Light regulates attachment, exopolysaccharide production, and nodulation in *Rhizobium leguminosarum* through a LOV-histidine kinase photoreceptor

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Edited by Robert Haselkorn, University of Chicago, Chicago, IL, and approved June 6, 2012 (received for review December 22, 2011)

Rhizobium leguminosarum **is a soil bacterium that infects root hairs and induces the formation of nitrogen-fixing nodules on leguminous plants. Light, oxygen, and voltage (LOV)-domain proteins are blue-light receptors found in higher plants and many algae, fungi, and bacteria. The genome of *R. leguminosarum* bv. *viciae* 3841, a pea-nodulating endosymbiont, encodes a sensor histidine kinase containing a LOV domain at the N-terminal end (R-LOV-HK). R-LOV-HK has a typical LOV domain absorption spectrum with broad bands in the blue and UV-A regions and shows a truncated photocycle. Here we show that the R-LOV-HK protein regulates attachment to an abiotic surface and production of flagellar proteins and exopolysaccharide in response to light. Also, illumination of bacterial cultures before inoculation of pea roots increases the number of nodules per plant and the number of intranodular bacteroids. The effects of light on nodulation are dependent on a functional *lov* gene. The results presented in this work suggest that light, sensed by R-LOV-HK, is an important environmental factor that controls adaptive responses and the symbiotic efficiency of *R. leguminosarum*.**

Light is not only the major source of energy in the biosphere but also an essential signal that controls growth, development, and behavior of many different organisms. All multicellular organisms have an array of sensory systems to detect light. Photosynthetic bacteria have both a photosynthetic apparatus that converts light into chemical energy and photoreceptor proteins that sense the wavelength and intensity of light and transduce this information into various cellular pathways. Sequencing of bacterial genomes showed that photoreceptor proteins are present in heterotrophic bacteria in addition to photosynthetic bacteria (1, 2). Photoreceptor proteins found in these microorganisms can be classified into six families, according to their amino acid sequence and their associated chromophore: phytochrome domains (linear tetrapyrrole); light, oxygen, and voltage (LOV) domains (flavins); blue-light photoreceptor (BLUF) domains (flavins); photoactive yellow protein (p-coumaric acid); and the sensory and light-harvesting rhodopsins (retinylidines).

LOV domains are a subset of the large and diverse PAS superfamily (3), which are implicated in cellular signaling processes across all kingdoms of life. The best-characterized LOV proteins are plant phototropins, which contain two N-terminal LOV domains (LOV1 and LOV2) and are involved in many responses of plants to blue light including phototropism, stomatal opening, chloroplast movements, and leaf solar tracking (4). About 20% of the sequenced nonphotosynthetic bacterial genomes contain genes codifying LOV proteins (5). However, the function of most of these proteins is still unknown.

The genomes of all *Brucella* species sequenced to date encode for a LOV protein harboring three domains: a LOV domain at the N terminus (the sensory domain), a histidine kinase (HK) at the C terminus (the output domain), and a PAS domain between them. LOV-HK from *Brucella abortus* binds FMN as a chromophore, shows a typical LOV domain absorption spectrum and photochemistry (cysteinyl-adduct formation), and exhibits blue-light–dependent HK activity (6). We have previously shown that light increases the virulence of *B. abortus* in a macrophage infection model. Deletion of the LOV-containing gene from *B. abortus*, or replacement of the LOV-domain conserved cysteine with alanine, abolishes the light-mediated virulence response to the same level as that observed in the dark (6).

Brucella and *Rhizobium* are closely related bacteria (7) that belong to the α-2 subdivision of Proteobacteria. Members of this group inhabit diverse ecological niches and are able to form close associations with eukaryotic hosts. Rhizobia are capable of establishing a nitrogen-fixing symbiosis within legume nodules, whereas *Brucella* spp. are facultative intracellular animal pathogens.

The interaction between rhizobia and legumes begins with an exchange of signals (8, 9); this "molecular talk" eventually results in the development of the root nodule primordium. For an effective symbiosis, bacteria must attach to root hairs, invade the infection thread, and be released into nodule host cells. Within the nodule, bacteria differentiate to bacteroids that fix atmospheric nitrogen (10). Several factors, including pH, ionic strength, bacterial motility, and chemotaxis, as well as the synthesis of the extracellular acidic exopolysaccharide (EPS) were proposed to influence rhizobial attachment to root hairs (11, 12). Results of several studies indicate that EPS may also affect root invasion and nodule development (8, 13). Although the biology of the legume–*Rhizobium* symbiosis has received more attention, the majority of rhizobia live in the bulk soil or in the competitive rhizosphere niche. In these environments, rhizobia must have mechanisms to survive and compete with other microorganisms (8). Production of a polymeric matrix made of polysaccharides to live in a biofilm could be a bacterial survival strategy on the soil particles or in the rhizosphere. The acidic EPS of *Rhizobium leguminosarum* is crucial for the formation of microcolonies and a structured biofilm in vitro as well as on the root hairs (14, 15).

The genomes of several *Rhizobium* strains harbor a *lov* gene encoding a predicted LOV protein that shares a high degree of identity with *Brucella* LOV-HK. Here, we present evidence showing that the *R. leguminosarum* LOV-HK regulates flagella synthesis, EPS production, biofilm formation, and nodulation in a light-dependent manner.

Results

R. leguminosarum **Has a LOV-HK Protein.** A predicted LOV-domain–containing protein, homolog to *Brucella* LOV-HK, was

Author contributions: H.R.B., D.M.P., G.P., R.A.B., A.Z., and F.A.G. designed research; H.R.B., D.M.P., G.P., M.d.C.C., and M.F. performed research; L.I.P. contributed new reagents/analytic tools; H.R.B., D.M.P., G.P., M.d.C.C., R.A.B., A.Z., and F.A.G. analyzed data; and H.R.B., D.M.P., G.P., A.Z., and F.A.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121292109/-/DCSupplemental.

identified in the *R. leguminosarum* bv. *viciae* strain 3841 (*Rle*) genome from a BLAST search (http://www.genedb.org). The gene pRL110320 is on a rather unusual megaplasmid involved in symbiosis, pRL11. The protein, here designated R-LOV-HK, shows 40% identity with the *B. abortus* homolog (Fig. 1*A*). Similar proteins to R-LOV-HK were also identified in other α-proteobacterial genomes (Fig. S1*A*).

The R-LOV-HK protein bears 345 residues and shows an N-terminal LOV domain followed by an HK domain (Fig. 1*A*). The comparison against the previously characterized *Brucella* LOV-HK (B-LOV-HK) shows that LOV is the most conserved domain with 60% identity, whereas the HK domain only has 30% identity (Table S1). Both HK domains belong to the HWE HK family that is usually associated with photosensory proteins (16). However, both LOV-HK proteins display important differences: B-LOV-HK has a sequence after the LOV domain predicted to be a characteristic Jα-helix followed by a PAS domain of unknown function whereas R-LOV-HK lacks both the predicted Jα-helix sequence and the PAS domain. Jα-helices are located directly next to some LOV domains in plants and bacteria and are involved in signal propagation (17). Proteins similar to R-LOV-HK from other α-proteobacteria were clustered into two groups defined by the HK family. One group has an HK domain belonging to the HWE family and the other group has an HK domain belonging to the HisK A2 family (18) (Fig. S1*B*). There is also a remarkable relationship between the Jα-helix and the HK domain: LOV-HK proteins with HWE domains do not bear a Jα-helix at the C terminus of the LOV domain, whereas LOV-HKs with HisK A2 exhibit an α-helix, although presenting differences in its length (Fig. S1*B*). Interestingly, R-LOV-HK displays a region of 29 residues at its N terminus (not present in *B. abortus* LOV-HK) that is predicted to be an α-helix exposed to the solvent similar to the Jα-helix from YtvA and not an amphipathic Jα-helix like that of LOV2. Structural studies are needed to describe the signaling elements of R-LOV-HK.

R-LOV-HK Has a Truncated Photocycle. To study the biochemistry of R-LOV-HK, a construct containing the LOV domain of R-LOV-HK (R-LOV) was successfully expressed in *Escherichia coli* and purified. The ground state absorption spectrum of R-LOV shown in Fig. 1*B* is consistent with that of other known LOV domains, with broad bands in the blue and UV-A regions, reflecting a bound flavin. These two bands exhibit the vibrational structure characteristic of other LOV domains such as phototropin LOV2 from *Avena sativa* or YtvA from *Bacillus subtilis* (19). R-LOV probably binds FMN as chromophore but we cannot exclude other flavins as cofactors. Following illumination with a short light pulse, R-LOV loses absorption in the blue region accompanied by the formation of a new maximal absorption band at around 390 nm (R-LOV-390), consistent with a light-driven cysteinyl adduct formation (Fig. 1*C*). The adduct state in R-LOV is stable for several hours, indicating that the flavin-cysteinyl adduct does not break spontaneously in the dark, in contrast with the adduct lifetimes of seconds to minutes for most other LOV domains (20). This truncation of the photocycle has been previously observed in full constructs of *Brucella* LOV-HK and in the three *Arabidopsis* LOV-domain–containing proteins FKF1, LKP2, and ZTL, which are involved in plant photo-periodic signaling (21). It has been suggested that adduct decay is base catalyzed and is initiated by abstraction of the N5 proton (19, 22). Supporting this mechanism, a recent report shows that in phototropin LOV2, the rate of adduct decay is enhanced by the presence of imidazole, presumably acting as an extrinsic base (23). However, we did not observe any back reaction for R-LOV-390 (adduct decay) in the presence of imidazole up to a 0.1 M concentration (Fig. 1*C*), suggesting either that the base cannot penetrate the R-LOV-390 structure or that this construct has additional structural constraints.

Sensing of Light Through R-LOV-HK in *Rle*. The development of a biofilm is one of the main adaptation responses of bacteria to environmental changes, especially when these changes involve stressful stimuli (24). In *Caulobacter crescentus*, sensing of blue light through LOV-domain HK has been associated with cell–cell and cell-surface attachment (25), suggesting that blue-light sensing may be involved in the regulation of bacterial surface determinants. To investigate the role of R-LOV-HK in bacterial attachment to an abiotic surface and the formation of biofilms in vitro, we generated a *lov* mutant of *Rle*. We first analyzed the effect of light on biofilm formation by measuring bacterial adherence to polystyrene. WT bacteria exposed to white light (15 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 48 h) showed a strong reduction in the adherence to polystyrene (Fig. 2*A*). In contrast, the *lov* mutant showed no significant differences in the biofilm biomass between light and dark conditions (Fig. 2*A*). The complemented strain expressing R-LOV-HK (*lov* +

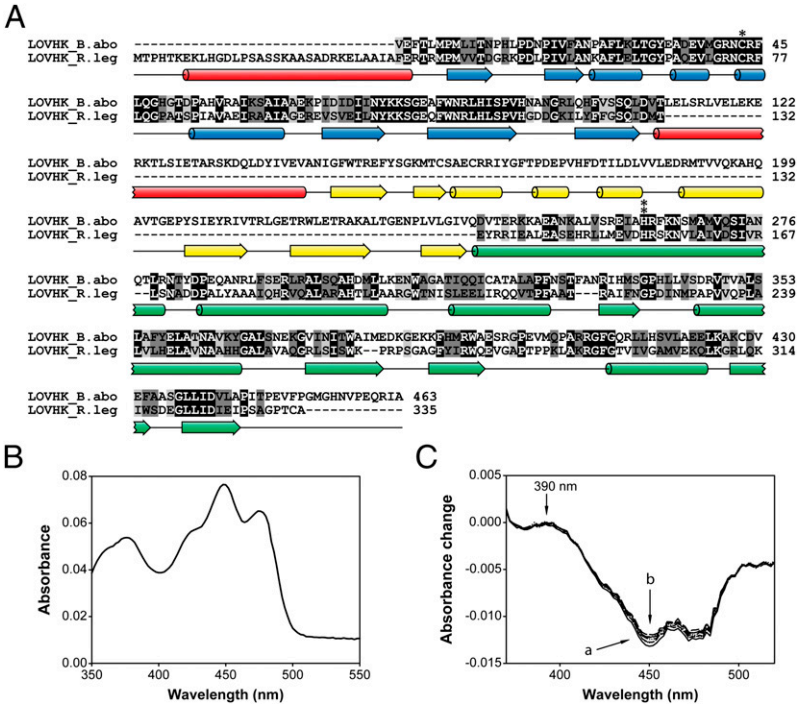

Fig. 1. *Rle* has a protein with LOV and HK domains and a truncated photocycle. (*A*) Sequence alignment between R-LOV-HK and B-LOV-HK. Identical amino acids are shaded in black and conserved amino acids in gray. The secondary structure elements are represented as cylinders (α-helices) and arrows (β-strands). The different domains are colored in blue (LOV), yellow (PAS), and green (HK). The predicted helices present at the N and C termini of the LOV domain are colored in red. The conserved cysteine residue present in all LOV domains (*) and the histidine residue that is autophosphorylated (**) are indicated. (*B*) Absorption spectrum of the R-LOV domain. (*C*) Light-induced absorbance changes. An absorption band around 390 nm (R-LOV-390) indicated by an arrow is consistent with a light-driven cysteinyl adduct formation. Trace a shows the absorption change about 1 s after a blue-light flash (1-ms duration, 400–500-nm bandwidth). Trace b is the absorption difference spectrum 7 h after the flash. The intermediate six traces are the difference spectra taken at 1-h intervals between traces a and b.

p*lov*) behaved like the WT strain; i.e., a clear reduction in biofilm formation was observed under the same light conditions. To further determine the dependence of this phenotype on a functional LOV protein, the ability of cloned *lov* carrying mutations in key predicted residues to complement the null *lov* mutant was analyzed. Two point mutations in conserved residues were generated: the residue C75, which is responsible for the formation of a light-induced covalent adduct to the FMN in the LOV domain (Fig. 1*A*) (19), and the predicted conserved H163 residue that is autophosphorylated by the HK domain (Fig. 1*A*) (16). These *lov* genes containing either the C75A or the H163A mutations were cloned under their own *lov* regulatory sequence in a replicative vector (yielding pC75A and pH163A plasmids) and conjugated into the insertional *lov* mutant strain, and adhesion to polystyrene was evaluated. Both pC75A and pH163A plasmids were unable to complement the adhesion phenotype. These observations confirm that light affects adherence through a functional R-LOV-HK protein (Fig. 2*A*). The expression of Myc-tagged wild-type, C75A, and H163A LOV variants in the *lov* mutant background was confirmed by Western blot analysis (Fig. S2). Remarkably, inactive LOV-HK C75A and H163A protein levels were much higher compared with the wild-type LOV levels, regardless the illumination conditions. The protein levels of wild-type LOV also remain unchanged in light/dark cultures. This observation suggests a negative regulation on the LOV-HK expression exists that depends on its own light-sensing and kinase activities.

In *Rle*, synthesis of the acidic EPS leads to the formation of a biofilm in vitro. Mutants unable to produce the EPS are impaired in biofilm formation (14). We wondered whether regulation of the EPS synthesis may account for the different amounts of biofilm biomass observed between light and dark conditions. WT, *lov* mutant, and *lov* mutant harboring p*lov*, pC75A, or pH163A were cultured in tryptone-yeast (TY) medium under different light conditions and extracellular EPS was quantified. The supernatants from the WT cultures showed a decreased amount of EPS under light conditions compared with the EPS produced in the dark. Significantly, though, the *lov* mutant strain produced similar amounts of EPS in light or darkness (Fig. 2*B*). The light-responsive phenotype was partially restored by reintroduction of an intact copy of the *lov* gene (p*lov*). The lack of full complementation under light conditions might be explained by a number of factors, including an unpredicted regulatory effect due to the expression *in trans* of the *lov* gene and a copy number effect from the p*lov* plasmid. As expected, *lov* + pC75A and *lov* + pH163A were unresponsive to light and displayed a phenotype similar to that of the *lov* mutant.

The flagellum is one of the factors that may influence initial attachment and biofilm development in several symbiont and pathogenic bacteria (26, 27). The ability of bacteria to swim or swarm by rotating their helical flagella represents another important adaptive mechanism as it provides optimal positioning of cells in the microenvironment (28). In *Sinorhizobium meliloti*, motility-impaired mutants that lack flagella have a reduced attachment to plastic surfaces (29). *Rle* has one or two subpolar flagella, which are responsible for swimming and swarming motility (11, 30). We investigated whether the abundance of flagellar proteins is modulated by light in *Rle* by SDS/PAGE of purified flagellar proteins (Fig. S3*A*). Some components of the flagella were more abundant in dark-grown than in irradiated cells, as previously observed in *Agrobacterium tumefaciens* (31). The observed protein bands were identified by mass spectrometry as FlaA (30.97 kDa) and FlaB (31.18 kDa) flagellin proteins and hook components FlgE (44.44 kDa) and FlgK (52.13 kDa), products of the RL0718, RL0719, RL0728, and RL0729 genes, respectively. All were induced in the dark (or repressed in the light) (Fig. S3). FlgE, FlaA, and FlaB have been shown to be present in the culture supernatant and/or in the flagellar filaments of *R. leguminosarum* strain 3841 (30, 32). The presence of flagella was evaluated by means of SEM. WT cells cultured in the dark displayed both attached and detached flagella (Fig. 2 *C* and *D*). Remarkably, under light conditions, bacteria displayed a strong reduction in the number of flagella compared with bacteria cultured in the absence

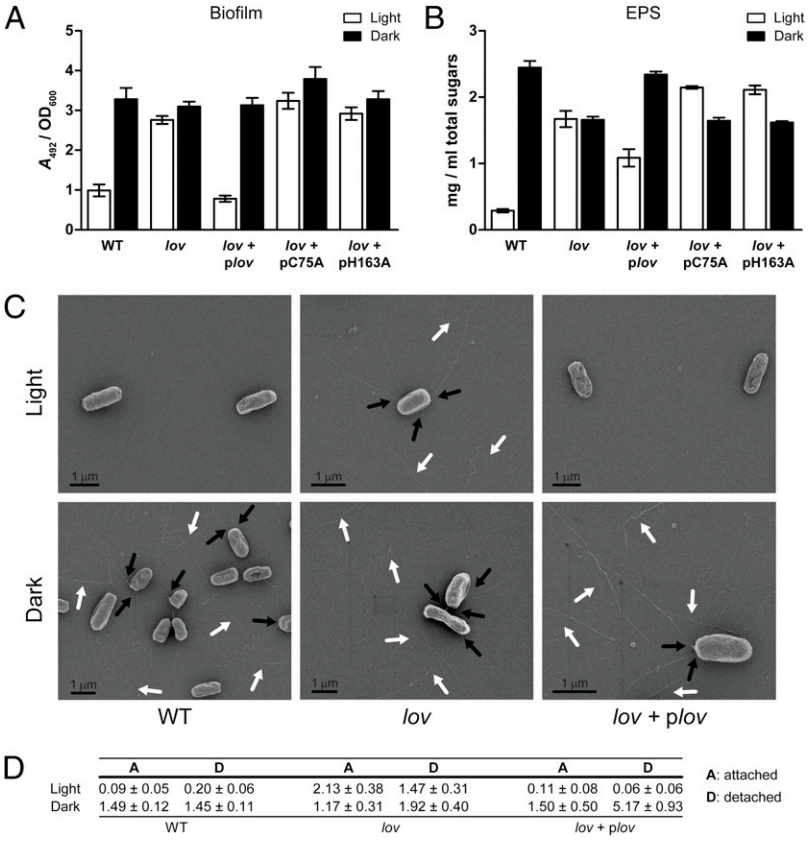

Fig. 2. Light regulates biofilm formation, EPS production, and flagella abundance in *Rle* through R-LOV-HK. (*A*) The adhesion to abiotic surfaces was measured by crystal violet staining. The absorbance of crystal violet (A_{492}) was measured at 492 nm and normalized by OD_{600}. Values are expressed as mean ± SD (n = 18). (*B*) Production of acidic EPS of *Rle*. Values are expressed as mean ± SD (n = 2). (*C*) *Rle* strains were visualized with SEM. Cell-detached (open arrows) and attached (solid arrows) flagella are indicated. (*D*) SEM quantification. Attached and detached flagella per cell from 10 representative fields of each sample were quantified. The average number of flagella per cell is indicated, assuming a Poisson distribution. The WT, *lov* mutant, and *lov*-complemented strains were analyzed. In *A* and *B* two *lov* strains harboring point-mutated LOV C75A and H163A were included in the assay. *A*–*C* are representative of three independent experiments performed under continuous light or darkness.

MICROBIOLOGY

of light (Fig. 2*D*). This difference was lost in the *lov* mutant strain and recovered in the complemented strain (Fig. 2 *C* and *D*). These observations suggest that sensing of blue light through R-LOV-HK down-regulates flagellar proteins. The detached flagellar structures that are not connected to any cell (Fig. 2*C*) are observed only in dark cultures of WT and complemented strains. However, they were observed in *lov* mutant bacteria in both light and dark conditions.

These differences in the presence of flagella and the previous findings from Oberpichler et al. (31) that light affects motility on some Rhizobiales strains, including a soil isolate of *R. leguminosarum*, led us to test whether there is a difference in the motility of *Rle* cells under light vs. dark conditions. We tested the swimming motility of WT *Rle* in soft agar plates by inoculating the bacteria in the center of the plate. When the plates were incubated under intense white light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$), WT bacteria showed a strong reduction in halo diameter compared with the plates kept in the dark (Fig. S4*C*). This striking difference prompted us to evaluate whether light intensity has an effect on bacterial growth. *Rle* cells were impaired to grow when light intensity was over 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ but this effect was not mediated by LOV-HK (Fig. S4*A*). Moreover, continuous illumination for 3 d with low light intensity allowed normal growth of both WT and *lov* mutant strains (Fig. S4*B*). Swimming motility of *Rle* was assayed under low light intensity (15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and showed no significant differences in halo diameters (Fig. S4*C*), suggesting that light affects the growth of *Rle* and that this effect is independent of R-LOV-HK. The swimming motility under permissive light intensities exhibits no measurable differences compared with that under dark conditions in both the WT and the *lov* mutant strains. A possible explanation for these observations is that under permissive light conditions the motility plate assay is not sensitive enough to show differences. Alternatively, flagella synthesis and motility are regulated by different mechanisms depending on whether bacteria are grown in a liquid or a semisolid medium. Several bacterial species were shown to have different flagella abundance and/or motility behavior in liquid and semisolid media (33–35).

To study the mechanism by which R-LOV-HK regulates flagellar abundance, the levels of *flaA* and *flgE* mRNA (encoded by the *flaA-B* and *flgE-K* operons, respectively, Fig. S3*B*) were quantified by RT-qPCR. To that end, WT or *lov* strains grown under continuous light or after a period of 3.5 h in the dark were analyzed. Our results show that light regulates in a negative fashion the expression of both *flaA* and *flgE* genes at the transcriptional level in a *lov*-dependent manner (Fig. 3 *A* and *B*).

Sensing of Blue Light Changes the Nodulation Capacity of *Rle*. Rhizobial EPS not only is required for biofilm formation but also plays an important role in host–symbiont interactions (8). Motility is also implicated in nodulation efficiency. It has been shown that different species of rhizobia, including *Rle*, display chemotaxis toward root exudates (36, 37). Chemotaxis of *Rle* cells is required to promote competitive nodulation of peas (11). As light regulates flagella formation and EPS production through R-LOV-HK, we were prompted to test the effect of light in the nodulation of pea plants. Bacteria grown under white light or dark conditions were used to infect pea roots. After 3 wk, total nodules were quantified and collected. Because effective (N_2 fixing) nodules can be discriminated by their reddish color due to the presence of leghemoglobin (38), reddish and white nodules of each plant were also quantified. The number of total (Fig. 4*A*) or reddish (Fig. S5*A*) nodules in plants inoculated with WT bacteria grown under light conditions was significantly higher than that in plants inoculated with WT bacteria grown in the dark. Interestingly, the number of total or reddish nodules induced by the *lov* mutant was similar regardless of the light/dark culture conditions and comparable to that of the WT grown in the dark (Fig. 4*A* and Fig. S5*A*). Conversely, the number of white nodules induced by irradiated WT was significantly lower than that induced by the WT strain under dark conditions (Fig. S5*B*). Consistent with these findings, the *lov* mutant formed a markedly higher number of white nodules compared with the WT (grown under any condition) and was independent of light/darkness (Fig. S5*B*). As expected, complementation of the mutant with the cloned *lov* gene restored light dependency of these nodulation features (Fig. 4*A* and Fig. S5 *A* and *B*).

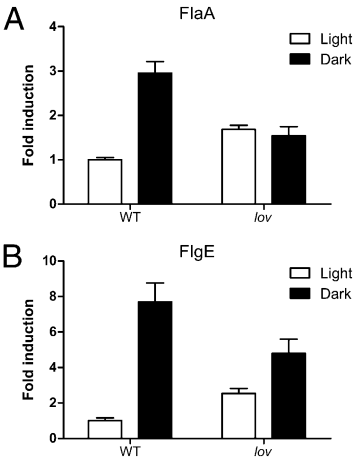

Fig. 3. Real-time quantitative RT-PCR. The expression of the flagellar genes (*A*) *flaA* and (*B*) *flgE* was assayed by RT-qPCR in *R. leguminosarum* 3841 and in the isogenic *lov* mutant strain in dark and light conditions using specific primers. Bacteria were grown in Y minimal medium at OD_{600} = 0.3 and harvested to extract total RNA. The data shown are mean ± SD of duplicate samples from one representative experiment and are reported as fold induction relative to expression of WT in light cultures. Initiation factor 1 (IF-1) was used as a housekeeping gene. The experiment was repeated two times with similar results.

The average number of rhizobia recovered from each nodule (cfu per nodule) was also dependent on light because intranodular bacterial counts were reduced over 10-fold in plants inoculated with WT *Rle* cultures kept in the dark compared with those under continuous illumination (Fig. 4*B*). Inoculation of pea roots with the *lov* mutant led to similar low bacterial counts in nodules, independently of the illumination conditions, and was comparable to those obtained with the WT strain cultured in the dark. Complementation with the p*lov* plasmid restored the WT phenotype (Fig. 4*B*). Fig. 4*C* shows a graphical display of how data are distributed. The horizontal axis represents the number of cfu per nodule and the vertical axis shows the fraction of nodules with this number of bacteria or less. This plot shows that the WT-light sample is shifted to higher values of cfu per nodule and is clearly separated from the rest. Using a two-sample Kolmogorov–Smirnov test, we found that the *lov*-light, *lov*-dark, and WT-dark samples are compatible, statistically identical; whereas all of them are different from the WT-light sample (the *P* value for the compatibility hypothesis is smaller than 10^{-11} for the three cases) (Fig. 4*D*). These observations indicate that sensing of light by R-LOV-HK increases the number of *Rle* bacteroids inside the nodules.

To better analyze differences in nodulation efficiency, a competence experiment was performed. WT, *lov*, and 1:1 mix cultures, grown in light or darkness, were used to inoculate pea plants and nodule occupancy was determined. The *lov* mutant was clearly outcompeted by the WT strain grown in light as well as in dark conditions (Fig. 4*E*). Interestingly, the mutant strain was found only in nodules occupied also by the WT strain (Fig. 4*E*). In addition, WT outnumbered *lov* mutant by 100-fold in light. In darkness this difference was even more remarkable (>10^5-fold) (Fig. 4*F*). One possible explanation for this unexpected result is that in light WT *Rle* partially rescues the *lov* mutant nodulation capacity, providing *in trans* some extracellular factors that help to increase nodulation efficiency of the *lov* mutant. Taken together, all these results confirm a preponderant role of R-LOV-HK in *Rle* symbiosis.

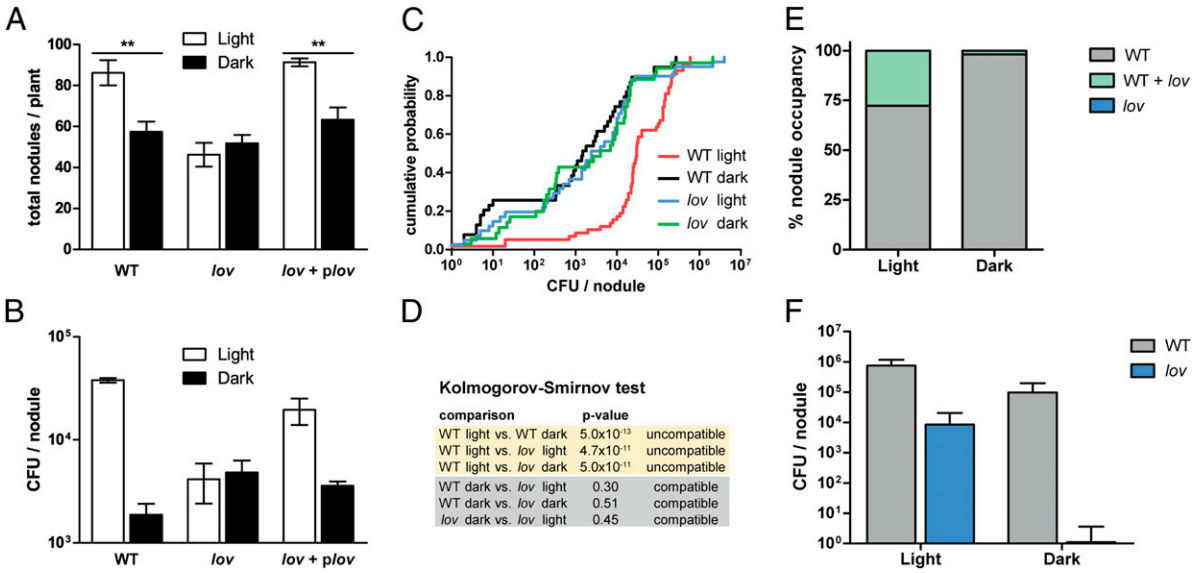

Fig. 4. Light modulation of symbiotic parameters in *Rle*. Pea plants were inoculated with white light- or dark-incubated cultures of *Rle* WT, *lov* mutant, or *lov* complemented strains After 21 d the numbers of (*A*) total nodules per plant and (*B*) cfu per nodule were determined. Values are expressed as mean ± SD ($n = 5$), representative of three independent experiments performed. Statistical analysis was carried out by performing a two-tailed Student's *t* test (**$P < 0.01$). (*C*) Cumulative probability of cfu per nodule data. The horizontal axis represents the number of bacteria per nodule and the vertical axes the fraction of nodules with this number of bacteria or less. (*D*) A two-sample Kolmogorov–Smirnov test was used to determine whether datasets are or are not compatible (statistically identical). (*E* and *F*) Competence assay. (*E*) The percentages of nodules occupied by the WT or the *lov* mutant or co-occupied by both strains, in light or dark conditions. (*F*) Intranodular counts for each strain are reported as cfu per nodule. Values are expressed as mean ± SD ($n = 5$).

Discussion

The results presented in this work show that R-LOV-HK regulates surface attachment, flagella synthesis, and EPS in response to light. Also, light modulates nodulation in a *lov*-dependent manner, implying that this signal is an important environmental factor that controls adaptation of *Rle* to the niche conditions. Besides, R-LOV-HK is required for competitive nodulation.

The *Rle* LOV domain construct shows a truncated photocycle (Fig. 1*C*) that resembles that of *Brucella* LOV-HK, which is in agreement with the high degree of identity between these two LOV domains (Fig. 1*A*). It has been suggested that specific structural features present in the *Brucella* LOV domain are responsible for the truncated photocycle (6). The *Rhizobium* LOV domain apparently controls the photochemical properties in a similar fashion. Despite the similarities in sequence and photochemistry, B-LOV-HK and R-LOV-HK have important differences that may implicate changes in the transduction mechanism. All LOV-HK genes that bear an HWE domain have a domain organization similar to that of *Rle*, lacking the Jα-helix and the PAS domain at the C terminus of the LOV domain and displaying an N-terminal α-helix (Fig. S1). Therefore, it is plausible that this N-terminal α-helix plays a similar function to that of the Jα-helix from other LOV domains.

The Rhizobiaceae family has 28 genomes sequenced to date but only 5 of them harbor LOV-domain–containing genes (*R. leguminosarum* 3841, *R. leguminosarum* WSM1325, *R. leguminosarum* WSM2304, *Agrobacterium* spp. H13-3, and *Agrobacterium vitis* S4 (Fig. S1). This observation is in good agreement with the scattered distribution of LOV domain proteins in many different bacterial species (5). The results presented in this work suggest that the adaptations driven by environmental stimuli could be advantageous for soil bacterial species that establish biological relationships with plants. Therefore, evolutionary forces could drive the conservation of the LOV module that allows the detection of blue light and modifies the lifestyle of the bacteria according to the environmental light conditions. In most species of this family, LOV domains or other blue-light receptors are absent. However, some species have other kinds of photoreceptors: BLUFs and/or bacteriophytochromes (photoreceptors that typically use bilin and sense red and far-red light) (20), suggesting that light sensing may have an important function for Rhizobiales. Future research will show whether LOV-domain proteins (blue light) and bacteriophytochromes (red/far-red light) regulate the same or different biological functions.

Here we show that light regulates biofilm formation, EPS production, and flagellar synthesis and that LOV-HK has an important role in mediating all these adaptations to changing environments. In *Rhizobium*, as in many other bacteria, the formation of biofilms is controlled by environmental factors (29, 39). Biofilm development and EPS synthesis are intimately related as has been shown earlier by Russo et al. (14). A mutation in *pssA* or deletion of several other *pss* genes involved in EPS synthesis completely abolishes the ability of *Rle* to develop a biofilm in vitro and severely affected bacterial attachment to root hairs (14, 15). The regulatory mechanisms and environmental factors that influence EPS production in *Rle* have not been extensively studied. However, the inhibitor PsiA, the regulator ExoR (40, 41), and the activator RosR (42) were shown to modulate EPS production. We have shown that light regulates the production of EPS and that R-LOV-HK is a light sensor involved in this process. Further experiments will determine whether there is a molecular link between light perception by LOV-HK and the above regulatory proteins that control the EPS synthesis and, if so, the nature of the link.

Light detected by LOV-HK reduces the number of flagella per cell, in agreement with a reduction in the amount of flagellin proteins and a marked reduction of the transcriptional level of at least two flagellar components. A similar effect of light on flagellar protein synthesis was previously observed in *A. tumefaciens* but this effect was not linked to any photoreceptor (31). In the same work, it was shown that light negatively regulates swimming motility in *A. tumefaciens*, *Rle*, and other rhizobia (31). We observed that light intensities over 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ affect *Rle* growth. These light conditions were comparable to those previously used (31). In plates incubated at light intensities that allow growth, no differences in the swimming halo diameters were observed (Fig. S4*C*). It is possible that in addition to light, the presence of flagella and motility are affected by other environmental factors such as carbon source, temperature, or medium viscosity (30) that could override the effect of light on *Rle* flagellar production. Our findings support the hypothesis that in *Rle*, both biofilm formation and flagella production are suppressed by light.

To understand the biological significance of these responses, a model could be envisaged in which the response of bacteria to light is integrated with other environmental factors. In the darkness and in response to chemical or physical gradients, an increase in the number of flagella and motility would allow bacteria to reach root hairs that are close to the soil surface. Once bacteria are well positioned, detection of light might reduce expression of both flagella and EPS, which are factors required for the formation of a biofilm. Bacteria become then competent for root hair infection (43). Reduction of EPS synthesis might be also important to appropriately regulate plant defense response. Alternatively, in the darkness and in response to nutrient limitation and other environmental signals, biofilm formation may be stimulated and bacteria might become enclosed in an extracellular matrix made of EPS and other components, resulting in a sessile and noninfecting community of cells. Besides, an increasing number of flagella in darkness might be also important for the first stages of biofilm formation, such as surface interaction and expansion of a robust biofilm (29).

Detection of light could also allow rhizobia to sense both the time of day and the position within the environment. These abilities could be particularly important to reach an optimal level of infection because it is known that light is one environmental cue that regulates the plant defense response against invading microorganisms (44) and, in turn, the plant defense response has a direct impact in the number of successful bacterial infections (45, 46).

The results presented in this work strongly suggest that light is an important environmental factor that controls several phenotypes in *Rle*. The light stimuli could be used as a unique tool to study the interaction of the symbiont *Rhizobium* with its plant host. The molecular components of the light-activated pathway controlled by LOV-HK and the genes regulated in the light response by rhizobia are currently being investigated in our laboratory.

Materials and Methods

Details of microbiological techniques and illumination conditions, generation of *lov* mutant and complemented strains, quantification of biofilm formation and EPS, nodulation assays, flagellar proteins purification, LOV-domain absorption changes, SEM and RT-qPCR are provided in *SI Materials and Methods*. Primers used for cloning, screening, and mutagenesis are listed in Table S2. All primers used for real-time RT-qPCR are listed in Table S3.

ACKNOWLEDGMENTS. We thank Dr. J. S. Tiffenberg for advising us in data analysis and Dr. F. Malamud for helpful discussions. We also thank Lic. F. Tricárico from Museo Argentino de Ciencias Naturales for critical-point drying and the MALDI-TOF service staff from Facultad de Ciencias Exactas y Naturales for their help in protein identification. This work was supported by grants from the Howard Hughes Medical Institute, Agencia Nacional de Promoción Científica y Técnica (PICT2008-1729), Universidad de Buenos Aires (UBACyT, X-240), and Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (PIP-545). R.A.B. is supported by NSF-0843662.

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