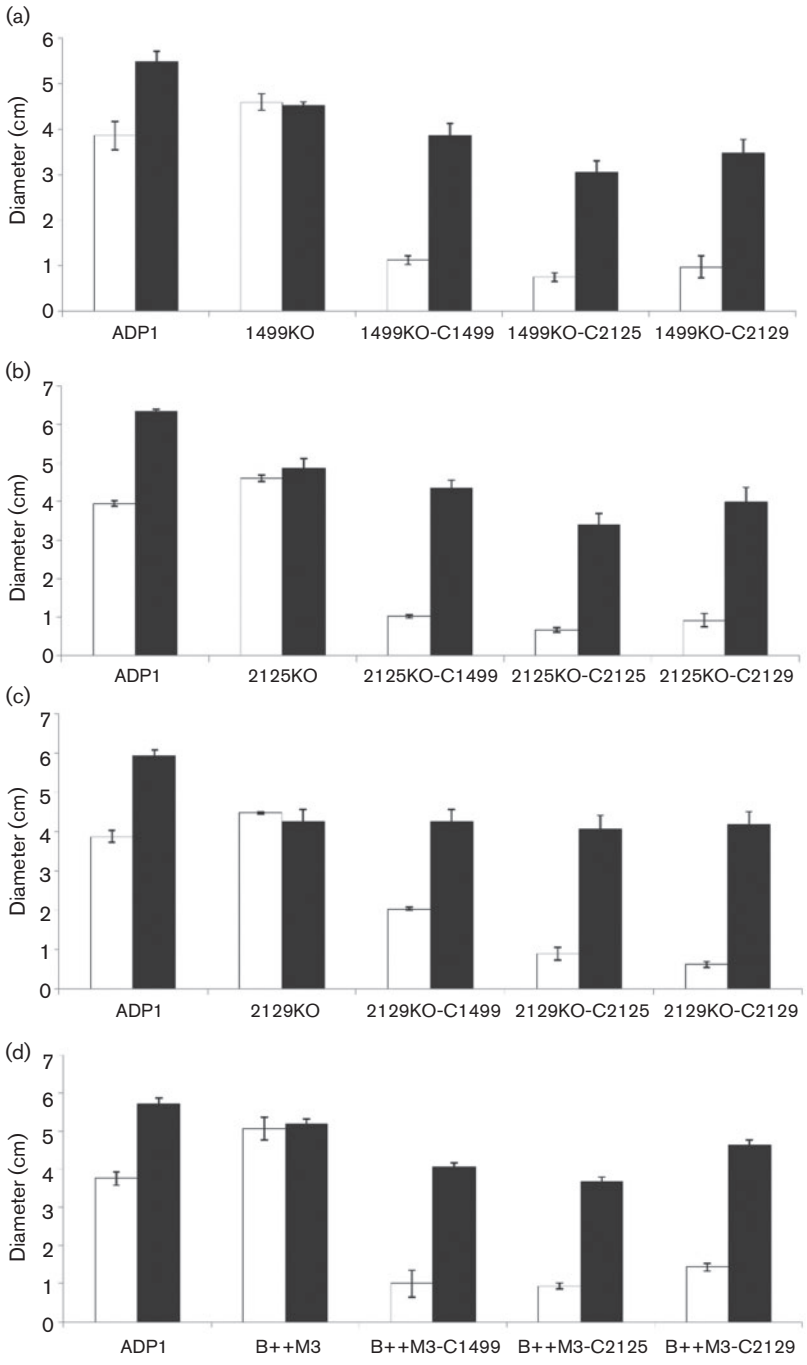

Fig. 4. Effect of blue light on motility of *A. baylyi* ADP1, its single knockout mutants (KO), its triple knockout mutant (B++M3) and their corresponding complemented strains (C). (a) Motility diameter measurements (mean ± SD, n=3) of *A. baylyi* ADP1, 1499KO, 1499KO-C1499, 1499KO-C2125 and 1499KO-C2129 after incubation in darkness (black bars) or under blue light (white bars) at 24 °C during 15 h for *A. baylyi* ADP1, and 21 h for the single knockout mutants and complemented strains. (b) Same assay and condition as (a) using strains *A. baylyi* ADP1, 2125KO, 2125KO-C1499, 2125KO-C2125 and 2125KO-C2129. (c) Same assay and condition as (a) using strains *A. baylyi* ADP1, 2129KO, 2129KO-C1499, 2129KO-C2125 and 2129KO-C2129. (d) Same assay and condition as (a) using strains *A. baylyi* ADP1, B++M3, B++M3-C1499, B++M3-C2125 and B++M3-C2129.

2007) and presumably in the plant pathogen *Pseudomonas syringae* (Losi, 2004). HKs with N-terminal GAF and/or Bhy domains (i.e. bacteriophytochromes) were identified in *Deinococcus radiodurans* and *P. aeruginosa* (Li *et al.*, 2010; Yang *et al.*, 2008) while orphan LOV domains, which lack an effector module, have been found e.g. in *Pseudomonas putida* (Jentzsch *et al.*, 2009).

The combination of domains present in the majority of bacterial light-sensing proteins studied to date gives an explicit suggestion for their function. However, this is not the case in representatives of the five species of the *Acinetobacter* genus, since all fully sequenced *Acinetobacter* genomes analysed in this work carry putative BLUF-domain-encoding genes consisting of a pfam04940 domain plus a ~55-amino acid long C terminus, without additionally recognizable sequence signatures. A similar gene architecture was found in *pixD* of *Synechocystis* sp. PCC 6803 (Yuan & Bauer, 2008) and *papB* of *Rhodopseudomonas palustris* (Kanazawa *et al.*, 2010). In this last strain biofilm formation is regulated via a blue-light-dependent modulation of the cellular c-di-GMP level, mediated by protein–protein interaction between the short

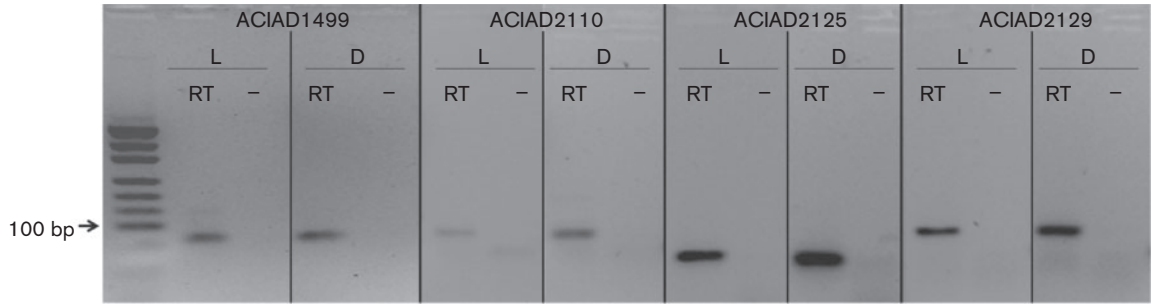

Fig. 5. Expression of four putative BLUF-coding genes of *A. baylyi* ADP1 (ACIAD1499 ACIAD2110, ACIAD2125 and ACIAD2129). Agarose gels at 2.5 % (w/v) show the RT-PCR (RT) products and their correspondent negative controls (–) from samples incubated in darkness (D) or under blue light (L). Effect of blue light on ACIAD1499, ACIAD2125 and ACIAD2129 transcripts levels was analysed with qPCR; the ratios of expression level measured at dark versus light were 2.69 ± 0.39; 1.65 ± 0.28 and 1.79 ± 0.21, respectively.

BLUF-domain-containing protein, PapB, and the EAL-domain-containing protein, PapA. Whether a similar complex between a photoreceptor protein and putative output proteins operates in *Acinetobacter* is currently unknown.

The four genes identified as ACIAD1499, ACIAD2110, ACIAD2125 and ACIAD2129 in the *A. baylyi* ADP1 genome were transcriptionally active both in light and in darkness, and none of the expressed BLUF-domain-containing proteins carried any accompanying structural motifs such as HK, response regulator, GGDEF, HYD, EAL, STAS, or helix–turn–helix type. Moreover, in *A. baumannii* ATCC 17978 the only BLUF-domain-encoding gene (*blsA*), which is also of the 'short' type, is close to an upstream gene encoding a putative BOF (binding of oligosaccharide) protein that has been suggested to synergistically contribute to a possible function (Mussi *et al.*, 2010). In *A. baylyi*, however, it is not possible to link the function of any of the BLUF-domain-containing proteins with any of the genes that are physically close in their genomic context. To date, only a *mutS* gene (upstream ACIAD1499) and two genes flanking ACIAD2125, corresponding to a (putative) siderophore biosynthesis protein and a ferredoxin, have been found at close distance.

Blue-light regulation of biofilm formation has been demonstrated in a number of non-phototrophs, namely *E. coli* (Tschowri *et al.*, 2009), *C. crescentus* (Purcell *et al.*, 2007) and *Idiomarina loihiensis* (van der Horst *et al.*, 2009). Surprisingly, we were not able to correlate the amount of biofilm formation to the presence of light in *A. baylyi* ADP1, as it has been reported in *A. baumannii* ATCC 17978 (Mussi *et al.*, 2010) and recently also in *A. baylyi* (Golic *et al.*, 2013).

On the other hand, we observed inhibition of twitching motility by blue light in a temperature-dependent manner, similar to the results previously described for *A. baumannii* (Mussi *et al.*, 2010). Furthermore, temperature has been found to strongly affect blue-light sensitivity, probably owing to differential expression of the proteins at different temperatures or due to involvement of a temperature-dependent monomer–dimer equilibrium (Losi & Gärtner, 2012).

The mechanism of light inhibition of twitching motility

A unique molecular mechanism underlying the blue-light mediated inhibition of twitching motility cannot be proposed yet. Blue light presumably converts the BLUF domains from their dark (i.e. basal) state to their signalling state. Upon illumination by blue light, the signalling state of each of the three proteins can then (i) interact with the pilus machinery to inhibit twitching motility or (ii) affect the expression of gene(s) that in turn would modulate twitching motility. Our current assays do not allow us to distinguish between these two possibilities. If the difference in growth rate between wild-type, knockouts and complemented strains is taken into account (approximately 20 % lower in the mutants due to marker insertions, plasmid copy number, etc.) it may be considered that (the rate of) twitching in the dark is unaffected by BLUF-domain deletion or complementation (Figs. 3 and 4).

Light decreases the expression level of all three BLUF domains involved in regulation of twitching motility (Fig. 5). This presumably will give rise to an overshoot in the extent of light inhibition of twitching motility, because the lower levels of transcripts will, in turn, also decrease the amount of signalling state of the BLUF domains in the light. The increased and persistent inhibition of twitching motility observed in the complemented strains (Fig. 4) is in agreement with this interpretation.

Redundancy of the BLUF-domain-encoding genes and additive effect of the encoded proteins

Strikingly, three of the four BLUF-domain-encoding genes, ACIAD1499, ACIAD2125 and ACIAD2129, but not ACIAD2110, are simultaneously and additively involved

in providing light sensitivity to twitching motility in *A. baylyi*. This conclusion is based on the observation that knocking out any of these three genes while the two others are actively present fully abolished the phenotype of light inhibition of twitching motility. For the gene labelled ACIAD2110, which groups in a different branch compared to the other three BLUF domains of *A. baylyi* ADP1, a different origin could be proposed, compatible with a horizontal gene transfer event, or a divergence occurring before the speciation event (Fig. 1). Nevertheless, the null phenotype associated with this gene was an unexpected result, in view of its similarity to the *blsA* gene identified in *A. baumannii* ATCC 17978, and the response of this organism to light (Fig. 5).

The three genes, ACIAD1499, ACIAD2125 and ACIAD2129, may be considered functionally redundant, as any of them, singly and independently, can restore the blue-light inhibition on motility phenotype in the triple knockout mutant, when expressed from a plasmid (Fig. 4d). In contrast to the situation in higher organisms, micro-organisms and particularly phages and viruses are very economical with the coding capacity of their DNA. Hence few examples of gene redundancy in bacteria are available. Exceptions are e.g. the seven *rrn* operons in *E. coli* (Stevenson & Schmidt, 2004), the four lactate dehydrogenases in *Lactococcus lactis* (Gaspar *et al.*, 2011), and the three *tcpD* detoxification genes for the degradation of haloaromatic compounds in *Cupriavidus necator* (Pérez-Pantoja *et al.*, 2009). In most cases there is a clear physiological function for this redundancy: high transcript levels that cannot be provided with a single promoter (in *E. coli* and *Lactococcus*), and differentiated selectivity of the separate promoters, or the advantage of rapid response in environments defined by fluctuations in resource availability, in the case of *Cupriavidus*. For the three BLUF domains in *A. baylyi* ADP1 such a reason has not been identified. Our data indicate that (i) the three BLUF-domain-encoding genes are relatively poorly expressed as compared to genes such as *recA* and *rrn*, (ii) all three BLUF-domain-encoding genes are functionally replaceable by each other with respect to the twitching motility response under blue light and (iii) all are moderately repressed by light. In contrast to higher plants, in which specific physiological responses may be regulated by up to four or five phytochromes (Franklin & Whitelam, 2005), the triple BLUF-domain based regulation of twitching motility in *A. baylyi* is the first example, to our knowledge, of the additive involvement of multiple photosensory receptors in the prokaryotic kingdom.

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