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Blue-Light-Activated Histidine Kinases: Two-Component Sensors in Bacteria

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Blue-Light–Activated Histidine Kinases: Two-Component Sensors in Bacteria

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Histidine kinases, used for environmental sensing by bacterial two-component systems, are involved in regulation of bacterial gene expression, chemotaxis, phototaxis, and virulence. Flavin-containing domains function as light-sensory modules in plant and algal phototropins and in fungal blue-light receptors. We have discovered that the prokaryotes *Brucella melitensis*, *Brucella abortus*, *Erythrobacter litoralis*, and *Pseudomonas syringae* contain light-activated histidine kinases that bind a flavin chromophore and undergo photochemistry indicative of cysteinyl-flavin adduct formation. Infection of macrophages by *B. abortus* was stimulated by light in the wild type but was limited in photochemically inactive and null mutants, indicating that the flavin-containing histidine kinase functions as a photoreceptor regulating *B. abortus* virulence.

LOV (light, oxygen, or voltage) domains are distributed in the three kingdoms of life (Eukarya, Archaea, and Bacteria) (*1*, *2*). In most cases, the LOV domain is the primary sensory module that conveys a signal to protein domains with known or putative functions as diverse as regulation of gene expression, regulation of protein catabolism, and activation of serine/threonine kinases in eukaryotes and histidine kinases in prokaryotes (*3*, *4*). The only two LOV-domain proteins from bacteria that have been studied are YtvA—a LOV-STAS (LOV–sulfate transporter and anti-sigma factor antagonist) protein from *Bacillus subtilis*—and a LOV protein (containing no other known domains) from *Pseudomonas putida* (*5*–*8*).

The best-characterized LOV domains belong to the plant blue-light receptors, the phototropins, and the closely related photoreceptor neochrome (*9*). The LOV-domain x-ray structure shows the flavin mononucleotide (FMN) chromophore noncovalently bound to the protein through hydrogen bonding and hydrophobic interactions, and the sulfur atom of a reactive cysteine to be within 4.2 Å of the C(4a) carbon of FMN (*10*). Light absorption by the LOV-domain flavin chromophore results in formation of a cysteinyl-flavin adduct in which the sulfur of the reactive cysteine forms a covalent bond with the C(4a) carbon of FMN. In the phototropin LOV domains, this stable bond spontaneously breaks in the dark (in many seconds), completing a photocycle (*11*). This process has been proposed to be base catalyzed (*12*–*14*). Cysteinyl adduct formation in the phototropins produces protein conformational changes (*15*, *16*) that activate a serine/threonine kinase domain, resulting in autophosphorylation (*9*). The activated phototropins mediate several blue-light responses in plants, including phototropism, chloroplast relocation, leaf expansion, and stomatal opening (*17*).

Sequence analysis predicts that the genomes of the human/animal facultative intracellular pathogen *Brucella melitensis* (*18*, *19*), the plant pathogen *Pseudomonas syringae* (*3*, *4*), and the marine bacterium *Erythrobacter litoralis* code for proteins that contain a LOV domain at their N terminus, with a histidine kinase occupying the C terminus (LOV-HK). Protein sequence and structural modeling [Swiss model (*20*)] of these bacterial LOV domains predict that all four LOV-HK proteins will bind a flavin and that all contain a cysteine within a few angstroms of the chromophore. Analysis of published genomes indicates 24 different sequenced bacteria contain genes that code for putative LOV-HKs (*1*, *4*).

The full-length proteins (489 amino acids) from *B. melitensis* (BM-LOV-HK) and *B. abortus* (BA-LOV-HK) contain three distinct domains: a LOV domain at the N terminus, followed by a PAS (Per- Arnt- Sim) domain in the intervening sequence, and a histidine kinase at the C terminus (Fig. 1). Although there are some silent mutations in the genes that encode the LOV-HK in the various *Brucella* species, the protein sequence is identical in *B. melitensis*, *B. abortus,* and *B. suis*. The two LOV-HK proteins from *E. litoralis* (346 amino acids and 368 amino acids in length for EL346-LOV-HK and

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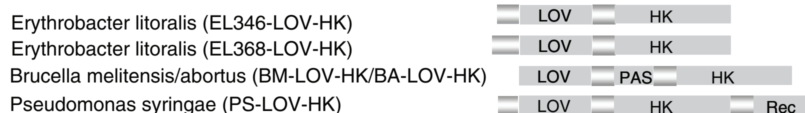

Fig. 1. Domain alignment of LOV histidine kinase proteins (LOV-HK).

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EL368-LOV-HK, respectively) contain only a LOV domain followed by a linking sequence and a C-terminal histidine kinase domain (Fig. 1). The LOV-domain protein from the plant pathogen *P. syringae* (PS-LOV-HK) (534 amino acids) contains a LOV domain at the N terminus, followed by a histidine kinase and a putative C-terminal receiver (Rec) domain (i.e., response regulator) (Fig. 1). All of these sensor/histidine kinase proteins are hypothesized to be involved in two-component signaling at some stage in the organism's life cycle.

We have cloned the genes encoding BM-LOV-HK, EL368-LOV-HK, EL346-LOV-HK and PS-LOV-HK, expressed them in *Escherichia coli,* and affinity-purified their full-length proteins. The four proteins exhibit characteristic LOV-domain absorption, with two broad absorption bands (450 nm and 370 nm) with secondary spectral peaks indicative of flavin in a tight-binding pocket (*16*) (Fig. 2A). The flavin chromophore was extracted from the four LOV-HKs and analyzed by thin-layer chromatography (TLC). All four LOV-HKs expressed in *E. coli* bind FMN as the flavin chromophore (table S1).

Light-induced absorption changes were measured to study the photochemistry of the isolated LOV-HKs. Illumination of the LOV-HKs with a 1-ms camera strobe flash produced a difference spectrum that indicates a loss of absorption from the 450-nm band, with some increase in absorption in the ultraviolet A (UVA) region (Fig. 2B). These difference absorption changes are characteristic of the formation of a flavin cysteinyl adduct that has a broad absorption band around 390 nm (*11*, *12*).

The BM-LOV-HK adduct state is extremely stable and does not decay measurably in 2 hours (Fig. 2C). This stability is unlike that of the other three LOV-domain proteins presented here, in which the cysteinyl adduct breaks spontaneously, with the chromophore/protein thermally returning to the ground state in the dark. The other three LOV-HKs complete a photocycle in which they decay thermally from the adduct state back to the ground state in the dark with half-lives of 28 (EL346-LOV-HK), 32 (EL368-LOV-HK), and 22 (PS-LOV-HK) min (Fig. 2C). In the phototropin LOV domains, the spontaneous adduct decay is thought to be base-catalyzed (*12–14*), possibly initiated by abstraction of the N5 proton. However, the putative proton acceptor has not been identified; sequence comparisons of the various LOV domains do not provide information about the residues controlling the relative rates of dark decay.

The four LOV-HKs incorporated ^{32}P from [γ-^{32}P]ATP (adenosine triphosphate) in a light-dependent manner at T = 0 min (Fig. 3 A-D), confirming that each functions as a light-activated kinase. The BM-LOV-HK-C69A mutant, which binds FMN but cannot form the cysteinyl adduct, did not activate the kinase in response to illumination (Fig. 3E). In addition, an assay testing the alkali and acid stability of the phosphate bond indicated that, in all the proteins, a phosphohistidine bond most likely formed upon kinase activation (fig. S1) (*21*).

The LOV-HKs we examined all contain a sensor/histidine kinase and therefore are presumably involved in two-component signaling. When activated, the histidine kinases operate by transiently phosphorylating a histidine residue on the kinase, and the phosphate from the activated kinase is transferred to an aspartate of a response regulator (*22*, *23*). The activated response regulators are generally involved in control of gene expression or, in the case of chemotaxis, in flagellar motor control (*22*, *23*). The PS-LOV-HK is unique among these four LOV kinases because its putative receiver (Rec) domain is covalently linked to the histidine ki-

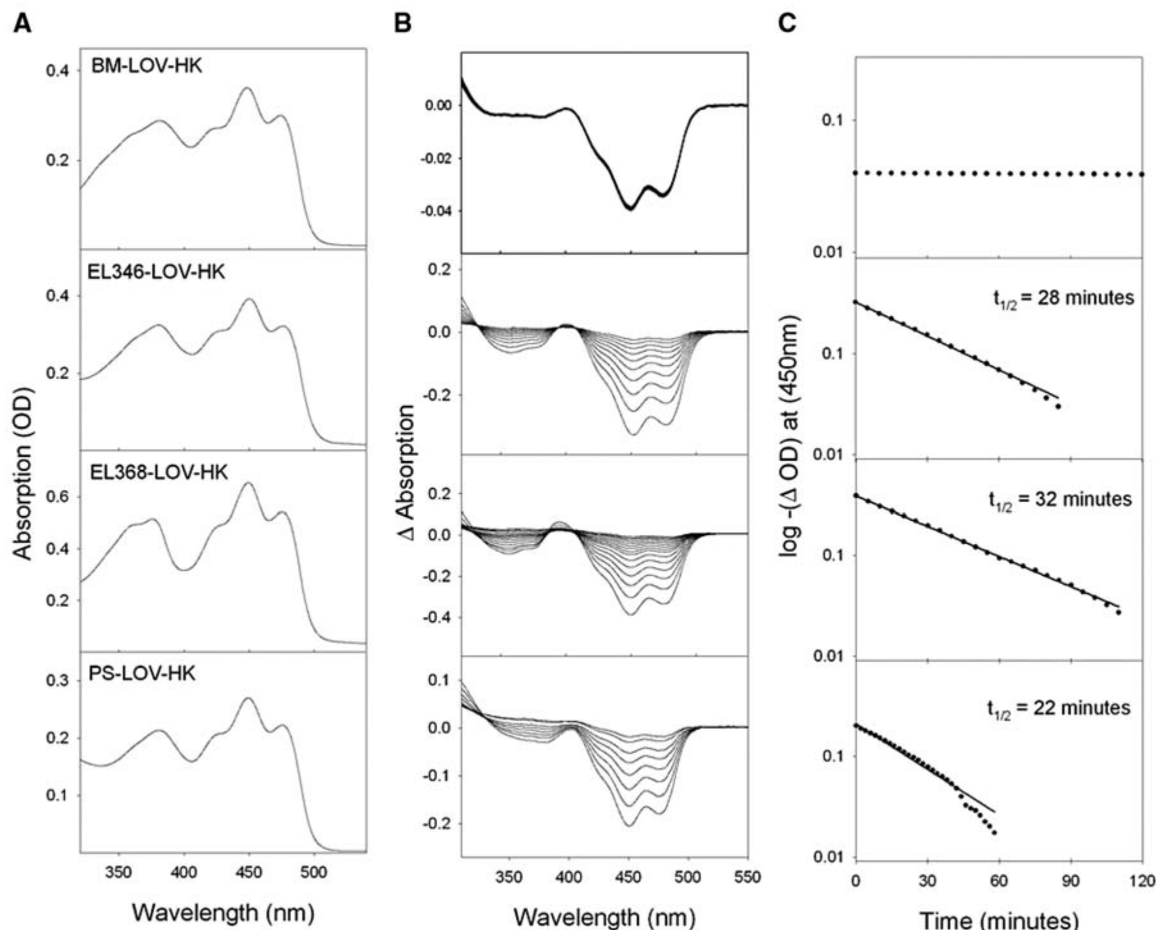

Fig. 2. Absorption spectra and absorption changes for full-length LOV-HKs. (**A**) Absorption of LOV-HKs showing flavin binding and characteristic LOV-domain absorption spectra. (**B**) Light-induced absorption difference spectra. BM-LOV-HK data collected every 5 min for 2 hours after light excitation. EL346-LOV-HK, EL368-LOV-HK, and PS-LOV-HK spectra collected every 10 min after light excitation. Difference spectra are typical of cysteinyl adduct formation characteristic of LOV-domain photosensory module photochemistry. (**C**) Absorption changes after light excitation measured at 450 nm. Decay is fit to a single exponential with the half-life shown on the graphs. Samples were all in phosphorylation buffer.

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nase at the C terminus (Fig. 1). In two-component signaling, the Rec should be the final acceptor of the phospho-relay chain. However, our in vitro activity assay (fig. S1) indicated that the histidine of the sensor kinase and not the aspartate on the response regulator domain was phosphorylated, because treatment of phosphorylated PS-LOV-HK with alkaline buffer to hydrolize phospho-aspartates (*21*) did not significantly reduce phosphorylation.

After irradiation, the BM-LOV-HK kinase activity correlates with the presence of the flavin cysteinyl adduct state (Fig. 3A). For the other three kinases, the amount of radioactive phosphate incorporated decreased with time in darkness (Fig. 3, B to D) corresponding to the breakage of the cysteinyl adduct (Fig. 2B), indicating that the LOV-domain adduct state is the signaling state that triggers activation of the histidine kinase. When a second light pulse was given 180 min after the first, phosphorylation was as strong as that from the initial light pulse, indicating that the decrease in phosphorylation over time after the initial light pulse was not due to protein degradation.

The kinase-domain activation results presumably from intramolecular propagation of a local structural perturbation generated by adduct formation. We do not know whether the LOV domains in microorganisms use a common intramolecular signaling mechanism that has been adapted to allow for simple exchanging of sensory modules to different output signaling domains. In the plant receptor phototropin, it seems that LOV-domain activation disrupts an adjacent amphipathic alpha helix (Jα), which then activates the kinase domain (*16*). Although protein modeling with the Swiss-model program (*20*) predicts a close resemblance to phototropin LOV domains, it does not predict an amphipathic Jα helix, which suggests that different intramolecular signaling mechanisms occur.

LOV domain photoactivity implies that light responses are important for these microorganisms (*3*, *4*) and possibly required for virulence (*24*). We found that the PS-LOV-HK gene was constitutively expressed in the *P. syringae* pathovar *syringae* strain DC3000 grown in minimal media (fig. S2), a condition known to induce the type III secretion/pathogenicity island (*25*). Conversely, we did find about a twofold increase in transcription of the BM-LOV-HK (BMEII0679) gene from *B. melitensis* at a pH (*26*) similar to that within infected macrophages (table S2).

Two-component signal transduction systems are involved in bacterial virulence (*24*); hence, we investigated the role of BA-LOV-HK in virulence by constructing an insertion-knockout mutant strain by the introduction of a kanamycin-resistant cassette into the gene (*27*). To determine whether the *B. abortus* LOV-HK mutant is able to replicate inside mammalian host cells, we infected J774A.1 murine macrophages with the wild type and the knockout bacteria and counted intracellular bacteria after infection

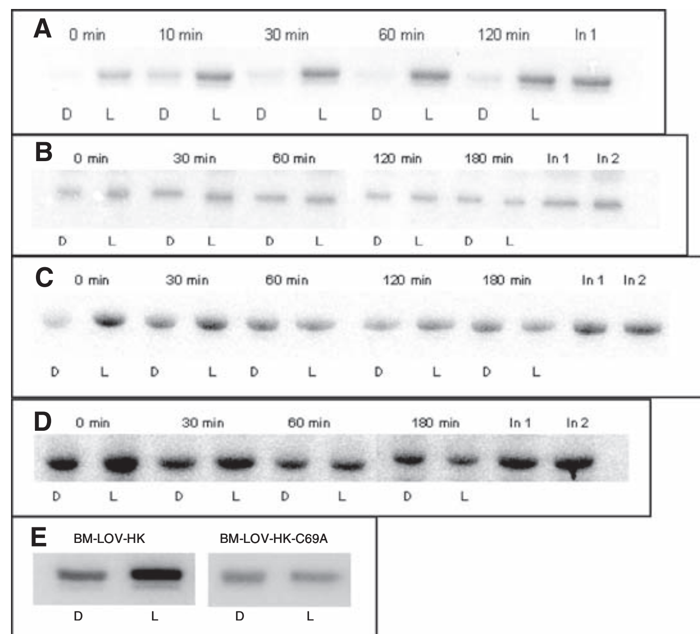

Fig. 3. LOV-HKs function as light-activated kinases. Autoradiogram showing the light-dependent phosphorylation of LOV-HKs. Samples were given a mock irradiation (D) or irradiated with a 1-min white light (L) pulse at a fluence of 2000 μmol m^{-2} s^{-2}. [γ-^{32}P]ATP was added 0, 30, 60, 120, and 180 min after the light pulse, and the samples were incubated for 4 min at room temperature. Samples were then fractionated with SDS–polyacrylamide gel electrophoresis (PAGE), and the gels were exposed to film. (In 1 indicates a sample in darkness for 180 min followed by a light pulse and addition of [γ-^{32}P]ATP. In 2 indicates a sample given light pulse followed by 180 min in darkness, followed by a second light pulse and immediate addition of [γ-^{32}P]ATP). All manipulations were carried out under dim red light. (**A**) BM-LOV-HK. (**B**) EL346-LOV-HK. (**C**) EL368-LOV-HK. (**D**) PS-LOV-HK. (**E**) BM-LOV-HK-C69A shows no light-induced activation of the kinase domain, consistent with the absence of light-induced photochemistry.

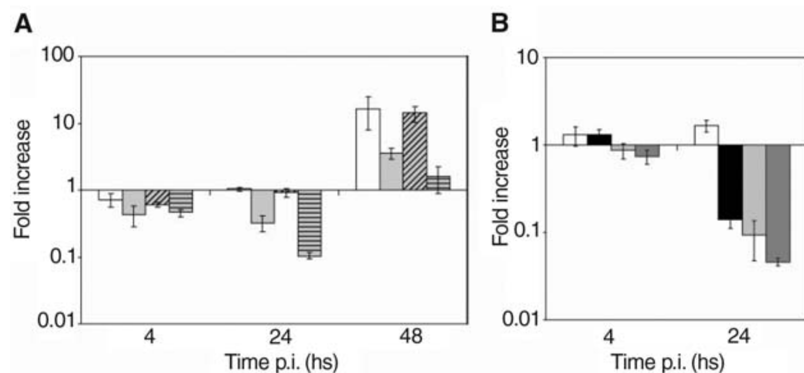

Fig. 4. LOV-HK protein from *B. abortus* is involved in virulence. Infectivity of *B. abortus* 2308 and *B. abortus* LOV-HK null mutant was tested in the J774A.1 murine macrophage cell line (**A**). At the indicated times (4, 24 and 48 hours), cells were lysed and the number of intracellular bacteria was determined by plating on tryptic soy agar. *B. abortus* 2308 (white), LOV-HK mutant (light gray), LOV-HK mutant complemented with plasmid pBBR-BaLOV expressing LOV-HK protein (diagonal hatched) and *B. abortus* LOV-HK mutant transformed with pBBR-BaLOV C69A expressing LOV-HK-C69A mutant protein (horizontal hatched). Data (mean ± standard deviation of duplicates) shown are representative of three independent experiments performed. Light affects *B. abortus* infection (**B**). *B. abortus* 2308 and LOV-HK bacteria given either dark or light treatment were used to infect macrophage cell line J774A.1. At the indicated times, the number of intracellular bacteria was determined by plating in tryptic soy agar. *B. abortus* 2308 light treatment (white) and dark treatment (black); LOV-HK knockout mutant light treated (light gray) and dark treated (dark gray). Data shown (mean ± standard deviation of duplicates) are representative of two independent experiments. In both graphs, data are presented as relative increase over 1-hour data. All CFU/ml (colony forming units) values were divided by the corresponding one-hour data.

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(Fig. 4A). Replication of the *B. abortus* LOV-HK mutant strain was less than the wild-type strain as early as 4 hours after infection. To confirm that this defect in replication was only due to the absence of the BA-LOV-HK gene, the LOV-HK mutant was complemented with a plasmid expressing the wild-type BA-LOV-HK gene. The complemented strain was able to rescue the mutant phenotype (Fig. 4A), confirming that the BA-LOV-HK gene is required for optimal replication of *B. abortus* in macrophages.

Light activation of the LOV-HK requires a reactive cysteine (C69). A C69A mutant in a *B. abortus* LOV-HK knockout background did not return the replication rate to the wild-type level (Fig. 4A), indicating that formation of the covalent adduct between FMN and the LOV protein is essential to restore kinase function. Thus, the biological role of the LOV-HK protein is associated with its capacity to sense light and transduce the signal to the output kinase domain. The number of intracellular bacteria increased slightly after 24 hours in culture in the light (Fig. 4B); however, in the dark, roughly an order of magnitude fewer bacteria survived, a survival rate no better than that of the BA-LOV-HK knockout mutant, which suggests clearly that the BA-LOV-HK photoreceptor serves as a virulence factor in *Brucella* spp. and that light may prepare the bacteria present in the aborted placenta for infection of the next host (*28*).

We have shown that four bacterial LOV-HKs bind a flavin chromophore that, upon illumination, forms the cysteinyl adduct characteristic of LOV domain photochemistry. Unexpectedly, the cysteinyl adduct in *Brucella* LOV-HK does not break spontaneously in the dark as it does in the other bacterial LOV-HK proteins and the plant phototropin LOV domains (*17*). Upon illumination in the presence of ATP, the kinases of all four LOV-HKs undergo autophosphorylation, likely on a conserved histidine, and are activated. The close correlation between cysteinyl-flavin adduct lifetime and kinase activity indicates that the adduct is the signaling state that activates the histidine kinase. Furthermore, the BA-LOV-HK appears to function as a photoreceptor that is directly related to *Brucella* survival and replication within macrophages.

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Supporting Online Material

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Temporal Fragmentation of Speciation in Bacteria

Adam C. Retchless and Jeffrey G. Lawrence*

Because bacterial recombination involves the occasional transfer of small DNA fragments between strains, different sets of niche-specific genes may be maintained in populations that freely recombine at other loci. Therefore, genetic isolation may be established at different times for different chromosomal regions during speciation as recombination at niche-specific genes is curtailed. To test this model, we separated sequence divergence into rate and time components, revealing that different regions of the *Escherichia coli* and *Salmonella enterica* chromosomes diverged over a ~70-million-year period. Genetic isolation first occurred at regions carrying species-specific genes, indicating that physiological distinctiveness between the nascent *Escherichia* and *Salmonella* lineages was maintained for tens of millions of years before the complete genetic isolation of their chromosomes.

The proper identification and delineation of bacterial species play critical roles in medical diagnosis, food safety, epidemiology, and bioterrorism mitigation. Human responses are guided by perceptions of the biological properties and capabilities of a named species, as well as by an understanding of its natural variability and potential to change. The biological species concept (BSC) considers a species to be a group of organisms that readily exchange genetic information only with each other (*1*). In eukaryotes, recombination—here defined as allelic exchange—is often tied to reproduction, whereby meiosis is followed by the karyogamy of two entire haploid genomes. Consequently, as new species arise, genetic isolation would occur simultaneously for all loci, meaning that all pairs of orthologous genes would be diverging for about the same amount of time. Whereas bacterial speciation is a complex process (*2–4*), the BSC has also been applied to bacteria such as *E. coli* (*5*). Bacterial recombination involves the occasional, unidirectional transfer of small DNA fragments from one strain into the homologous locus of another strain. Because only a small portion of

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