The general stress response of Bacillus subtilis

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Publication date
2013

Citation for published version (APA):

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Chapter 3: Modeling the functioning of YtvA in the general stress response in *Bacillus subtilis*

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3.1. Abstract
The blue-light photoreceptor YtvA activates the general stress response (GSR) of *Bacillus subtilis* by activating a large protein complex (the stressosome). We have constructed a model for YtvA’s photocycle, and derived an equation for the fraction of YtvA in the light-induced signaling state at a given light intensity. The model was verified experimentally *in vitro* on wild-type YtvA and on an R63K mutant with faster recovery kinetics. Application of the model to the activation of the GSR at various light intensities *in vivo* revealed that the GSR is more sensitive to light than would be expected based on YtvA’s *in vitro* kinetics. These results were confirmed with the R63K mutant and a slower-recovering V28I mutant. Additionally, we have demonstrated the presence of a near-UV-light-induced branching reaction that converts the signaling state of YtvA to the dark state. Extension of the model with this reaction shows that it does not contribute significantly to the *in vivo* blue-light response. The model represents an important step towards a complete systems biology model of the GSR.

3.2. Introduction
Photoreceptor proteins are involved in many important regulatory processes in all kingdoms of life. Several different photoreceptor classes have been identified, of which the input domains can bind a small chromophore such as a flavin, retinal or bilin, allowing these proteins to absorb in the UV/visible region of the electromagnetic spectrum (461). When they absorb light there are typically significant changes in the absorption spectrum of the photoreceptor protein which can readily be observed with spectroscopic techniques *in vitro*. Hence, a large body of literature is available on the kinetic properties and intermediate species of photoreceptor proteins. However, it is often hard to relate effects of perturbations in their kinetic properties (brought about *e.g.* through site-directed mutagenesis) to *in vivo* phenotypes.

YtvA is a blue-light photoreceptor from the common soil bacterium *Bacillus subtilis* (16, 277). YtvA has two domains: an N-terminal LOV (light, oxygen, voltage) domain and a C-terminal STAS (sulfate transporter and anti-sigma-factor antagonist) domain. LOV domains are members of the family of PAS-domains (Per-ARNT-Sim), and were first identified in phototropins found in plants. LOV domains are also found in an increasing number of bacteria (249, 279, 280, 367, 462). LOV domains bind a flavin mononucleotide (FMN) in a binding pocket with a highly conserved sequence motif. In the case of YtvA this results in a molecule with maximal absorbance at 448 nm in the dark. Illumination with blue light triggers a photocycle (Figure 1A). After formation of the triplet excited state of FMN through internal conversion on the ns timescale (D* in Figure 1A), a thiol adduct is formed between cysteine 62 and the C4a atom of the FMN on the µs timescale. The resulting signaling state (S), which has an absorbance maximum at 390 nm, thermally converts back to the dark state (D) with a recovery lifetime of 2600 s (at 25°C) (278).
YtvA is an interesting target for systematic quantitative studies because the full-length protein can readily be purified and its biological function is well-characterized. As a soil organism, *B. subtilis* can respond in multiple ways to fluctuating environmental conditions. One of the most important of these is the general stress response (GSR), which activates 150 to 200 genes in response to a wide range of stresses (179, 372, 378). These stresses can be subdivided into two groups: energy stresses (starvation) and environmental stresses (such as NaCl shock, ethanol shock or heat shock). YtvA renders the environmental GSR sensitive to blue light. When YtvA is overexpressed, low intensities of blue light result in strong GSR activation (16). At wild-type expression levels the effects of YtvA are more modest. Nevertheless, GSR activation induced by NaCl-shock is enhanced by light (16, 448), and a clear transient light-dependent activation of the GSR is seen if a negative regulator of YtvA is knocked out (463).

The signaling network that regulates the environmental GSR is shown in Figure 1B. A comprehensive description of this network goes beyond the scope of this paper, but extensive reviews have been published elsewhere (178). Briefly, the activity of the GSR is regulated by the alternative transcription factor SigmaB, which in turn is regulated by a partner-switch consisting of the anti-sigma factor RsbW and the anti-anti-sigma factor RsbV (Rsb: regulator of SigmaB). The activity of the partner switch is controlled through
the phosphorylation state of RsbV, which needs to be in the non-phosphorylated form to capture RsbW and release SigmaB. The phosphatase responsible for the environmental GSR is RsbU, which is activated and turns on the GSR when bound to RsbT. To prevent activation in unstressed cells, RsbT is captured by a large protein complex called the stressosome (78, 106, 292). The stressosome consists of multiple copies of RsbS and of multiple copies of proteins from the RsbR family, which has at least four members (5). Both RsbS and the RsbR proteins contain a C-terminal STAS domain, as does YtvA. YtvA has been co-purified with other stressosome components (144) and has recently been shown to displace RsbRA in stressosomes in vitro independently of its illumination state (215).

We have used YtvA to study the influence of changes in kinetic parameters of a photoreceptor (YtvA) on its biological function (GSR activation), linking microscopic rate constants to biological phenotypes in a systems biology approach. We have constructed a detailed kinetic model of the photocycle of YtvA to model the fraction of protein that is in the light-induced signaling state at a given light intensity. This model was verified using the isolated protein in vitro. We have then compared this to experimentally obtained data for the activation of the GSR, which was determined using a well-characterized and often-employed reporter-enzyme assay (16, 18, 178). The model was further tested with two mutants of YtvA with altered kinetics: R63K, which has a faster recovery of the dark state than the wild-type protein (451); and V28I, which has a slower recovery (528). Additionally, we have demonstrated the presence of a light-induced branching reaction, emerging from the signaling state of YtvA, and examined the possible influence of this branching reaction on the biological activity of YtvA.

3.3. Results

3.3.1. Modeling the activation of YtvA

The known reactions and microscopic properties of the photocycle of YtvA (Figure 1A) were incorporated into a kinetic model, schematically shown in Figure 2A. In this model, which was based on a previously published general photocycle model for PYP (182), $k_{exD}$ describes the rate of excitation, $k_{pe}$ the rate of photocycle entry, $k_{re}$ the rate of recovery of the dark state, and $Q_{yd}$ the quantum yield of photocycle entry. All parameters, save for $k_{exD}$, have been determined experimentally in vitro (see the legend of Figure 2 and Table I). Using these parameters, it is possible to derive differential equations for the concentration of every species (equations 1-3), which can be used to numerically simulate the temporal dynamics and steady-state levels of the species distribution of YtvA.

For the rate of excitation we assumed that every photon absorbed will first lead to formation of the excited state (D*), after which a certain fraction (the quantum yield of photocycle entry) will continue in the photocycle, while the rest will fall back to the dark state (D) without entry into the photocycle (e.g. by fluorescence or heat release). This assumption enables the use of the Lambert-Beer law for absorption, to derive the number
of photons absorbed per time unit, from which the $k_{exD}$ reaction rate can be derived (equation 4; see Materials and methods for details).

$$\frac{d[D]}{dt} = -k_{exD} \cdot [D] + \left( \frac{1}{Q_{yD}} - 1 \right) \cdot k_{pe} \cdot [D^*] + k_{re} \cdot [S]$$  \hspace{1cm} \text{Eq. 1}

$$\frac{d[D^*]}{dt} = k_{exD} \cdot [D] - \frac{k_{pe}}{Q_{yD}} \cdot [D^*]$$  \hspace{1cm} \text{Eq. 2}

$$\frac{d[S]}{dt} = k_{pe} \cdot [D^*] - k_{re} \cdot [S]$$  \hspace{1cm} \text{Eq. 3}

$$k_{exD} = \frac{I_0 \cdot (1 - 10^{-\varepsilon_D l [D]})}{I \cdot [D]} \approx I_0 \cdot \varepsilon_D \cdot \ln(10)$$  \hspace{1cm} \text{Eq. 4}

In its full form, $k_{exD}$ is dependent on the concentration of the protein in the dark state ([D]), the extinction coefficient of the dark state of the protein for the light source used ($\varepsilon_D$), the path length ($l$) and the light intensity ($I_0$). When the rate equation is approximated using a Taylor expansion, the dependency on [D] can be eliminated (equation 4), which allows one to analytically derive fractions for all three protein species in steady state (i.e. when equations 1-3 are equal to zero; see Materials and methods for the derivation).

$$f_D = \frac{1}{1 + Q_{yD} \cdot k_{exD} \cdot \left( \frac{1}{k_{pe}} + \frac{1}{k_{re}} \right)}$$  \hspace{1cm} \text{Eq. 5}

$$f_{D^*} = \frac{1}{1 + k_{pe} \cdot \left( \frac{1}{Q_{yD} \cdot k_{exD}} + \frac{1}{k_{re}} \right)}$$  \hspace{1cm} \text{Eq. 6}

$$f_S = \frac{1}{1 + k_{re} \cdot \left( \frac{1}{Q_{yD} \cdot k_{exD}} + \frac{1}{k_{pe}} \right)}$$  \hspace{1cm} \text{Eq. 7}

When equations 1-3 are applied using the parameters for YtvA, determined by Losi et al. during their initial in vitro characterization of the protein (277, 278), results such as shown in Figure 2B-D are obtained. In these simulations, all proteins at time point zero are in the dark state. They are then illuminated with different light intensities (the different lines). The results of the simulations match expectations. For example, higher light intensities result in greater accumulation of the light-induced signaling state S. Furthermore, the contribution of the excited state D* to the protein species distribution can be neglected, as expected based on its rapid conversion to the dark state (D) or the light-induced signaling state (S).
Figure 2: Quantitative model for the photocycle of YtvA. A. Representation of the relevant reaction rates and states of YtvA in the model. B-D. Changes in time of the fraction of the different protein species (D, D* and S, respectively) as simulated using equations 1-3 for various light intensities: 0.01 (dotted lines), 0.1 (square-dotted lines), 1 (short-dashed lines), 10 (long-dashed lines), and 100 (solid lines) μEinsteins·m⁻²·s⁻¹. Note that the lines in panel C are not visible because there is no significant contribution of D* on this timescale. E. Distribution of the three species of YtvA in steady state, using either steady-state equations 5-7 (symbols) or differential equations 1-3 (lines, no symbols). The solid line and filled circles represent the dark state (D), the dotted line and open circles the light-induced signaling state (S), and the open diamonds the excited state (D*; the line corresponding to this species is indistinguishable from the x-axis). The two black bars at the top indicate a crude estimation of relevant laboratory light intensity (the left bar) and daylight intensity (the right bar) in the Netherlands at noon (see Materials and methods). F. Effect of changes in the recovery rate on the steady-state fraction of protein in the light-induced signaling state (S). The solid line represents a recovery lifetime (1/kₚₑ) of 2600 s, the lines to the right lower recovery lifetimes (dot-dash: 260 s, dot-long dash: 26 s and dot-dot-long dash 2.6 s), and the lines to the left higher recovery lifetimes (dashed: 2.6 · 10⁵ s, short-dashed: 2.6 · 10⁴ s and dotted: 2.6 · 10⁶ s). B-F. The parameters were taken from Losi et al. (277, 278) for wild-type YtvA in vitro: quantum yield (Qₚₒ; 0.30), photocycle entry rate (kₚₑ; 5 · 10⁵ s⁻¹) and recovery rate (kₚₑ; 1/2600 s⁻¹ at 25 °C), with the exception of panel F where the recovery rate was varied. Light intensities correspond to illumination with a blue LED (λ_max = 464 nm) that was used for experiments (see below).

Figure 2E summarizes the results of the numerical simulations by taking only the protein species distribution at steady state (lines). The symbols in this figure show the results of the application of the simplified steady-state equations 5-7 to the same
Table I: Parameters for the photocycle model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>R63K</th>
<th>R63K</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantum yield ($Q_{vo}$)</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
<td>(277)</td>
</tr>
<tr>
<td>Photocycle entry rate ($k_{pe}$)</td>
<td>$5 \times 10^5$ s$^{-1}$</td>
<td>-</td>
<td>-</td>
<td>(278)</td>
</tr>
<tr>
<td>Recovery rate ($k_{re}$)</td>
<td>$1.52 \times 10^3$ s$^{-1}$</td>
<td>$1.50 \times 10^2$ s$^{-1}$</td>
<td>$1.43 \times 10^3$ s$^{-1}$</td>
<td>This study</td>
</tr>
<tr>
<td>Recovery lifetime (1/$k_{re}$)</td>
<td>658 s</td>
<td>66 s</td>
<td>699 s</td>
<td>This study</td>
</tr>
<tr>
<td>Temperature of the experiment</td>
<td>37°C</td>
<td>37°C</td>
<td>19°C</td>
<td>This study</td>
</tr>
</tbody>
</table>

parameters. Since both simulations overlap, the validity of the simplification of $k_{vo}$ for a protein with properties like YtvA is confirmed.

One of the advantages of this model is its ability to predict the consequences of changes in parameters. For example, Figure 2F shows the predicted influence of changes in the recovery rate on the accumulation of the signaling state at various light intensities. Such predictions are relevant in view of the availability of mutants of YtvA with altered recovery rates (see below).

3.3.2. Application of the model to in vitro light titrations

To validate the model, purified YtvA was incubated in a temperature-controlled cuvette at 37°C under illumination with different light intensities. When a steady state was reached, the fraction of YtvA in the light-induced signaling state (S) was determined from the spectrum of the sample by treating the spectrum as a linear combination of the dark state and the light-induced signaling state (symbols in Figure 3A).

To simulate the predicted behavior of the protein, the quantum yield and photocycle entry rate were taken from literature, while the recovery rate was measured in our experimental setup since it strongly depends on temperature (Table I). The analytical solutions (equations 5-7) were applied to simulate the predicted fraction of the protein in each of the three states (lines in Figure 3A). It is clear from the figure that the simulations match the observed values very well without the need for a fit for any of the parameters.

To assess the robustness of the model, a mutant of YtvA with drastically altered recovery kinetics was assayed as well. The R63K mutation was previously shown to accelerate recovery at 20°C by approximately 8-fold (451). The mutation was transferred to the same full-length YtvA construct used for the wild-type protein, and the process was repeated on purified R63K protein (Figure 3B). The quantum yield and photocycle entry rate were assumed to be the same as for wild type, and the recovery rate was again determined under the same conditions as the light-intensity titration. As predicted by our model, the very fast recovery kinetics of this mutant did not allow saturation of formation of the light-induced signaling state (S) with our light source at 37°C (circles in Figure 3B). Therefore, the temperature was lowered to 19°C (diamonds in Figure 3B). The model was able to predict the behavior of the mutant at both temperatures without the need for a fit. Thus, our model gives an accurate and robust description of the species distribution of YtvA in vitro.
Figure 3: Fraction of the protein in the light-induced signaling state (S) or in the dark state (D) in steady state when YtvA is illuminated with different light intensities in vitro. A. Wild-type YtvA at 37°C. B. The R63K mutant at 19°C (diamonds) and 37°C (circles). A-B. Open symbols show the measured fractions of the light-induced signaling state; filled symbols the measured fractions of the dark state. Dotted and short-dashed lines show simulated fractions of the light-induced signaling state; solid and long-dashed lines simulated fractions of the dark state. Note that, for display purposes, the data obtained without light (i.e. at a light intensity of 0) have been plotted at a light intensity of 0.01 because a logarithmic scale cannot display 0. The data are a composite of multiple measurement series on different days and on different samples.

3.3.3. Light-intensity dependence of YtvA-mediated GSR activation

Next, we wanted to investigate the behavior of YtvA in vivo. YtvA transmits a signal to the stressosome when illuminated by blue light, which in turn activates the general stress response (GSR). We showed previously that there is a correlation between light intensity and the level of GSR activation (18). However, the precise shape of this correlation was not investigated. Using our model, we can now investigate if the fraction of YtvA in the light-induced signaling state correlates with the activation of the GSR.

An established reporter-enzyme assay, based on the genomic insertion of a fusion of the GSR-dependent ctc promoter with the gene coding for the β-galactosidase, was used for these experiments. With this system we previously showed the light-dependent activation of the GSR by overexpressed YtvA and by YtvA at wild-type expression levels under selected conditions (16, 463), which was confirmed by microarray analysis (18). Cells were grown in the presence of IPTG to induce overexpression of YtvA. After serial dilutions (see Materials and methods for details) the light was turned on, and a sample was taken after 120 min to ensure that the species distribution of YtvA had enough time to reach a steady state. Dark controls were always included to ensure that there was no light-independent activation of the GSR.

Again, we used both wild-type YtvA and the R63K mutant with an approximately 10-fold accelerated recovery rate under our conditions (Table II). A third mutant, V28I, was included as well. The V28I mutation was previously shown to slow down recovery by
approximately 4-fold in the isolated LOV domain of YtvA (528). The mutation was transferred to the full-length construct and its recovery was determined to be approximately 8-fold slower in vitro under our experimental conditions (Table II).

The results (Figure 4, symbols) resemble the shape of the predictions from our model for all three mutants. Fits of the model with only the recovery rate as a variable—since this parameter is most likely to be influenced by intracellular conditions (see Discussion)—were then simulated (Figure 4, dashed lines), from which an estimation of the in vivo recovery rate was derived (Table II). Although the values obtained in vitro and in vivo are quantitatively different, they clearly show a trend: the R63K mutant with faster recovery in vitro also requires more light in vivo for a maximal activation of the GSR, while the opposite is true for the slower recovering V28I mutant. Thus, the model qualitatively describes the effect of the altered kinetics of YtvA in vivo.

3.3.4. The signaling state of YtvA shows a light-induced branching reaction

Above, we have modeled the photocycle of YtvA as a three-step process. However, the light-induced signaling state also absorbs visible light. Many other photoreceptors have photoactive signaling states, leading to processes that are often referred to as ‘branching’

<table>
<thead>
<tr>
<th>Mutant</th>
<th>In vitro $k_{re}$</th>
<th>In vitro $1/k_{re}$</th>
<th>In vivo (fit) $k_{re}$</th>
<th>In vivo (fit) $1/k_{re}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$1.52 \cdot 10^{-7}$ s$^{-1}$</td>
<td>658 s</td>
<td>$3.47 \cdot 10^{-7}$ s$^{-1}$</td>
<td>2881 s</td>
</tr>
<tr>
<td>R63K</td>
<td>$1.50 \cdot 10^{-7}$ s$^{-1}$</td>
<td>66 s</td>
<td>$9.34 \cdot 10^{-8}$ s$^{-1}$</td>
<td>1071 s</td>
</tr>
<tr>
<td>V28I</td>
<td>$1.98 \cdot 10^{-7}$ s$^{-1}$</td>
<td>5059 s</td>
<td>$9.61 \cdot 10^{-8}$ s$^{-1}$</td>
<td>10405 s</td>
</tr>
</tbody>
</table>

Figure 4: Light-intensity dependence of GSR activation with wild-type YtvA and two site-directed mutants (open circles), and fits with the photocycle model with only the recovery rate as free variable (dashed lines). YtvA was overexpressed prior to the experiment in all strains (see the text). Note that, for display purposes, the data obtained without light (i.e. at a light intensity of 0) have been plotted at a light intensity of 0.01 because a logarithmic scale cannot display 0. A. Wild-type YtvA. B. The R63K mutant. C. The V28I mutant.
reactions (e.g. in PYP (181, 305), rhodopsins (24, 176, 396, 440), and phytochromes (397)). The presence of such a branching reaction has also been shown for another LOV domain, in which absorption of a near-UV photon results in very fast recovery of the absorption spectrum to the dark state (226). While Losi and co-workers did not detect a similar reaction during their initial characterization of YtvA, they did state that it may have escaped detection if the yield of this reaction is low (278). Therefore, we wondered if the presence of such a reaction could explain the different recovery rates in vitro and in vivo.

To first prove the existence of the light-induced branching reaction, the light state of YtvA was accumulated by illumination with blue light while its absorption at 450 nm was monitored with low-intensity probe light. The blue lamp was then turned off, resulting in characteristic slow thermal recovery of the dark state (Figure 5, dotted line). At this point, the sample was illuminated with a 6 ns flash of 355 nm laser light, a wavelength at which both the dark state and the light state absorb well. Since the dark state was almost fully depleted prior to the laser flash the majority of the light was absorbed by the signaling state. This resulted in very fast recovery of the dark state spectrum in a small fraction of the sample (dashed line), demonstrating the presence of a photo-induced branching reaction. The difference between both signals (solid line) illustrates this fast recovery. Note that the gradual decrease of the relative absorption after induction of the branching reaction can be explained by diffusion of a small amount of YtvA in the light-induced signaling state into the measurement beam.

The quantum yield of this branching reaction was estimated to be 0.2 in the LOV2 domain of phy3 from Adiantum (226). However, its contribution appears to be much lower in YtvA, consistent with the fact that this reaction escaped attention in the original characterization of YtvA (278).

To make a quantitative estimate of this quantum yield we examined the steady-state

![Figure 5: The signaling state of YtvA displays a light-induced branching reaction. Absorption at 450 nm in time was recorded after accumulation of the signaling state (S) by prolonged illumination with blue light (464 nm). The blue light was switched off at time zero, after which the light-induced branching reaction was induced with a short (6 ns) laser pulse of 355 nm after 7 s. The dotted line displays thermal recovery of the dark state without the laser-induced branching reaction, the dashed line the recovery with laser-induction of the branching reaction, and the solid line shows the difference between both traces.](image-url)
distribution of the dark state and the light-induced signaling state of YtvA under conditions where the thermal recovery can be neglected (i.e. at high light intensity and/or at low temperature (226)). Under such conditions, the photocycle of YtvA is purely driven by the light source, with the conversion of the dark state to the light-induced signaling state competing with the back-conversion to the dark state. In steady state, both rates must be equal so that equation 8 must hold, in which \( N_D \) and \( N_S \) represent the number of photons absorbed by the dark state and the light-induced signaling state, respectively. From this, one can derive equation 9 which yields the quantum yield of the light-induced branching reaction \( \langle Q_{\alpha} \rangle \). Note that the ratio of the population of the dark state and the light-induced signaling state can easily be determined from the UV/Vis absorption spectrum in steady state at saturating light intensities, and that the ratio between absorbed photons is equal to the ratio of spectral overlap of either state with the spectrum of the actinic light source.

\[
[S] \times N_S \times Q_{\alpha S} = [D] \times N_D \times Q_{\alpha D} \tag{Eq. 8}
\]

\[
Q_{\alpha S} = \frac{[D]}{[S]} \times \frac{N_D}{N_S} \times Q_{\alpha D} \tag{Eq. 9}
\]

To estimate the quantum yield of the light-induced branching reaction we used a light source centered at 401 nm for which the ratio \( N_D/N_S \) is equal to 0.92, indicating that both species absorb its light almost equally well. YtvA was illuminated with saturating light intensities, from which a \([D]/[S] \ra \approx 0.06\) was derived. Together with the known value for \( Q_{\alpha D} \) (0.3) we used equation 9 to estimate the value of \( Q_{\alpha S} \) to be 0.02.

### 3.3.5. The light-induced branching reaction of YtvA does not significantly contribute to its in vivo function

It is straightforward to extend our photocycle model with the light-induced branching reaction. As shown in Figure 6A three additional reactions and parameters have to be included, with \( k_{exS} \) as the rate of excitation of \( S \) (similar to \( k_{exD} \)), \( Q_{\alpha S} \) as the quantum yield of the branching reaction and \( k_{be} \) as the rate of branching entry. These additional reactions complicate the differential equations (equations 10-13). Using the same assumption for \( k_{exD} \) and \( k_{exS} \) as made previously, they can nevertheless be solved into equations for the steady-state fractions of the different species of YtvA (equations 14-16).

\[
\frac{d[D]}{dt} = -k_{exD} \cdot [D] + \left(\frac{1}{Q_{\alpha D}} - 1\right) \cdot k_{pe} \cdot [D^*] + k_{re} \cdot [S] + k_{be} \cdot [S^*] \tag{Eq. 10}
\]

\[
\frac{d[D^*]}{dt} = k_{exD} \cdot [D] - \frac{k_{pe}}{Q_{\alpha D}} \cdot [D^*] \tag{Eq. 11}
\]

\[
\frac{d[S]}{dt} = k_{pe} \cdot [D^*] - (k_{re} + k_{exS}) \cdot [S] + \left(\frac{1}{Q_{\alpha S}} - 1\right) \cdot k_{be} \cdot [S^*] \tag{Eq. 12}
\]
\[
\frac{d[S^*]}{dt} = k_{exS} \cdot [S] - \frac{k_{be}}{Q_{yS}} \cdot [S^*]
\]  
Eq. 13

\[
f_D = \frac{1}{1 + k_{exD} \cdot Q_{yD} \cdot \left(\frac{1}{k_{pe}} + \frac{k_{be} + k_{exS} \cdot Q_{yS}}{k_{be} \cdot (k_{re} + k_{exS} \cdot Q_{yS})}\right)}
\]  
Eq. 14

\[
f_{D^*} = \frac{1}{1 + k_{pe} \cdot \left(\frac{1}{k_{exD} \cdot Q_{yD}} + \frac{k_{be} + k_{exS} \cdot Q_{yS}}{k_{be} \cdot (k_{re} + k_{exS} \cdot Q_{yS})}\right)}
\]  
Eq. 15

\[
f_S = \frac{1}{1 + (k_{re} + k_{exS} \cdot Q_{yS}) \cdot \left(\frac{1}{k_{exD} \cdot Q_{yD}} + \frac{1}{k_{pe}}\right) + \frac{k_{exS} \cdot Q_{yS}}{k_{be}}}
\]  
Eq. 16

\[
f_{S^*} = \frac{1}{1 + \frac{k_{be}}{k_{exS} \cdot Q_{yS}} \cdot \left(\frac{k_{re} + k_{exS} \cdot Q_{yS}}{k_{exD} \cdot Q_{yD}} + \frac{k_{re} + k_{exS} \cdot Q_{yS}}{k_{pe}} + 1\right)}
\]  
Eq. 17

To simulate the influence of the branching reaction on the proteins’ species distribution the model was applied using the same parameters as in Figure 2F. The value of \(k_{be}\) was taken to be equal to \(k_{pe}\)—the laser flash experiment revealed very fast kinetics and faster kinetics do not influence the outcome significantly (data not shown). The quantum yield \(Q_{yS}\) was taken to be 0.02 (see above). The simulation shows an effect at high light intensities that effectively lowers the fraction of the light-induced signaling state \(S\) that can be accumulated (Figure 6B). However, the effect is so small that application of this branching model to the \textit{in vivo} light titration data (Figure 4) results in less than 1% change of the fitted recovery rate for all three mutants (data not shown).

3.4. Discussion

3.4.1. Construction of a model for the photocycle of YtvA

We have constructed a mathematical model for the photocycle of the blue-light photoreceptor YtvA based on its known properties and kinetics. Differential equations were used to simulate the temporal changes in protein species distribution during illumination (equations 1-3; Figure 2). From this model we analytically derived equations for the fraction of the protein in each species in steady state (equations 5-7). These equations were applied to light-intensity titration experiments \textit{in vitro} to validate the assumptions made in the model. The model predicted the behavior of wild-type YtvA \textit{in vitro} extremely well without a need for fitting, which was also true for a mutant with altered recovery kinetics (Figure 3).
It is interesting to note that the steady-state equations for the dark state and the signaling state (equations 1 and 3) can be simplified to equations 28 and 29. Also, when some reasonable assumptions are made, a simple analytical expression can be derived to approximate the temporal changes in protein species distribution when light conditions change (equation 30). From there another expression can be derived to determine the time it takes to reach a certain percentage of a new steady-state value after a change in light conditions (equation 31). Together, these equations form a toolkit for the prediction of required conditions for a given experiment that involves a photoreceptor (see Materials and methods and Supplementary Information for the derivation and notes on usage).

3.4.2. Application of the model to in vivo data

YtvA has the added advantage of having a well-resolved biological role in inducing the general stress response (GSR) of B. subtilis. This enabled us to indirectly measure the activation of YtvA through a reporter-enzyme. The results (Figure 4) closely resemble the shape the model predicts. Most variation likely derives from biological variation and inaccuracy in the determination of the average light intensity in a shaking Erlenmeyer flask. The data show a clear correlation with in vitro data: the strain with the faster-
recovering R63K mutant of YtvA is less sensitive to light than wild type, while the strain with the slower-recovering V28I mutant is more sensitive instead.

Intriguingly, the sensitivity of the *in vivo* response to light is quantitatively different from the sensitivity of YtvA itself to light. The results of the fits suggest slower recovery kinetics *in vivo* than *in vitro*. As a result, the GSR is more light-sensitive than would be predicted based on the *in vitro* kinetics of YtvA (Table II). This is exciting, because it suggests that the signal transduction network of the GSR may be highly optimized to increase its sensitivity to harmful high-energy light, while still maintaining a reasonable time resolution. A rationale for this may be the relatively modest molar extinction coefficient of oxidized flavins (~1.25 · 10[^4] M[^−1] · cm[^−1] at the absorption maximum) compared to other photoreceptors that mostly have a several fold higher extinction coefficient (257, 304, 393).

We cannot derive the *in vivo* feature responsible for this effect from our data because our model does not include all reactions of the signaling pathway. One of the possibilities is that the response to blue light is a cooperative response, as suggested for other environmental stresses that are signaled through the stressosome (291). Alternatively, the curve may be shifted, or its shape may be altered, by the involvement of signal-amplification and feedback loops such as the induction of expression of SigmaB (and its primary regulators RsbV and RsbW) upon stress, the induction of RsbX (a negative regulator of the environmental GSR) or the stressosome component RsbRD (216, 372, 463). A third possibility is involvement of stress-induced degradation of the reporter enzyme (LacZ), as was recently suggested based on an artificial system (267), which may also alter the shape of the curve. Finally, the environment of YtvA in the cell may not be well-represented by *in vitro* buffer conditions. In general, buffers do not necessarily resemble *in vivo* conditions (147, 154, 466). However, we observed variations in the recovery rate of less than 10% *in vitro* when the salt concentration was increased up to 300 mM (enough for a GSR stress *in vivo*) or when the pH was varied between 7 and 10 (data not shown), suggesting that YtvA is relatively insensitive to buffer conditions. Still, YtvA is proposed to be a part of the stressosome *in vivo*, which may significantly alter its local environment and consequently its microscopic rate constants, e.g. by stabilization of the light-induced state.

Our results also imply that most experiments on the GSR in *B. subtilis* for which the light climate was not specified, presumably had a significant fraction of YtvA in the light-activated signaling state (Figure 2E). Thus, this work emphasizes the need to define the light climate in future experiments—even if those experiments do not directly deal with light. The basic equations 28-31 are intended to help with the definition of the optimal experimental light climate.
3.4.3. YtvA has a light-induced branching reaction that does not significantly impair its function

Previously, YtvA was not thought to have a light-induced branching reaction such as found in the LOV2 domain of phy3 of Adiantum (226, 278). Our data clearly show the presence of such a reaction (Figure 5), although its quantum yield is low (approximately 0.02).

An extension to our model shows that the effect of this branching reaction in vivo, when YtvA is illuminated with a blue LED such as in our experiments, can be neglected (Figure 6). However, it is worthwhile to note that illumination of YtvA with a light source closer to the UV is predicted to have larger effects. Thus, UV light may have a significant effect on the biological function of YtvA. Additionally, our model predicts that the function of LOV domains such as LOV2 of phy3 of Adiantum, which was reported to have a quantum yield of the branching reaction of 0.2 (226), would be significantly affected (Figure 6, dashed line).

3.4.4. Future directions for GSR research

We consider YtvA the ideal tool for the study of the GSR in B. subtilis because it is the only stress signal which can easily, rapidly and reversibly be controlled. The construction of a model for its photocycle is a significant step towards a kinetic model of the entire signal transduction pathway that regulates the GSR. At present, this is not feasible because there are still too many unknown factors, including the nature and strength of other input signal(s) of the stressosome. Nevertheless, progress has been made with computational models of the RsbV-RsbW-SigmaB partner switch module and the energy stress response (203, 270), and it will be interesting to combine these two approaches to generate a full systems biology model. Ultimately, such quantitative models for the GSR may contribute to a better understanding of the process of signal integration, as much as—and complementary to—the chemotaxis system (50, 243).

3.5. Conclusion

Our model is constructed such that it is applicable to every photoreceptor which can be described with a three-state cyclic model. However, the ease of extension of the model with the light-induced branching reaction also shows that the model can easily be modified to accommodate photoreceptors with more complicated photodynamics. This means that the model will be useful e.g. for estimation of the light intensity required to achieve a desired effect (given a photoreceptor with known characteristics), and estimation of key kinetics based on preliminary experiments. Additionally, the model will be useful to estimate which parameter can be most effectively changed to achieve a desired effect, which will be useful for e.g. application in optogenetics.
3.6. Materials and methods

3.6.1. Derivation of the photocycle model

The model for the photocycle of YtvA was based on the approach outlined in Hendriks and Hellengwerf 2009 (182) for PYP, with modifications where required. Our model is schematically shown in Figure 2A. The parameters are: \( k_{exD} \), the rate of excitation; \( k_{pe} \), the rate of photocycle entry; \( k_{re} \), the rate of recovery of the dark state; and \( Q_{yD} \), the quantum yield of photocycle entry. Using this, it is possible to derive differential equations for every species (equations 1-3), which can be used to numerically simulate the species distribution of YtvA.

The model is based on the assumption that every absorbed photon leads to the formation of the excited state (D*), which then continues into the photocycle to an extent depending on the quantum yield (182). This allows the application of the Lambert-Beer law for absorption (equation 18-19) with \( A \) the absorption, \( I_0 \) and \( I \) the light intensities before and after absorption by the sample, \( \varepsilon_0 \) the molar extinction coefficient of the protein, \( l \) the path length and \( [D] \) the concentration of the dark state of the protein.

\[
A = \log \frac{I_0}{I} = \varepsilon_D \cdot l \cdot [D] \quad \text{Eq. 18}
\]

\[
I = I_0 \cdot 10^{-\varepsilon_D \cdot l \cdot [D]} \quad \text{Eq. 19}
\]

The number of photons absorbed is equal to the difference between the light intensity before and after the sample. This light intensity can most easily be expressed in mEinstein \( \cdot \) cm\(^{-2} \cdot \) s\(^{-1} \) (10\(^7\) \( \mu \)Einstein \( \cdot \) m\(^{-2} \cdot \) s\(^{-1} \)). Thus, to obtain the rate of formation of D* (\( \nu_{D*} \)), the light intensity can be multiplied by the illuminated area (\( a \)). Dividing this by the volume of the sample (\( a \cdot l \)) gives the rate of formation of D* in M \( \cdot \) s\(^{-1} \) (equation 20).

\[
\nu_{D*} = a \cdot \frac{I_0 - I}{a \cdot l} = \frac{I_0 - I}{l} = \frac{I_0 \cdot \left(1 - 10^{-\varepsilon_D \cdot l \cdot [D]}\right)}{l} \quad \text{Eq. 20}
\]

Taking into account that the rate of formation of D* is equal to \( k_{exD} \cdot [D] \), this results in equation 21 for \( k_{exD} \). The dependence of \( k_{exD} \) on \( [D] \) makes it impossible to obtain analytical solutions for the fraction of protein in any of the three states at steady state. Thus, \( k_{exD} \) was approximated by converting the power 10 part of this equation to an exponent (equation 21), after which a Taylor expansion for an exponential function (equation 22) was used to obtain equation 23. This simplification is especially valid at low values of \( [D] \), but also holds well for higher \( [D] \) values in this system (see, e.g., Figure 2E).

\[
k_{exD} = \frac{I_0 \cdot \left(1 - 10^{-\varepsilon_D \cdot l \cdot [D]}\right)}{l \cdot [D]} = \frac{I_0 \cdot \left(1 - e^{-\varepsilon_D \cdot l \cdot [D] \cdot \ln 10}\right)}{l \cdot [D]} \quad \text{Eq. 21}
\]

\[
e^x = \sum_{n=0}^{\infty} \frac{x^n}{n!} = 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \frac{x^4}{4!} + \ldots \quad \text{Eq. 22}
\]
\[ k_{exD} \approx \frac{I_0 \cdot (1 - (1 - \varepsilon_D \cdot l \cdot [D] \cdot \ln 10))}{I \cdot [D]} = I_0 \cdot \varepsilon_D \cdot \ln 10 \]  

Eq. 23

In this equation, the molar extinction coefficient \((\varepsilon_D)\) has to be calculated for the entire spectrum of the light source (see below).

With the dependency of \(k_{exD}\) on \([D]\) removed, it is possible to derive equations for the steady-state fraction of YtvA in any of the three states (when equations 1-3 are zero). For example, setting equation 3 to zero, results in equation 24. Similarly, setting equation 2 to zero results in equation 25, and setting equation 1 to zero and expressing \([D^*]\) in \([S]\) via equation 24 therein leads to equation 26.

\[ \frac{d[S]}{dt} = k_{pe} \cdot [D^*] - k_{re} \cdot [S] = 0 \]
\[ k_{pe} \cdot [D^*] = k_{re} \cdot [S] \]

Eq. 24

\[ [D^*] = \frac{k_{re}}{k_{pe}} \cdot [S] \]

Eq. 25

\[ [D] = \frac{k_{pe}}{Q_{yD} \cdot k_{exD}} \cdot [D^*] \]

Eq. 26

The fraction in any of the three states is given by the concentration in that state divided by the total concentration, as in equation 27 for the dark state (D).

\[ f_D = \frac{[D]}{[D] + [D^*] + [S]} \]

Eq. 27

Expressing concentrations \([D^*]\) and \([S]\) in \([D]\) using equations 25-26 allows one to divide out the concentration \([D]\), which simplifies to equation 5. A similar approach leads to equations 6-7.

Simulations of steady-state fractions of each of the YtvA species, as well as of time traces based on the differential equations (such as in Figure 2), were done using Matlab R2010b (MathWorks).

Equations 5 and 7 can be further simplified by noting that the term \(1/k_{pe}\) is in general much smaller than the term next to it (\(1/k_{re}\) in equation 5 and \(1/(Q_{yD} \cdot k_{exD})\) in equation 7), and can therefore be neglected. Reorganizing the resulting equations then gives equations 28-29.

\[ f_D = \frac{1}{1 + Q_{yD} \cdot k_{exD} \cdot \left( \frac{1}{k_{pe}} + \frac{1}{k_{re}} \right)} \approx \frac{k_{re}}{Q_{yD} \cdot k_{exD} + k_{re}} \]

Eq. 28
As the contribution of $[D^*]$ is negligible (See Figure 2C), one can make the approximation that the total amount of protein is equal to $[D] + [S]$. In addition, it is reasonable to assume that the change in $[D]$ mirrors the change in $[S]$ ($d[D]/dt = -d[S]/dt$). Using these approximations it is possible to derive a simple analytical expression for the temporal changes in protein species distribution for a specific light condition (equation 30) (182). From this, an expression can be derived for the time it takes to reach a certain percentage of the new steady-state value after a change in light conditions (equation 31). In equation 30, $c_{tot}$ represents the total concentration of protein and $[D]_0$ the concentration of the dark state at time zero. In equation 31, $t$ represents the time (in seconds) until $f_D$ (the fraction of protein in the dark state) has reached a fraction of $f_{steady}$ state of the steady-state value of $f_D$. For example, if $f_{steady}$ is 0.95, $t$ represents the time it takes for 95% of the change in $f_D$ due to the new light intensity to have taken place.

$$f_D = \left( \frac{[D]_0}{c_{tot}} - \frac{k_{re}}{Q_{yD} \cdot k_{extD} + k_{re}} \right) e^{-\left( \frac{Q_{yD} \cdot k_{extD} + k_{re}}{k_{re}} \right) t} + \frac{k_{re}}{Q_{yD} \cdot k_{extD} + k_{re}}$$  \hspace{1cm} \text{Eq. 30}

$$t = \frac{\ln(1 - f_{steady})}{(Q_{yD} \cdot k_{extD} + k_{re})}$$  \hspace{1cm} \text{Eq. 31}

For a detailed derivation and notes on how to use these equations, see the Supplementary Information. Note that these simplified equations were not used for the simulations shown in the manuscript.

### 3.6.2. Extension of the photocycle model to include a light-induced branching reaction

The addition of a light-induced branching reaction to our model extends it with three additional reactions (Figure 6A). Compared to the full basic model outlined above, the extra parameters are: $k_{extS}$, the rate of excitation of $S$; $Q_{yS}$, quantum yield of the branching reaction; and $k_{be}$, the rate of branching entry. Similar to before, this can be used to set up differential equations to describe the temporal dynamics of each protein species involved (equations 10-13).

In this model, the form of $k_{extS}$ is equal to $k_{extD}$, with the exception that now the molar extinction coefficient of the light-induced signaling state ($\epsilon_s$) is used (see below for the calculation). In steady state, when equations 10-13 are zero, it is again possible to express the concentrations of the protein species in each other, resulting in equations for the steady-state fractions (equations 14-17). For example, the steady-state fraction of the light-induced signaling state $S$ can be derived by expressing $S^*$ in $S$ through equation 13, resulting in equation 32. Substituting this result in equation 12 allows the expression of $D^*$.
in S (equation 33), and substituting both results in equation 10 allows the expression of D in S (equation 34). These three equations can then be combined to derive the fraction of the protein that is in state S in steady state (equation 16).

\[
[S^*] = \frac{k_{es} \cdot Q_{ys}}{k_{be}} \cdot [S] \quad \text{Eq. 32}
\]

\[
[D^*] = \frac{k_{re} + k_{es} \cdot Q_{ys}}{k_{pe}} \cdot [S] \quad \text{Eq. 33}
\]

\[
[D] = \frac{k_{re} + k_{es} \cdot Q_{ys}}{k_{ed} \cdot Q_{yd}} \cdot [S] \quad \text{Eq. 34}
\]

A similar approach can be used to derive the other steady-state fractions (equations 14-17).

3.6.3. Determination of the effective extinction coefficient of YtvA for a given light source

The rates of excitation for the dark state \(k_{ed}\) and the light-induced signaling state \(k_{es}\) in the branching model depend on the extinction coefficient for the respective protein species \(\epsilon\) and the light intensity used \(I_0\). However, as is often the case, the light source used emits a peak over a range of wavelengths for which the extinction coefficient is not constant. Thus, both \(\epsilon\) and \(I_0\) are functions of the wavelength \(\lambda\), and the expression should be an integral over \(\epsilon(\lambda) \cdot I_0(\lambda)\).

The wavelength-dependent extinction coefficient for the dark spectrum was determined by normalizing the absorption spectrum to \(\epsilon_{450} = 12,500 \ \text{M}^{-1} \cdot \text{cm}^{-1}\): the extinction coefficient of FMN that was also used for the quantum yield determination by \(277\). An isosbestic point was used to normalize the light-induced spectrum to the dark spectrum to obtain \(\epsilon(\lambda)\) for this species.

The emission spectrum of the blue LED with a \(\lambda_{max}\) of 464 nm that was used for all experiments was determined using an Ocean Optics USB4000 spectrophotometer. Because the total light intensity over all emitted wavelengths \(I_0(\text{total})\) was measured experimentally, \(I_0(\lambda)\) was split into two factors: \(I_0(\text{total}) \cdot f(\lambda)\), where \(f(\lambda)\) represents the fractional contribution of wavelength \(\lambda\) to the total intensity. In effect \(f(\lambda)\) is the emission spectrum of the light source normalized to a total area of 1. The area of overlap between \(\epsilon(\lambda)\) and \(f(\lambda)\) was used as the ‘effective molar extinction coefficient’, resulting in \(\epsilon_D = 9516 \ \text{M}^{-1} \cdot \text{cm}^{-1}\) and \(\epsilon_S = 963 \ \text{M}^{-1} \cdot \text{cm}^{-1}\) for the light source used in these experiments.

The two black bars that denote a crude estimate of relevant light intensities in the lab and of sunlight in the Netherlands at noon in Figure 2E were included for comparative purposes. The light intensity of the lighting in our lab was estimated using a LI-COR LI-250 light meter and a LI-COR Quantum Sensor, and its spectrum was measured using an Ocean Optics USB4000 spectrophotometer. The measured light intensity was then corrected to represent a light intensity with comparable activity with regard to YtvA activation as the
blue LED for which the data in the figure were simulated. Similarly, the solar light intensities were estimated based on the average solar irradiance at noon in De Bilt, the Netherlands in December (lower limit) and June (upper limit) (481), combined with the solar irradiation spectrum from ASTM Standard G173-03 (global tilted) reproduced with SMARTS v2.9.2 (168).

3.6.4. Plasmid and strain constructions

Strains, plasmids and primers used in this study are listed in Table III. DNA manipulations and other molecular genetic techniques were carried out using standard procedures. The sequences of all constructed plasmids were verified.

Plasmids suitable for expression and isolation of YtvA mutants were constructed using Escherichia coli XL1-Blue as cloning host. E. coli M15/pREP4 was then transformed with the constructed plasmids to create production strains. The ytvA(R63K) mutation was amplified using primers pQE30ytvAFW and pQE30ytvARV from a template plasmid carrying the mutation (a kind gift of Wolfgang Gärtner). The PCR product was then digested with BamHI and Sall and ligated into similarly digested pQE-30 to create pYN006. The ytvA(V28I) mutation was constructed using the QuickChange method. Template pQE-30-ytvA was amplified using primers JBS190 and JBS191 to create plasmid pJA024.

Plasmids suitable for expression of YtvA in B. subtilis were also constructed using E. coli XL1-Blue as cloning host. E. coli MC1061, a strain that creates plasmid multimers, was subsequently transformed with the constructed plasmids. The ytvA gene carrying the R63K mutation was amplified from pYN006 with primers OB1 and OB2, digested with Sall and SphI, and ligated into similarly digested pDG148 to create pYN012. The ytvA(V28I) mutation was constructed in a two-step PCR procedure. First, overlapping fragments of the mutated gene were amplified using primers OB1 and JBS191, and JBS190 and OB2. Second, both fragments were used as a template in a PCR with flanking primers OB1 and OB2. The fragment was digested with Sall and SphI and ligated into similarly digested pDG148 to generate pJBS1140 (V28I).

B. subtilis strain PB565 (5), a derivative of the wild-type PB2 (46), contains a ytvA null mutation and an integration in a neutral site (the amyE gene) of a fusion between the SigmaB-dependent ctc promoter and the lacZ reporter gene (which encodes for a β-galactosidase). This strain was transformed with pYtvA, pYN012 or pJBS1140, isolated from E. coli MC1061, to generate the strains used for GSR activation assays.

3.6.5. Protein isolation

E. coli M15/pREP4 with the appropriate plasmid (pQE-30-htvA for wild-type YtvA, pYN006 for the R63K mutant or pJA024 for the V28I mutant) was grown overnight in 20 ml Production Broth (PB: 20 g/l Bacto trypton, 10 g/l yeast extract, 5 g/l NaCl, 8.7 g/l K2HPO4 and 5 g/l glucose) supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin. The next day, 1 l of PB was inoculated with the overnight culture and grown at 37°C, with shaking at 250 rpm. When an optical density (OD) at 600 nm of 0.4 to 0.6 was reached, 1
mM (final concentration) of IPTG was added to induce expression, and the culture was allowed to grow overnight at room temperature. Cell-free extracts were prepared by sonication in buffer A (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0) supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche), DNAse, RNAse and lysozyme.

Poly-histidine-tagged protein was purified using a HisTrap HP 5 ml column (GE Healthcare) and an ÄKTA FPLC system (GE Healthcare) using a linear gradient from 20 to 500 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, pH 8.0. Fractions containing the protein were collected and dialyzed against 20 mM Tris-HCl, 10 mM NaCl, pH 8.0. The protein was then further purified on a 6 ml Resource Q column (GE Healthcare) using a linear gradient from 0 to 1 M NaCl in 20 mM Tris-HCl pH 8.0. Again, fractions containing the protein were dialyzed against 20 mM Tris-HCl, 10 mM NaCl, pH 8.0. Protein purity was checked via SDS-PAGE.

### Table III: Strains, plasmids and primers used in this study.

<table>
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<th>Strain, plasmid or primer</th>
<th>Genotype, characteristic or sequence</th>
<th>Reference</th>
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</tr>
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<td>ytvA∷ery amyE∷(Pctc-lacZ cat) trpC2</td>
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*Resistance cassettes: bla for ampicillin resistance, cat for chloramphenicol resistance, ery for erythromycin resistance, and kan for kanamycin resistance. MCS: multiple cloning site. In primer sequences, restriction sites are underlined while altered bases for mutagenesis are in bold.*
3.6.6. *In vitro* photocycle measurements and data analysis

All *in vitro* photocycle measurements were done with an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies) using a Kraayenhof-cuvette (248) with temperature control, stirring, and light-input ports. Measurements were carried out in buffer (20 mM Tris-HCl, 10 mM NaCl, pH 8.0) at temperatures as described in the text. Where applicable, samples were illuminated with a blue LED ($\lambda_{\text{max}} = 464$ nm, full width at half-maximum (FWHM) = 25 nm) of which the intensity could be controlled. Recovery kinetics were fitted to an exponential function using Microsoft Excel 2010 and non-linear regression analysis, essentially as described in Brown (2001) (56). The spectrum of YtvA in the light-induced signaling state was obtained by bleaching with high intensity white light from a SCHOTT KL 1500 LCD source, while the spectrum of YtvA in the dark state was determined after full recovery. Light intensities were measured using a LI-COR LI-250 light meter and a LI-COR Quantum Sensor.

For the measurements with varying light intensities (Figure 3) whole spectra were monitored until a steady state was reached. To ensure that results of different experiments and samples were comparable, each spectrum was baseline-corrected by setting the average absorption between 525 and 535 nm to zero, and subsequently normalized to the isosbestic point at 410 nm. The fraction of protein still in the dark state was then calculated by treating the spectrum as a linear combination of similarly corrected full dark and full light spectra. The R63K mutant of YtvA was treated in the same way. However, this mutant was slightly sensitive to bleaching at 37°C and high light intensities. To correct for this, dark spectra were determined after each measurement, and the calculations of the fraction of the protein in the dark state and in the light-induced signaling state were adjusted accordingly. In addition, fresh protein samples were used whenever significant bleaching was detected. Bleaching was not an issue for the R63K mutant at 19°C, nor for the wild type at 37°C.

3.6.7. *In vivo* GSR activation assays and data analysis

For all experiments, Tryptic Soy Broth (TSB) medium was used, supplemented with 0.5% (w/v) glucose, 5 μg/ml chloramphenicol and 10 μg/ml kanamycin. Cells were continuously kept at 37°C in a water bath. To ensure sufficient aeration, cells were always shaken at 250 rpm in an orbital shaker with no more than 10 ml culture in a 100-ml Erlenmeyer flask.

To initiate an experiment, 10 ml of medium was inoculated and cells were allowed to grow overnight. Such cultures were diluted to an OD at 600 nm of 0.05 and allowed to grow until an OD$_{600}$ of approximately 0.6. At this point, expression of (wild-type or mutant) YtvA was induced by the addition of IPTG to a final concentration of 1 mM. Next, cells were allowed to grow until they reached an OD$_{600}$ of approximately 2.0 (late exponential growth phase), when they were again diluted to an OD$_{600}$ of 0.05. This dilution was divided over 16 flasks which were incubated until an OD$_{600}$ of approximately 0.5 was reached. A control sample was taken (time point 0), and the light was switched on. Additional
samples were then taken after 120 minutes. Samples were immediately flash-frozen in liquid nitrogen and stored at −80°C. Note that experiments were stopped before the cells reached stationary phase to prevent activation of the GSR via the energy stress branch of the upstream activation network.

Illumination was provided by blue LEDs with a $\lambda_{\text{max}}$ at 464 nm. All experiments were carried out in a dark room to prevent uncontrolled illumination before time point 0. Each experiment included at least two dark controls, which were wrapped tightly in aluminum foil. Samples of dark controls and of cells prior to time point 0 were taken in complete darkness or with minimal background illumination from a red LED ($\lambda_{\text{max}} = 632$ nm), which was previously shown not to activate YtvA (16, 18). Light intensities were measured with a LI-COR LI-250 light meter and a LI-COR Quantum Sensor.

$\beta$-galactosidase activities were measured and expressed in Miller units according to a previously described protocol (16). The resulting GSR activation at varying light intensities was analyzed in Microsoft Excel 2010 using non-linear regression analysis, essentially as described in Brown 2001 (56). Data were fitted to the analytically derived equation for the fraction of protein in the light-induced signaling state (S) (equation 7 or 16). The recovery rate ($k_{\text{re}}$) was left free, while all other parameters were fixed to the values specified in the text.

3.6.8. Light-induced branching reaction
The presence of the light-induced branching reaction was demonstrated using an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies). A quartz cuvette with a path length of 1 cm at room temperature without stirring was used. A sample of YtvA was illuminated with a blue LED ($\lambda_{\text{max}} = 464$ nm, FWHM = 25 nm) at maximum light intensity to completely convert YtvA to the light-induced signaling state. When the light source was removed YtvA slowly recovered to the dark state, which was monitored at 450 nm with a time resolution of 0.1 s. The branching reaction was immediately induced by photo-excitation with the third-harmonic of light from a Continuum Surelite I-10 YAG laser with an output intensity of 100 mJ at 355 nm and a pulse duration of 6 ns.

The quantum yield of the light-induced branching reaction was estimated from the steady-state distribution of the dark state (D) and the light-induced signaling state (S) at saturating light intensities of a light source absorbed by both species, as explained in the Materials and methods section. Experiments were carried out in the same setup as described above for the in vitro photocycle measurements. A temperature of 25°C was used to ensure that thermal recovery would be negligible. As the light source, a custom-made LED with a $\lambda_{\text{max}}$ of 401 nm (FWHM of 12 nm) and a variable intensity was used. Data were processed analogous to the blue light in vitro experiments described above, using Matlab R2010b (MathWorks) and Microsoft Excel 2010.
3.7. Note added in preparation
While this paper was in the preparation and submission process Losi et al. published an intriguing paper on super-resolution microscopy using YtvA in which the existence of the branching reaction is also convincingly demonstrated (281). Their data confirms our observation that the quantum yield of this branching reaction is low, which further solidifies our conclusions with respect to the biological implications.

3.8. Acknowledgements
The authors would like to acknowledge the help of Jos C. Arents and Francis K. Fordjour with experiments, to thank Wolfgang Gärtner for kindly providing a strain, and to thank Chris de Koster for critical reading of the manuscript.

3.9. Author contributions
JBvdS and YN performed experiments; JH, JBvdS, KJH and YN developed models; JBvdS, YN and KJH designed experiments; and JBvdS wrote the paper.

3.10. Conflict of interest
The authors declare that they have no conflict of interest.
Chapter 3: Supplementary information

Modeling the functioning of YtvA in the general stress response in *Bacillus subtilis*

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3.11. Analytical approximation of the temporal changes in protein species distribution

Note that several equations from the main text are repeated in the Supplementary Information to present a coherent story. For clarity, they retain the numbering of the main text (‘equation #’), while new equations are labeled ‘supplementary equation #’.

3.11.1. Introduction

The temporal behavior of the kinetic photocycle model presented in Figure 2A can be described using three differential equations (equations 1-3). These equations can be used to simulate the temporal changes in the protein species distribution of a photoreceptor such as YtvA (Figure 2B-D; see the main text). However, for the purpose of predictions of routine experiments, differential equations can be too cumbersome. Therefore, it is of interest to obtain a simple analytical expression for the temporal changes in protein species distribution.

\[
\frac{d[D]}{dt} = -k_{exD} \cdot [D] + \left( \frac{1}{Q_{yD}} - 1 \right) \cdot k_{pe} \cdot [D^*] + k_{re} \cdot [S] \quad \text{Eq. 1}
\]

\[
\frac{d[D^*]}{dt} = k_{exD} \cdot [D] - \frac{k_{pe}}{Q_{yD}} \cdot [D^*] \quad \text{Eq. 2}
\]

\[
\frac{d[S]}{dt} = k_{pe} \cdot [D^*] - k_{re} \cdot [S] \quad \text{Eq. 3}
\]

3.11.2. Required approximations to simplify the model

To obtain such an expression, we followed a procedure analogous to the one outlined in Hendriks & Hellingwerf 2009 (182). We assumed that the contribution of the excited state D* to the species distribution of YtvA is negligible. Our simulations with parameters for YtvA confirm that D* does not accumulate significantly (Figure 2C). This leads to supplementary equation 1, where \(c_{tot}\) represents the total concentration of the photoreceptor. In light of this, it is also reasonable to assume that the change in [D] mirrors the change in [S], as shown in supplementary equation 2.

\[c_{tot} = [D] + [S] \quad \text{Supp. eq. 1}\]

\[\frac{d[D]}{dt} = -\frac{d[S]}{dt} \quad \text{Supp. eq. 2}\]

3.11.3. Derivation of an equation for the temporal change in concentration of the dark state

Substituting equations 1 and 3 into supplementary equation 2 allows the derivation of an expression for D* in D (supplementary equation 4).

\[-k_{exD} \cdot [D] + \left( \frac{1}{Q_{yD}} - 1 \right) \cdot k_{pe} \cdot [D^*] + k_{re} \cdot [S] = -k_{pe} \cdot [D^*] + k_{re} \cdot [S] \quad \text{Supp. eq. 3}\]
\[ [D^*] = \frac{Q_{yD} \cdot k_{exD}}{k_{pe}} \cdot [D] \]  

Supp. eq. 4

Substituting this result and the fact that \([S]\) is equal to \(c_{tot} - [D]\) (as follows from supplementary equation 1) into equation 1 results in supplementary equation 5, which can be rewritten to supplementary equation 6.

\[
\frac{d[D]}{dt} = -k_{exD} \cdot [D] + \left( \frac{1}{Q_{yD}} - 1 \right) \cdot k_{pe} \cdot \frac{Q_{yD} \cdot k_{exD}}{k_{pe}} \cdot [D] + k_{re} \cdot (c_{tot} - [D]) \]  

Supp. eq. 5

\[
\frac{d[D]}{dt} = \alpha \cdot [D] + k_{re} \cdot c_{tot} \quad \text{with} \quad \alpha = \left( Q_{yD} \cdot k_{exD} + k_{re} \right) \]  

Supp. eq. 6

This equation can be rewritten and integrated to obtain a solution for \([D]\). Note that this uses the same assumption that \(k_{exD}\) is independent of \([D]\) as was made for the derivation of the analytical equations for the steady-state fractions (see the main text and in particular equation 4).

\[
\frac{d[D]}{dt} = \alpha \cdot \left( [D] + \frac{k_{re} \cdot c_{tot}}{\alpha} \right) \]  

Supp. eq. 7

\[
\frac{1}{[D] + \frac{k_{re} \cdot c_{tot}}{\alpha}} d[D] = \alpha \cdot dt \quad \Rightarrow \quad \int_{[D]_0}^{[D]} \frac{1}{[D] + \frac{k_{re} \cdot c_{tot}}{\alpha}} d[D] = \alpha \cdot \int_0^t dt \]  

Supp. eq. 8

\[
\frac{[D] + \frac{k_{re} \cdot c_{tot}}{\alpha}}{[D]_0 + \frac{k_{re} \cdot c_{tot}}{\alpha}} = e^{\alpha \cdot t} \]  

Supp. eq. 9

\[
[D] = \left( [D]_0 - \frac{k_{re} \cdot c_{tot}}{Q_{yD} \cdot k_{exD} + k_{re}} \right) e^{-\left( Q_{yD} \cdot k_{exD} + k_{re} \right) t} + \frac{k_{re} \cdot c_{tot}}{Q_{yD} \cdot k_{exD} + k_{re}} \]  

Supp. eq. 10

Herein, \([D]_0\) represents the starting concentration of the protein in the dark state. The fraction of the protein in the dark state can easily be derived from supplementary equation 10.

\[
f_D = \frac{[D]}{c_{tot}} = \left( \frac{[D]_0 - k_{re}}{Q_{yD} \cdot k_{exD} + k_{re}} \right) e^{-\left( Q_{yD} \cdot k_{exD} + k_{re} \right) t} + \frac{k_{re}}{Q_{yD} \cdot k_{exD} + k_{re}} \]  

Eq. 30

If the time is set to infinite in equation 30, the exponential part of the equation is canceled, and the equation simplifies to the steady-state equation for the fraction of protein in the dark state (equation 28), as expected.

Using the same parameters for YtvA as used in Figure 2, we compared the outcome of equation 30 (solid line in Supplementary Figure 1) to simulations with the differential equations (crosses). Both simulations overlap, verifying the validity of the assumptions.
3.11.4. Derivation of an equation for the time needed to reach a given percentage of steady state

Another interesting application of equation 30 is in rewriting it to obtain an expression for the time it takes before a certain percentage of steady state is reached. If \( f_{\text{steady state}} \) is taken to be the fraction (between 0 and 1) of the change in response to the given perturbation in light intensity that has taken place, then the relationship in supplementary equation 11 holds.

\[
f_{\text{steady state}} = 1 - e^{-(Q_{yD}k_{exD} + k_{re})t}
\]

Supp. eq. 11

This can be rewritten to obtain equation 31.

\[
\ln(1 - f_{\text{steady state}}) = -(Q_{yD}k_{exD} + k_{re})t
\]

Supp. eq. 12

\[
t = \frac{\ln(1 - f_{\text{steady state}})}{-Q_{yD}k_{exD} - k_{re}}
\]

Eq. 31

It is important to note that \( f_{\text{steady state}} \) represents the fraction of the change in \( f_D \) that will take place as a result of the perturbation in light intensity. Thus, an \( f_{\text{steady state}} \) of 0.95 is equal to 0.95 \( \Delta f_D \) (see Supplementary Figure 2). It should also be noted that it is not possible to use this formula to calculate the time it takes to reach steady state, as a true steady state takes an infinite time to reach.
**Supplementary Figure 2.** Illustration of the value of $f_{\text{steady state}}$ in the same simulation as in Supplementary Figure 1. The solid line is the same as in Supplementary Figure 1 (the result of applying equation 30), while the dotted line represents the value of $f_{\text{steady state}}$. 