**REVIEW ARTICLE**

**Optogenetics in bacteria – applications and opportunities**

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One sentence summary: This review highlights the diversity of optogenetic approaches in prokaryotes and aims to provide microbiologists with a guide for the application of light for precise, reversible and non-invasive control of biological processes in bacteria.

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**ABSTRACT**

Optogenetics holds the promise of controlling biological processes with superb temporal and spatial resolution at minimal perturbation. Although many of the light-reactive proteins used in optogenetic systems are derived from prokaryotes, applications were largely limited to eukaryotes for a long time. In recent years, however, an increasing number of microbiologists use optogenetics as a powerful new tool to study and control key aspects of bacterial biology in a fast and often reversible manner. After a brief discussion of optogenetic principles, this review provides an overview of the rapidly growing number of optogenetic applications in bacteria, with a particular focus on studies venturing beyond transcriptional control. To guide future experiments, we highlight helpful tools, provide considerations for successful application of optogenetics in bacterial systems, and identify particular opportunities and challenges that arise when applying these approaches in bacteria.

Keywords: synthetic biology; optogenetic interaction switches; two-component systems; protein interactions; biotechnology; fluorescence; light-sensing domains

**INTRODUCTION**

Optogenetics has its origin in the 1970s when researchers found that bacterial rhodopsins act as ion pumps that are activated by visible light (Oesterhelt and Stoeckenius 1971; Oesterhelt 1976). During that time, Francis Crick challenged neuroscientists to come up with a system that would allow the precise stimulation of single neurons, as opposed to the large areas stimulated by electrodes (Crick 1979; Siegel and Callaway 2004; Deisseroth 2011). At the time, this was perceived as an almost impossible task. However, a breakthrough was made in the seemingly distant field of plant and microbial biology, when the group of Peter H. Quail developed a light-controlled gene promoter (Shimizu-Sato et al. 2002) based on the plant phytochrome B-phytochrome interacting factor 3 (PhyB-PIF3) photoreceptor system (Ni, Tepperman and Quail 1999), and three years later, Boyden and coworkers found that expression of an algal channel-rhodopsin made neurons respond to light (Boyden et al. 2005). Since then, optogenetics has found wide range of application in eukaryotic systems, extending from neuroscience (Boyden 2011; Yizhar et al. 2011) to areas as diverse as cardiology (Entcheva and Kay 2020) and regenerative medicine such as bone repair (Sato et al. 2018) or restoration of muscle function (Bryson et al. 2014).
The first optogenetic setup in bacteria was established in 2005, when Levskaya and colleagues developed a synthetic sensor kinase, based on the photoreceptor Cph1 and the histidine kinase EnvZ, to achieve red light-controllable gene expression of LacZ in Escherichia coli (Levskaya et al. 2005). Since then, the number of optogenetic applications in prokaryotes has markedly increased. This review will summarize these applications and provide an overview of the range of optogenetic approaches applied in bacteria to date. To facilitate the development of even more diverse applications of optogenetics in prokaryotes in the future, we will discuss the specific requirements, challenges, and opportunities of applying optogenetics in bacteria. A characterization of optogenetic base systems, including their light spectrum and dynamics, a list of useful tools for optogenetic setups in bacteria, and guidelines for selecting the optimal system for specific approaches are aimed at helping microbiologists evaluate these systems and design experiments to address individual biological questions. We want to draw particular attention to studies that go beyond the control of gene expression, which is currently the main application area of optogenetics in bacteria, and showcase its great potential for the direct control of diverse biological processes with light.

**OPTOGENETIC SYSTEMS**

Optogenetics combines optical and genetic techniques to design and apply light-sensitive proteins in order to control cellular processes within living organisms. Light-reactive proteins or protein domains are often plant-derived, such as phytochromes, cryptochromes, light-oxygen-voltage sensing (LOV) and ultraviolet B resistance locus 8 (UVR8) domain proteins, but also originated in bacteria, archaea, algae, and higher animals, for example channelrhodopsins, halorhodopsins, cyanobacteriocromes, cryptochromes, phytochromes, and additional LOV domain proteins (Endo and Ozawa 2017). Upon illumination with a specific wavelength, the light-sensing domains undergo a conformational change. The resulting modulation of protein properties can lead to association/dissociation with an interaction partner or partial folding/unfolding of the protein structure, which then controls downstream biological processes.

Optogenetic systems constitute excellent tools for the direct control of biological systems in a highly time- and space-resolved manner with minimal intervention. They allow for easy and often reversible manipulation of protein functionality and localization (Guglielmi, Falk and De Renzis 2016), metabolism (Berry and Wojtovich 2020), intracellular interaction of enzymes and substrates (Huang et al. 2020), or processes like gene expression (de Mena, Rizk and Rincon-Limas 2018). Its fast and easy tunability gives light an advantage over chemical inducers and other environmental triggers like pH or temperature and enables more precise spatiotemporal control (Deisseroth 2011; Liu et al. 2018), even up to a single-cell level (Chait et al. 2017). By modulating the amplitude or pulse-width of the light source (Baumschlager and Khammash 2021) or by combining different optogenetic approaches (Tabor, Levskaya and Voigt 2011; Fernandez-Rodriguez et al. 2017), even more complex modulation of expression can be achieved. Different optogenetic systems react to light across the whole visible spectrum and extending into the infrared range, which can be preferable in sensitive systems due to its lower energy. While the vast majority of applications were originally developed in eukaryotes, optogenetics increasingly finds its way into prokaryotic systems.

In this review, we define optogenetic base systems as domains, proteins or pairs thereof that react to light and are modified and applied for optogenetic applications. In most cases, the direct reaction to illumination occurs in a bound chromophore cofactor, which features the conjugated electron system that can absorb photons. The resulting change in conformation is then transmitted to the protein core. Optogenetic base systems comprise a diverse collection of proteins, which can be generally divided into channel proteins, widely used in neurobiology, and intracellular proteins, which are the predominant class for applications in prokaryotes. Channel systems are mostly based on opsins, which are light-driven ion pumps or channels carrying the chromophore retinal, or on opsin variants (channelrhodopsins and halorhodopsins) (Terakita 2005; Fenno, Yizhar and Deisseroth 2011). Intracellular optogenetic base systems include proteins from the LOV, blue-light-utilizing flavin adenine dinucleotide (BLUF), cryptochrome (CRYs) (all reacting to blue light), phytochrome (PHY, red-light-responsive), and UVR8 (reacting to ultraviolet light) families (Van Der Horst and Hellingwerf 2004; Mögl ich and Moffat 2010; Pudasaini, El-Arab and Zolotowski 2015). These base systems can then be either adapted or simply combined with other proteins or domains in a modular manner, extending light control to usually non-light-reactive proteins. An excellent example for this approach is the development of the light-responsive histidine kinase YF1, part of a much-used optogenetic two-component system (TCS) for transcriptional regulation. Möglich and colleagues structurally aligned a LOV-based photoreceptor, Bacillus subtilis YtVA (Losi et al. 2002) and an oxygen-sensing histidine kinase, Bradyrhizobium japonicum FixL, both of which feature N-terminal sensor and C-terminal output domains. Testing different recombination points in a central α-helical domain, they identified fusion proteins conferring light-dependent activation of the cognate response regulator FixJ (Möglich, Ayers and Moffat 2009). The resulting TCS YF1/FixJ has since been evolved further to allow for bidirectional control of protein expression (see chapter 3.1.2). Fig. 1 provides an overview of optogenetic base systems that have been used in bacteria and their respective activation wavelength, which covers the visible spectrum from violet to red with a particular density of systems reacting to blue light. Readers are encouraged to refer to specialized reviews (Scherbakov et al. 2015; O’Banion and Lawrence 2018) for details on the biophysical mechanisms of these and other base systems.

**OPTOGENETIC APPLICATIONS IN BACTERIA**

The modularity of optogenetic systems, which allows control of protein behavior by combining proteins of interest (POI) with light-reactive base systems, has led to the development of a large variety of different systems, many of which build on each other through both linear evolution and combination.

Optogenetic applications can be roughly grouped into intramolecular and intermolecular systems. While intramolecular systems consist of only one light-sensitive protein, intermolecular systems have additional interaction partners (Fig. 2).

In intramolecular systems, absorption of light leads to a conformational change in the light-sensitive part of the protein, which is transmitted intramolecularly to an effector domain and modulates the conformation or accessibility of an active center, a substrate-binding pocket, or a regulatory domain. The design of intramolecular optogenetic systems requires knowledge about the behavior of the optogenetic base system as well as the POI’s structure-function relationship (see paragraph “Intramolecular control” for details).

Intermolecular systems, also called optogenetic interaction switches, consist of two or more molecules whose binding is
influenced by a conformational change in at least one of the interaction partners, in analogy to chemical dimersizers (Fegan et al. 2010). These systems mostly exploit light-induced homodimerization or -dissociation, although examples of light-induced oligomerization exist. In this review, 'optogenetic interaction switch' will be used exclusively for the control of intermolecular interactions, which can be used to directly activate (or, in special cases, inactivate) protein function via dimerization or oligomerization, to recruit a POI to its site of action, or, conversely, to sequester it from its site of action, such as the cytosol (Fig. 2).

Related aims can be achieved with different optogenetic systems. A prime example is the large field of translational control (discussed in the next paragraph), which has been accomplished by intra- and intermolecular optogenetic systems. Similarly, direct protein control (see paragraph "Direct (post-translational) control of protein function by illumination") can be achieved by intramolecular optogenetic actuators, although most published systems rely on intermolecular interactions, such as optogenetic interaction switches.

In the last years, the utilization of optogenetics to investigate biological processes in bacteria has markedly increased (Table 1). To date, the most established application of optogenetic systems in prokaryotes is the light-mediated control of gene expression.

Control of expression
Optogenetic systems regulating the expression level of proteins can be divided into one-component systems (OCS) and two-component systems (TCS). OCS comprise different optogenetic base systems designed to mediate expression control, for example by light-dependent dimerization and recruitment of transcription repressors or activators, or direct mediation of translational control (on the RNA level) by steric blocking. TCS consist of a light-activatable histidine kinase (HK) and a corresponding response regulator (RR) that is phosphorylated by the HK upon illumination and regulates downstream processes like gene expression (Schmidt et al. 2014). The vast majority of published optogenetic approaches for expression control target gene transcription; readers that are specifically interested in this aspect are encouraged to also refer to the excellent recent review from Baumslag and Khammash (Baumslag and Khammash 2021).

One-component systems
Blue-light mediated control over protein binding or dimerization most often relies on LOV domains due to their relatively small size (17–20 kDa), robustness and well-studied properties (reviewed in Zayner and Sosnick 2014; Pudasaini, El-Arab and

| Optogenetic base system | Off | Cofactor(s) | Domain size | Light source | Action upon illumination | Reference |
|-------------------------|-----|-------------|-------------|--------------|-------------------------|-----------|
| Cyanobacteriochromes    |     |             |             |              |                         |           |
| UirS/R                  | Pcb | UirS: 97 kDa | 77 kDa      | 30 kDa       | cAMP synthesis          | (Blain-Hartung et al. 2018) |
| cPAC                    |     |             |             |              |                         |           |
| Magnets                 | s   | FMN, FAD, RF| 30 kDa      | 77 kDa       |                         |           |
| VVD                     |     | FMN, FAD    | 77 kDa      | 20 kDa       |                         | (Aso et al. 2009) |
| AsLOV2                  | s   | FMN         | 77 kDa      |              |                         |           |
| ILD                     | s   | FMN         | 77 kDa      |              |                         |           |
| LOVTRAP                 | s   | FMN         | 77 kDa      |              |                         |           |
| BphP1                   | s   | FMN         | 77 kDa      |              |                         |           |
| BphS                    | h   | PCB         | 77 kDa      |              |                         |           |
| BLUF domain-based       |     |             |             |              |                         |           |
| bPAC (Blac)             | s   | FMN, FAD    | 40 kDa      |              |                         |           |
| EL222                   | s   | FMN         | 40 kDa      |              |                         |           |
| RSyLOV                  | s   | FMN         | 40 kDa      |              |                         |           |
| LOVTRAP                 | s   | FMN         | 40 kDa      |              |                         |           |
| Cryptochrome-based      |     |             |             |              |                         |           |
| CRY2-CIB1               | min | FAD         | 40 kDa      |              |                         |           |
| CyaB/CyaR               | >h  | PCB         | 40 kDa      |              |                         |           |
| Phytochrome-based       |     |             |             |              |                         |           |
| Cph1                    |     | PCB         | 40 kDa      |              |                         |           |
| BphS                    | h   | BphS        | 40 kDa      |              |                         |           |
| BphF1/PpR2              | min | BphP1       | 40 kDa      |              |                         |           |
| Light (bPacD)           | >h  | BphP2       | 40 kDa      |              |                         |           |

Figure 1. Optogenetic base systems used in bacteria. Left, graphical representation of main optogenetic base systems used in bacteria. Activation wavelength and position in visible spectrum indicated; word size represents number of applications (font size = square root of number of applications listed for bacterial host in optobase.org (Kolar et al. 2018) for all systems with applications in bacteria as of August 2021). Right, key properties of these optogenetic base systems. FMN, Flavin mononucleotide; FAD, flavin adenine dinucleotide; RF, riboflavin; PCB, phycocyanobilin.
Zoltowski 2015). EL222, one of the most frequently applied one-component blue light-responsive bacterial transcription factors, combines an N-terminal LOV domain with a C-terminal helix-turn-helix (HTH) DNA-binding domain (Motta-Mena et al. 2014; Jayaraman et al. 2016), which allows for the direct control of gene expression by binding to DNA upon illumination. EL222-mediated gene expression was established in a wide range of applications. Controlled expression of CheZ, a regulator of flagellar rotation, resulted in negative phototaxis in E. coli (Zhang, Luo and Poh 2020), while bacterial division and cell shape were perturbed by control of expression of FtsZ, a key player in bacterial cell division (Ding et al. 2020). In Sinorhizobium meliloti, EL222-controlled exopolysaccharide (EPS) synthesis was used to control the resulting biofilm formation (Pirhanov et al. 2021).

By fusing a LOV domain from Rhodobacter sphaeroides (RsLOV) to a transcriptional repressor, a different blue light-controllable gene expression system (eLightOn) was designed and, similar to the cases above, used to influence bacterial motility and cell division, respectively (Li et al. 2020). Romano and colleagues combined the LOV-derived VVD domain with the transcriptional regulator AraC in order to make it responsive to blue light, and created a set of blue-light-inducible AraC dimerization-based systems for expression control in E. coli (BLADE) (Romano et al. 2021). As a proof of concept, this system was used to light-control the arabinose pathway itself (Romano et al. 2021). By fusing the VVD domain to the E. coli DNA repressor LexA, an optogenetic setup for blue-light mediated gene control (LEVl) with a high on/off ratio (up to 10 000-fold) was developed. As applications, light-induced cell death (expression of the toxic protein CcdB) and motility control (expression of CheZ) were presented (Chen et al. 2016).

Based on a phytochrome from Idiomarina, a small near infrared (NIR) one-component system termed iLIGHT was developed that can be packed in adeno-associated viruses for easy transduction of eukaryotic cells and enabled light controlled gene expression with up to 115-fold dynamic range in bacteria and mouse tissue (Kaberniuk et al. 2021).

In a light-controlled, though not fully optogenetic approach¹, Chou and colleagues used a photoactivatable chemical ortho-nitrobenzyl caging group to block the active side of T7 RNA polymerase (RNAP) in E. coli. Upon UV-light illumination, the caging group was released and T7 polymerase activity restored (Chou, Young and Deiters 2010). A disadvantage of this setup is the irreversibility of the reaction, unlike for photoactivatable protein domains. Therefore, Pu and colleagues engineered a split-T7 RNA polymerase (split-T7-RNAP) where two non-functional polymerase domains were fused to different optogenetic interaction switches including the pair of Avena sativa LOV2 (iLID) and SspB Nano (iLID system, (Guntas et al. 2015)). Illumination with blue light induced dimerization of the interacting domains, restoring T7-RNAP function and activating gene expression (Pu, Zinkus-Boltz and Dickinson 2017). Han and colleagues developed a similar split-T7-RNAP setup to control gene expression by either dimerization (Magnets) or allosteric blocking (VVD) (Han, Chen and Liu 2017). To further improve previous setups, a Magnet-based split-T7-RNAP setup was combined with photodegradable compounds (anyhydrotetracycline/tetracycline) to

¹ The photocaged tyrosine was introduced by amber stop codon suppression, which requires the presence of additional enzymes and external addition of ortho-nitrobenzyl tyrosine
Table 1. Optogenetic applications in bacteria. Optogenetic applications in prokaryotes. Examples are subsequently ordered by the type of control mechanisms (subheadings), light range, optogenetic system and chronologically. Where applicable, the left column indicates both the modification of the system and the application, separated by a dash. The central column indicates the optogenetic system used and, where applicable, the base system, according to the classification used in Fig. 1, which used the optobase.org classification (in brackets) and the controlled protein or domain (orange font).

| Application in bacteria                                      | Bacterial host | Optogenetic system (base) | Light source on/off | Reference                               |
|-------------------------------------------------------------|----------------|---------------------------|---------------------|-----------------------------------------|
| Development of dual-color light-controlled gene expression  | E. coli        | CcaS/R green/red          | blue/dark           | (Senoo et al. 2019)                     |
| Development of a two-color light-controlled gene expression  | E. coli        | CcaS/R green/red          | blue/dark           | (Senoo et al. 2019)                     |
| Flux control between glycolysis and the methylglyoxal pathway | E. coli        | CcaS/R green/red          | blue/dark           | (Senoo et al. 2019)                     |
| Adaptation of CcaS/R gene circuit to B. subtilis—enhanced   | S. meliloti     | CcaS/R green/red          | blue/dark           | (Testard et al. 2019)                    |
| Regulation of glycolytic flux                               | E. coli        | CcaS/R green/red          | blue/dark           | (Testard et al. 2019)                    |
| Adapation of CcaS/R gene circuit to P. putida—PCB synthesis | P. putida      | CcaS/R green/red          | blue/dark           | (Hartsough et al. 2020)                  |
| Expression of colonic acid—secreted by E. coli in gut, affects C. elegans longevity | E. coli        | CcaS/R green/red          | blue/dark           | (Hartsough et al. 2020)                  |

Optogenetic system (base) and Light source on/off in brackets.
Table 1. Continued

| Application in bacteria | Bacterial host | Optogenetic system (base system) | Light source on/off | Reference |
|-------------------------|---------------|----------------------------------|--------------------|-----------|
| Development of a red-light gene control system based on Cph1 and histidine kinase EnvZ | E. coli | Cph8 (Cph1) | red/off/red | Levskaya et al. 2005 |
| Combination of direct and diffusion-based optogenetic control—dark/light edge detection by expression of black pigment | E. coli | Cph8 (Cph1) | red/dark/red | Habor et al. 2009 |
| Engineered Cph1-based histidine kinase library—control of gene expression | E. coli | Cph1 | red/dark/red | Ma et al. 2017 |
| Development of different TCS (controllable with light, nitrate, glucose) | E. coli | light | red/NIR | Schmidl et al. 2019 |
| Development of NIR-controlled GCS system (light)—smaller and packable in adenovirus | E. coli | iLight | red/NIR | Kaberniuk et al. 2021 |
| Development of a light-activatable split-intein T7-RNAP—control of lycopene synthesis | E. coli | PhyB/PIF3 Split-VMA intein | red | Raghavan et al. 2020 |
| Development of most red light shifted gene control system | E. coli | BphP1/PpsR2 | NIR/red | Xiong et al. 2018 |
| Second messenger conversion | | | | |
| First description of blue-light activatable adenylate cyclase (BlaC) from Beggiatoa—adaptation of guanylate cyclase (BlgC) for cAMP/cGMP synthesis | E. coli | BlaC (bPAC) | blue/dark | Ryu et al. 2010 |
| Blue-light activatable adenylate cyclase (bPAC) from the soil bacterium Beggiatoa—control of cAMP synthesis | E. coli | bPAC | blue/dark | Stierl et al. 2011 |
| cAMP synthesis module for Pseudomonas—as control of twitching motility and host cell infection (mouse) | P. aeruginosa | cPAC | blue/dark | Xie et al. 2021 |
| Development of cyanobacteriochrome-based photoswitchable adenylate cyclase (cPAC)—control of cAMP synthesis—LacZ expression as readout | E. coli | cPAC | blue/green | Wain-Hartung et al. 2018 |
| Conversion of c-di-GMP—characterization of a motaxis in Azospirillum brasilense | A. brasilense | Bps; EBS | NIR/blue | O’Neal et al. 2017 |
| Conversion of c-di-GMP—regulation of motility and biofilm in E. coli and chemotaxis in A. brasilense | E. coli; A. brasilense | Bps; EBS | NIR/blue | Ryu et al. 2017 |
| Conversion of c-di-GMP—bioprinting defined structures of bacteria biofilm | E. coli | Bps; EBS; BPhP1 | NIR/blue | Huang et al. 2018 |
| Conversion of c-di-GMP—control of biofilm for mitigating biofouling and water purification purpose | E. coli | Bps; EBS | NIR/dark | Mukherjee et al. 2018 |
| Synthesis of c-di-GMP—control of biofilm formation to catalyze the biotransformation of indole into tryptophan | E. coli | Bps | NIR/dark | Hu et al. 2019 |
| Post-translational control | | | | |
| Proof of concept for engineering light-regulatory activities by interface design at conserved allosteric sites—light-activatable dityrosine reductase | E. coli | AsLOV2 DHFR | blue/dark | Lee et al. 2008 |
| Development of optogenetic Cas9—allosteric inhibition by dimerization (dark), release and activation (light)—induced DNA cleavage activity | E. coli | RdLOV Cas9 | blue/dark | Richter et al. 2013 |
| Oligomerization of intein domains mediated through conformational change—functional protein self-splicing and activation | E. coli | AsLOV2 Npu intein | blue/dark | Jones et al. 2016 |
| Formation of cytosolic oligomers—control amyloidogenesis—control arrest of cell growth | E. coli | AsLOV2 WH1 | blue/dark | Cinaldo 2019 |
| Light-induced non-amyloidogenetic tools (LIPOPS) based on dimerization—control of cell death | E. coli | AsLOV2; CRY2 | blue/dark | He et al. 2021 |
| Light-controlled enrichment of proteins in cytosolic compartments formed by liquid-liquid phase separation—up to 15-fold enrichment of POI | E. coli | CRY2/CIB1s | blue/dark | Huang et al. 2020 |
| Optogenetic protein expressed on outer surface of bacteria—attachment to optogenetic interaction protein containing surface—control of biofilm formation | E. coli | Magnets | blue/dark | Chen and Wegner 2017; Chen and Wegner 2020 |
| Optogenetic protein expressed on outer surface of bacteria—control of aggregation, Hg²⁺ sensing and biofilm formation | E. coli | Magnets | blue/dark | Chen et al. 2020 |
| Split-recombinase for DNA modification | E. coli | VVD; Magnets Cre; Flp | blue/dark | Sheets et al. 2020 |
| Optogenetic mediated oligomerization of multimeric structures—assembly of dodecamer nitirase | E. coli | iLID | blue/dark | Yu et al. 2017 |
| Membrane tethering of essential component of the type III secretion system—spatiotemporally control of protein delivery into eukaryotic host cells | Y. enterocolitica | LOVTRAP; iLID SctQ | blue/dark | Lindner et al. 2020 |
| Membrane tethering of POI for protein purification | E. coli | iLID | blue/dark | Tang et al. 2021 |
| Optogenetic protein expressed on outer surface of bacteria—attachment, transport and release of cargo proteins | E. coli | PhyR/PIF6 | red/off/red | Sentürk et al. 2020 |
increase optogenetic controllability and provide an optochemical
gene expression system in E. coli (Baumenschlager, Rullan
and Khammash 2020). Based on a fusion of the red-light controllable PhyB-PIF3 dimerization switch with a split-intein and
a split-T7-RNAP, Raghavan and colleagues accomplished a red
light (∼660 nm)-induced dimerization that activated the intein
self-splicing effect and released the restored and functional T7-
RNAP. This application was established for the biochemical pro-
duction of lycopene in E. coli (Raghavan, Salim and Yadav 2020).

Finally, optogenetic control of gene expression can also be
achieved by influencing protein binding to RNA—the Nakamura
multispecies photoreceptor PAL combines a LOV sensor
domain and an RNA-binding domain to bind short RNA stem-
loops upon illumination, which decreases expression of the cor-
responding reporter gene up to 85% (Weber et al. 2019).

Two-component systems
Two-component systems usually consist of a membrane-bound
histidine kinase (HK) sensing an environmental stimulus and a
corresponding response regulator (RR) mediating a cellular
response. The first two-component optogenetic gene expression
module was designed by combining the photoreceptor Cph1
from Synechocystis sp. with the E. coli HK EnvZ. The resulting
light-controllable HK Cph8 allowed for red light-controllable
gene expression of LacZ, controlled by the cognate RR OmpR in
E. coli (Levskaya et al. 2005). This TCS was also used for con-
trolled LuxR expression within a bacterial edge detection setup,
where E. coli were engineered to detect the border between dark
and light conditions in a bacterial lawn, in analogy to edge
detection algorithms in image processing (Tabor et al. 2009).
In this landmark approach, expression of a black pigment was
induced by the combination of two factors: direct illumination
(more precisely the absence of darkness, implemented by the
dark-activated transcription of the λ-phage repressor cl, which
itself negatively controls expression of the transcription factor
LuxR) and proximity to darkness (indicated by presence of the
diffusible quorum signal and LuxR activator 3-oxohexanoyl-
homoserine lactone (AHL), produced by siblings in the dark)
(Tabor et al. 2009). Researchers highlighted the range of RR
DNA-binding domains within TCS by domain-swapping to cre-
ate, amongst others, a modified system with 1300-fold dynamic
range (Schmidl et al. 2019). Ma and colleagues used the photore-
ceptor Cph1 to develop an engineered HK library, consisting of 16
HK-RR TCS to serve as a toolbox for red-light controllable gene
expression in E. coli and other bacteria (Ma et al. 2017).

Based on the YtvA-based TCS YF1/FixJ, a one-plasmid sys-
tem was developed that allows blue-light mediated transcriptional
control in both directions, repression (pDusk) and activa-
tion (pDawn). In the latter, the insertion of the λ-phage repres-
or cl into the pathway inverts signal polarity and allows light-
activatable gene expression (Ohlendorf et al. 2012). Wang and
colleagues used the pDusk plasmid to achieve blue-light mediated
expression of lysin, a bacteriophage-derived protein disrup-
ting the bacterial cell wall, for controlled cell lysis in E. coli
(Wang et al. 2018). Other researchers modulated biofilm for-
mation by light-dependent expression of either the biofilm degrad-
ing phosphodiesterase PA2133 in P. aeruginosa (Pu et al. 2018)
or the outer membrane adhesin Ag43 in E. coli (Jin and Riedel-Kruse
2018). pDawn was further established as part of a synthetic tool-
box for bioengineering purposes in Vibrio natriegens (Tschirhart
et al. 2019) and for applications with a focus on biochemical pro-
duction (Box 1). Optogenetic regulation of gene expression with
TCS has also been applied in infection biology, where a blue
light-controllable sensor kinase, a chimera of a LOV domain and
the native sensor kinase GacS, was used to control the expres-
sion of virulence factors in P. aeruginosa and subsequently modu-
late infection in the model system Caenorhabditis elegans (Cheng
et al. 2020).

Box 1. Applying optogenetics for biological compound pro-
duction.
Optogenetic approaches are increasingly used to control
microbial industrial production, where a tight balance
between culture growth phase and bioproduction phase
is necessary to maximize production (reviewed in Pouzet
et al. 2020). Optogenetic control of bioproduction is often
based on light-controlled gene expression with the pre-
sented gene circuits pDawn (Ohlendorf et al. 2012), EL222
(Motta-Mena et al. 2014) or other TCS that enables a switch
between bioproduction and growth phase by changing the
light conditions (Senoo et al. 2019; W u et al. 2021). Light-
mediated applications in microbial bioproduction include
metabolites like muconic acid (W u et al. 2021), mevalonate
or isobutanol (Lalwani et al. 2021); important enzymes, such as
β-glucosidase (Chang et al. 2017); or antimicrobial drugs
(Sankaran et al. 2019). Some applications allow control of
the metabolic flux by switching between two different gly-
colytic pathways (Tandar et al. 2019) or between glycol-
sis and the methylglyoxal pathway (Senoo et al. 2019).
To automate and optimize optogenetic-driven bioproduction
in bacteria, bioreactor devices that allow cultivation, illu-
mination and real-time feedback control for the equilib-
rium between growth and production phase were devel-
oped (Milias-Argeitis et al. 2016; Wang and Yang 2017).
The optogenetic control of bioproduction is sometimes also
coupled to induced microbial lysis, to provide an efficient
release of the product into the medium (Miyake et al. 2014;
Chang et al. 2017).

One of the most widely used systems is CcaS/R, a modified
cyanobacterial TCS comprising a light-regulated HK that is acti-
 vated and deactivated in response to green and red light, respec-
tively, to phosphorylate its cognate RR and transcription activ-
ator CcaR (Tabor, Levskaya and Voigt 2011). This TCS was also
applied in other bacteria like the cyanobacterium Synechocys-
tis sp. (Abe et al. 2014), Pseudomonas putida (Hueso-Gil et al. 2020),
and Bacillus subtilis (Castillo-Hair et al. 2019), and further modi-
fied to achieve an increased dynamic range (up to 600-fold for
gene expression control in E. coli) by reducing leakiness under
‘off’ conditions (Ong and Tabor 2018). CcaS/R-mediated gene
expression control was used for diverse applications in bacteria
like the regulation of metabolic flux (Senoo et al. 2019; Tandar
et al. 2019) (see Box 1 for further details), light-controlled bac-
terial lysis (Miyake et al. 2014), easier harvesting of bacteria by
expression of a self-aggregating antigen (Nakajima et al. 2016),
and for colanic acid production in E. coli, where the optogenetic
control allowed for the controlled synthesis in the C. elegans
 gut, resulting in a beneficial effect on gut metabolism and longer
worm life (Hartsough et al. 2020).

TCS that can be controlled with ultraviolet (UV) light, such as
UirS/R (Ramakrishnan and Tabor 2016) were designed for a
wide spectral range of orthogonal light-controllable TCS and for
multichromatic control of gene expression (Tabor, Levskaya
and Voigt 2011; Schmidl et al. 2014; Fernandez-Rodriguez et al.
2017). To achieve an even more red light-shifted two-component-like
optogenetic gene control system, the Rhodopseudomonas bacte-
riophytochrome BphP1 and its cognate RR PpsR2 were modi-
fied; near infra-red light at ∼760 nm activates BphP1, which then binds the transcription repressor PpsR2 and activates gene expression in E. coli, while red light at ∼660 nm accelerates the return to the ‘off’ state (Cng, Olson and Tabor 2018).

Control of second messenger conversion

About ten years ago, a light-dependent adenylate cyclase (bacterial photoactivated adenyl cyclase, bPAC) containing a BLUF domain was identified and isolated from the soil bact-
erium Beggiatoa and integrated into E. coli for blue light-
dependent cyclic adenosine monophosphate (cAMP) synthe-
sis, a key tool for future investigations of signal transduc-
tion pathways (Ryu et al. 2010; Stierl et al. 2011). By mutat-
ing critical residues of this enzyme, Ryu and colleagues further developed a blue light-controllable guanylate cyclase (BgcC) for light-mediated synthesis of cyclic GMP (cGMP) in E. coli (Ryu et al. 2010). Light-dependent cAMP synthesis, mediated by bPAC, was later applied in P. aeruginosa to control twitching motility and host cell infection (Xia et al. 2021). The development of a cyanobacteriochrome-based photoswitchable adenylate cyclase (cPAC) that allows activation and deactivation with blue and green light, respectively, further extended the optogenetic tool-
box of controlled cAMP synthesis in bacteria (Blain-Hartung et al. 2018). To generate a regulatory c-di-GMP synthesis and degrada-
tion module, a NIR light-sensitive diguanylate cyclase (BphS) (Ryu et al. 2014) and a counteracting phosphodiesterase EB1 (Ryu et al. 2017), responding to blue light, were combined with the aim of controlling biofilm formation in E. coli in a bidirectional man-
er (Ryu et al. 2017; Mukherjee et al. 2018) and chemo-/aerotaxis in Azospirillum brasilense (O’Neal et al. 2017; Ryu et al. 2017). The resulting control over biofilm production was then in turn used to catalyze the biotransformation of indole into tryptophan (Hu et al. 2019). Huang and colleagues designed a similar optogenetic setup, using another phosphodiesterase (BlrP1) for blue light-
controlled c-di-GMP synthesis in P. aeruginosa to induce bioprinting of defined structures of bacterial biofilms with high spatial resolution (∼10 μm) (Huang et al. 2018).

Direct (post-translational) control of protein function by illumination

Beyond the control of gene expression or second messenger conversion, researchers become increasingly interested in using light to control the localization and function of matured target proteins. A key advantage of this approach is that by bypassing the time needed for gene transcription and protein expres-
sion, it offers faster and more direct control over biological processes, compared to optogenetically controlled gene expres-
sion (Liu et al. 2003). Whether this inhibition is a direct steric effect of RsLOV dimer-
ization or allosteric, a light-activatable dihydrofolate reductase (DHFR) was designed in E. coli, by coupling it to the photoactivat-
able LOV2 domain of Avena sativa. Upon blue light illumination, the LOV domain, located between two DHFR domains, undergoes a conformational change that leads to a small, but signifi-
cant (1.6–2.0-fold) change in DHFR catalysis rate (Lee et al. 2008). In a variation of this approach, Jones and colleagues fused a LOV domain to a split intein-extein module to achieve controlled self-splicing upon blue-light-triggered conformational change of the LOV2 linker protein, followed by the release of the functional extein (POI) (Jones, Mistry and Tavassoli 2016). The combination of split-inteins with LOV also allows trans-splicing, which can be used for the light-regulated recombination of mature fusion proteins (such as swapping a membrane-bound and a cytosolic protein domain) (Wong, Mosabir and Truong 2015).

Intermolecular control

Light-induced conformational change can be used to regulate the affinity to other proteins, which allows the optogenetic control of protein di- and oligomerization. To date, there are few examples where the interaction of more than two proteins was exploited: LOV2-dependent oligomerization of the prion-
like WH1 protein upon blue light illumination in E. coli was used to induce the irreversible formation of cytotoxic amyloid oligomers, which lead to growth arrest (Giraldo 2019), while the oligomerization of CRY2-linked receptor-interacting protein kinases upon illumination (termed light-induced non-apoptotic tools, LIPOPS) initiated pathways leading to killing of bacteria, as well as necroptosis and pyroptosis, which was used to reverse tumor progression in a mouse model (He et al. 2021).

Most post-translational optogenetic applications in bacteria are based on dimerization events, where one interacting pro-
tein (often referred to as bait) is reversibly recruited to its part-
ner (anchor) upon illumination. Dimerization can then directly influence protein activity, or can be used to relocalize a pro-
tein to its fixed interaction partner, which in turn can activ-
ate (recruitment) or inactivate (sequestration) the POI (Fig. 2). As mentioned earlier, engineering a protein to directly influ-
ence its activity by illumination requires knowledge about its structure-function relation, which so far has limited the num-
ber of applications. Using the RsLOV domain, Richter and col-
leagues developed a light-controllable version of the endonu-
clease Cas9, whose function is inhibited under dark conditions. Whether this inhibition is a direct steric effect of RsLOV dimer-
ization on Cas9, or caused by restricted binding of other cellular factors remained open. Illumination with blue light restores Cas9 function, which led to a DNA cleavage in E. coli (Richter et al. 2013), an approach with the potential to increase the spatiotem-
poral control of genome editing. Furthermore, optogenetic inter-
action switches were established that restore the function of split-proteins upon light-induced assembly of the interaction
partners. A light-induced dimerization tool of split recombinases based on VVD or Magnets was used to induce DNA modification events, such as the excision of a transcription terminator region and resulting induction of gene expression with blue light within two hours (Sheets, Wong and Dunlop 2020).

Several recent studies highlighted the use of optogenetics to control the intracellular localization of target proteins. An innovative approach applied light-induced phase separation (photoactivated switch in E. coli, PHASE) to increase the proximity of enzyme and substrate within the same phase and therefore facilitate enzymatic reactions. The system, based on the blue light dimerization switch CRY2-CIB1 (Liu et al. 2008), resulted in up to 15-fold enrichment of POIs and roughly two-fold enhanced rates of enzymatic reactions upon illumination (Huang et al. 2020). Even higher on/off rates can be achieved for systems targeting proteins that, at least temporarily, localize to the cytosol, and can therefore be inactivated by sequestration, e.g. to the bacterial membrane. By using the complementary blue light optogenetic interaction switches LOVTRAP (Wang et al. 2016) or iLID (Guntas et al. 2015) to tether or release an essential cytosolic component to and from the bacterial inner membrane, almost complete activation or suppression of the bacterial type III secretion system (T3SS) function upon illumination (light/dark ratio of secretion of > 50 and < 0.02, respectively) was achieved (Lindner et al. 2020). This light-induced translocation of effectors by sequestration of endogenous components of the T3SS (LITESEC-T3SS) was used for the light-controlled delivery of pro-apoptotic protein cargos into cancer cells (Lindner et al. 2020). iLID-based membrane tethering was also used in an optogenetic protein purification method (mem-iLID) to isolate the membrane-tethered POI with the membrane fraction in the light and then release it by incubation in the dark (Tang et al. 2021). Optogenetics is not restricted to the control of intracellular processes, but can also be used to target inter-bacterial or bacteria-cargo interaction. Expression of at least one component of optogenetic interaction switches on the outer surface of the bacterial membrane allowed controlling diverse biological activities. The Magnet system was used by Chen and Wegner to regulate biofilm formation through light-dependent cell-cell aggregation (Chen and Wegner 2017, 2020). In combination with a mercury-activated luciferase (Sciuto et al. 2019), this was applied for the sensing of mercury ions (Chen et al. 2020). The red/far-red interaction switch PhyB-PIF6 was used for the subsequent binding (upon red-light illumination), transport (by motile bacteria) and release (upon far-red illumination) of extracellular cargo linked to the other part of the dimerization switch (Sentürk et al. 2020). The above examples and further optogenetic applications in bacteria are listed in Table 1.

### TOOLS FOR OPTOGENETIC INVESTIGATIONS IN BACTERIA

Optogenetics greatly benefits from specific tools, both for the design and the practical implementation of the experiments. Although most optogenetic setups are tailored to the specific research demands and many labs develop their systems in a do-it-yourself manner (Pouzet et al. 2020), bioinformatic resources and physical devices designed to facilitate the investigation of biological processes with optogenetics in smaller environments like bacteria are increasingly being developed. Most resources and databases are open access and easy to adapt to individual demands. One of the most extensive and very intuitive optogenetic databases is optobase.org, which was founded by Weber and colleagues and provides manually curated information about optogenetic systems and their characteristics, publications (searchable by color range, base system or host organism), as well as additional tools such as an application search (Kolar et al. 2018). Other recommendable resources are the comprehensive Addgene guide on optogenetics (addgene.org/guides/optogenetics), and the Optogenetic Resource Center by the Deisseroth lab (web.stanford.edu/group/dlab/optogenetics). With the increasing interest in the application of optogenetics in bacteria, the toolbox of technical resources that are specific to or particularly useful in supporting such investigations has also been expanded. One of the first such platforms was presented by Gerhardt and colleagues, who engineered a light plate apparatus (LPA) to illuminate different culture setups in a 24-well plate format (Gerhardt et al. 2016). This setup was later improved by integrating microfluidics and an automated closed-loop feedback control system that allows independent control of samples in a 96-well plate (Soifer et al. 2021). Optogenetic control of bacteria down to the single-cell level was achieved by combining microfluidics with fluorescence microscopy in a computer-interfaced control setup that can visualize and control dynamic processes for up to 200–400 cells in four to eight fields of view (Chait et al. 2017). For larger culture volumes, Steel and colleagues developed Chi.Bio, an open-source platform that provides computational control of light and media parameters as well as temperature settings for the implementation of pre-designed and even feedback-controlled optogenetic experiments with bacteria up to a 25 ml culture scale (Steel et al. 2020). Other researchers presented mathematical and computational models that correlate optical inputs with output models like gene expression, allowing to accurately predict the output even for multiplexed systems and in turn to estimate the optimal illumination parameters for a desired output (Olson et al. 2014; Olson, Tzouanas and Tabor 2017). Pulsed illumination setups (Hennemann et al. 2018) and frequency modulating oscillation (Mahajan and Rai 2018) further support the fine-tuning of optogenetic systems. To bring light into living tissues, a crucial prerequisite for many biomedical applications of optogenetics, photoactivatable printed hydrogels or nanocarriers were developed, which can also be incorporated into the human body and guide light signals to certain sites of action. This technique allowed the remotely light-controlled drug expression and secretion by E. coli encapsulated in a hydrogel (Feng et al. 2020) and has been applied to treat ulcerative colitis (Cui et al. 2020) and for repression of subcutaneous tumors (Yang et al. 2020).

### CONSIDERATIONS FOR THE SUCCESSFUL APPLICATION OF OPTOGENETICS IN BACTERIAL SYSTEMS

The vast majority of optogenetic applications have been developed in eukaryotic systems. In the following, we therefore want to present core considerations for the successful implementation of optogenetics in bacteria. We will discuss specific limitations and challenges of optogenetic applications in bacteria and highlight areas of potential future applications of optogenetics in microbiology beyond currently established research tools. With this section, we want to inspire and support microbiologists to develop individual optogenetic setups and experiments for their specific biological question.
Light as a trigger for biological processes

Using light to control biological processes in bacteria comes with new challenges and considerations. To apply optogenetics to control biological processes with a high spatiotemporal resolution and dynamic range, specific aspects have to be taken into account, including the kinetics, directionality and sensitivity to ambient light of different optogenetic systems, as well as the wavelength-dependent penetration depth and possible phototoxicity. The choice of a suitable base system is therefore an important consideration.

Kinetics and directionality of optogenetic systems

Most optogenetic systems return to their ‘dark’ state within seconds to minutes after illumination ceases (Fig. 1). On the one hand, rapid deactivation of the system in the dark can be beneficial, for example in clinical studies where the bacteria need to be activated at a specific site, but relocation of the activated bacteria would lead to side effects. On the other hand, this feature can complicate long-term studies. Accordingly, the required duration and frequency of illumination is a key variable of different optogenetic base systems and an important factor for their successful application in bacteria. Constant illumination protocols are easier to set up and are mostly used to provide the highest possible binding/unbinding range and therefore achieve a strong on/off ratio. Pulsed illumination setups have the advantage of varying the optogenetic output by modulating light pulse-width or amplitude (Hennemann et al. 2018; Dietler, Stabel and Möglich 2019). In combination with a feedback control software, which measures system output like expressed protein levels, variation of illumination can be used to balance bacterial growth and bioproduction in order to maximize long-term yields (Endo and Ozawa 2017; Pouzet et al. 2020). Pulsed illumination can also decrease the risk of phototoxic effects on bacterial cultures. However, when applying pulsed illumination setups, users have to consider the fast off kinetics of some optogenetic systems (Fig. 1). Pulsed illumination with longer intervals can sometimes relax the control over biological processes, compared to constant illumination. Therefore, it is recommendable to screen for the best-suited illumination for each setup. The direction of the optogenetic switch also influences the need for illumination. In many settings, triggering an event by (short) illumination will be preferable to triggering it by darkness. Depending on the desired output, optogenetic switches that either dissociate or associate upon illumination are therefore preferable in such cases (see chapter “Addressing specific research questions with direct optogenetic control” for details).

Sensitivity to ambient light

Ambient light during cultivation and handling may partially activate optogenetic systems (Ochoa-Fernandez et al. 2020). Even the relatively low level of laboratory light can lead to residual activation of sensitive systems. In the few studies that explicitly tested the influence of ambient light, both iLID (Lindner et al. 2020) and VVD (Sheets, Wong and Dunlop 2020) were only activated to a low degree by ambient light, while LOVTRAP displayed stronger background activation (Lindner et al. 2020).

Phototoxicity

High-energy light at shorter wavelengths ranging from UV to blue can have damaging effects on eukaryotic cells (Gentile, Latonen and Laiho 2003) and many bacterial species (Yin et al. 2013; Pereira et al. 2014). Studies have shown an influence of blue light on growth for model organisms like B. subtilis or P. aeruginosa (De Lucca et al. 2012; El Najjar et al. 2020), as well as the light-induced production of carotenoid pigments in Myxococcus xanthus, induced by blue-light-generated singlet oxygen (Burchard and Dworkin 1966; Burchard and Hendricks 1969; Galbis-Martinez et al. 2012; reviewed by Padmanabhan et al. 2021). In most studies, however, the relatively low light intensities required for the activation of optogenetic systems did not influence bacterial growth, division and specific function. Nevertheless, this should be tested for each individual case.

The varying penetration depth of light of different wavelengths is worth considering as well, especially for the application of optogenetics in bulk volumes or in clinical samples. Red and near-infrared light generally penetrates biological tissues well (Cheong, Prahl and Welch 1990; Stolik et al. 2000; Jacques 2013) and is less absorbed by common growth media, which is advantageous in these cases.

Direct control of biological processes by optogenetics

While the application of optogenetics to control bacterial gene expression is becoming an established method, future applications can aim at a more direct protein-based control of biological processes (Brechun, Arndt and Woolley 2017) with widespread application potential: most proteins can in theory be controlled by structural changes, dimerization events (e.g. split-protein domains) or control of localization through optogenetics. The most widely used tool to control the function of initially non-light-dependent proteins or complexes are optogenetic interaction switches, which can be used to control the binding of a POI, fused to one domain of the interaction switch (the bait), to the other domain (the anchor) upon illumination. Some proteins are activated at a specific cellular localization, for example at the membrane (Levkaya et al. 2009 as an example in eukaryotes), or at the bacterial poles (Laloux and Jacobs-Wagner 2014). More often, forced relocation (sequestration) will inactivate the target protein or a complex that requires its presence. Release of the POI can then re-activate the target function. Pre-requisites for this approach are (a) the functionality and stability of a fusion of the POI to one of the optogenetic interaction domains, (b) an at least temporary presence of the POI in the cytosol to allow for sequestration and (c) a loss of function of the POI when tethered to the anchor protein. The latter is usually the case for proteins with an essential reaction/interaction interface that is blocked by the interaction with the anchor, or a specific site of action (e.g. as part of a protein complex with a specific localization) (Lindner et al. 2020). In eukaryotic systems, anchor proteins can be recruited to the cell membrane (Gil et al. 2020) or to organelles like mitochondria (Wang et al. 2016), the endoplasmic reticulum (He et al. 2017) or peroxisomes (Spiltoir et al. 2016). Bacteria are much less compartmentalized than eukaryotic cells; the primary target for optogenetic recruitment of cytosolic target proteins is therefore the bacterial membrane (Lindner et al. 2020; Tang et al. 2021).

Addressing specific research questions with direct optogenetic control

Compared to the optogenetic control of transcription, direct (post-transcriptional) optogenetic control requires a few additional considerations. In this paragraph, we want to delineate key aspects for planning and implementing experiments to directly control localization and activity of a POI by optogenetics.
While some of these points also apply to controlling transcription, readers who are specifically interested in this aspect should also refer to the information listed in Fig. 1 and the recent review by Baumschlager and Khammash (Baumschlager and Khammash 2021).

**Does the POI tolerate fusions, and where?**

The size of optogenetic interaction domains differs considerably (see Fig. 1 for details). For hetero-dimer interaction switches, the choice of which interaction domain is fused to either the POI or a localization target (e.g. a membrane anchor) may influence the success of the experiment. In most existing studies, the smaller interaction domain (e.g. Zdk1, SspB Nano, PIF) was fused to the POI (which may be sensitive to sterical hindrance by larger fused domains) and acts as a bait; accordingly, the larger interaction domain (AsLOV2, iLID, PhyB) was used as the anchor domain (Toetchter et al. 2011; Wang et al. 2016; Lindner et al. 2020; Tang et al. 2021). In line with this reasoning, Tang and colleagues did not observe binding of the optogenetic interaction switch, when the smaller interaction domain SspB was used as a membrane anchor and the larger interaction partner iLID was expressed as the cytosolic part (Tang et al. 2021). While many interaction domains can be freely combined at both termini, the orientation of the protein fusion may also influence the success of optogenetic control. As an example, Toetchter and colleagues noted that for the PIF6/PhyB switch, the larger interaction domain PhyB works best as an N-terminal fusion component, whereas the smaller domain PIF6 does not show any preference for N- or C-terminal fusions (Toetchter et al. 2011; Tang et al. 2021). Short, flexible peptide linkers (often 5–20 amino acids long and glycine-rich) between the interaction domain and the POI are frequently used to increase the chances to retain function of both the interaction switch and the POI. In any case, the expression level and stability of the fusion protein should be tested by Western blot, as flexible linker domains are subject to proteolysis in some bacteria.

**Where should the POI be anchored, and should this happen in the light or the dark?**

The incorporation of the anchor domain requires the fusion to a protein or motif that confers the desired location. For membrane tethering, an easy and robust option is to attach the anchor domain to an N-terminal transmembrane helix (Lindner et al. 2020; Tang et al. 2021). As for the bait protein, linker domains can increase the binding capacity of the anchor, and expression and stability should be confirmed. Obviously, the direction of the switch is important. Most optogenetic interaction switches have a dark ground state that can be influenced by illumination, and then recovers over time in the dark. Fortunately for users, systems that associate and that dissociate upon illumination both exist (e.g. iLID or Magnets for association and LOVTRAP for dissociation, see Fig. 1), which even allows to create parallel systems for light-dependent activation and deactivation of a target.

**How fast does the switch need to occur, how stable should the switched state be, and is light-regulated switching in both directions required?**

The kinetics of optogenetic control depend on the base system and the dynamics of the target system. Most optogenetic base systems react to light within a very short time (seconds and below), while their recovery in the dark differs widely, ranging from seconds to hours (Fig. 1). In several cases, mutant libraries with varying binding affinities have been developed, e.g. for the LOVTRAP and iLID systems (Wang et al. 2016; Zimmerman et al. 2016; Tang et al. 2021), which greatly improves both the range of possible applications and the possibility to troubleshoot and optimize applications (see below). If active bidirectional photo-switching is required, phytochrome- or cyanobacteriochrome-based systems (such as PhyB/PIF or Cph1), where association and dissociation of the interacting proteins are induced by illumination with different wavelengths, need to be used.

**Dynamic range**

Optogenetic interaction switches display affinity changes between the light and dark state; no switch features complete vs. non-existing binding in the respective states. The sequestration of a target protein to the membrane anchor therefore depends on expression levels and binding affinities of the interaction partners. While a low anchor/POI ratio or low affinity of the interaction domains may prevent the sequestration of a sufficient fraction of the cytosolic POI in the binding state, too much anchor or high affinity to the POI may lead to insufficient cytosolic concentrations of POI even in the unbinding state, and subsequently affect bacterial fitness or function of the POI. Determining the ‘sweet spot’ of anchor/cytosolic bait expression ratio is therefore instrumental for tight control over downstream processes within optogenetic dimerization switch applications (Lindner et al. 2020). Notably, there is an upper limit to the number of bait proteins that can be sequestered. In one of our studies, a protein present in more than 1000 copies could be efficiently membrane-anchored at an anchor expression level that could still be increased > 4-fold without visible detrimental effect on the bacteria ([Lindner et al. 2020] and unpublished), suggesting an upper limit of at least several thousand bait proteins. However, this number will strongly depend on the optogenetic base system used, the expression system and of course the host bacterium. For proteins with a large copy number, phase separation-based systems (Huang et al. 2020) might offer an alternative, although their capacity and dynamic range remain to be determined. While suitable pre-experiments (see below) can help to estimate the dynamic range of a base system and anchor in a given bacterial species, adjustments to the final experiments may be required for best results (see ‘Troubleshooting and optimization below’). Notably, such adjustments are greatly facilitated by a tunable anchor expression system and a base system featuring versions of different affinity. For example, efficient sequestration of a natively expressed cytosolic protein to the membrane was only possible using the V416L version of the LOVTRAP AsLOV2 protein (Kawano et al. 2013; Lindner et al. 2020), while screening and even directed mutagenesis of iLID SspB versions allowed Tang and colleagues to optimize light-controlled protein purification (Tang et al. 2021).

**Are there any restrictions to the wavelengths that can be used?**

While most bacteria appear to tolerate the low light intensities required for optogenetic experiments well, sensitive species can be harmed, especially by blue light. When working with these species or for experiments in animal tissue or bulk volumes, red- to infrared-sensitive systems may be preferable due to the lower photon energy and (generally) deeper penetration of light in this part of the spectrum (also see “Light as a trigger for biological processes”, above). For experiments involving additional fluorescent proteins or dyes, the compatibility of
their excitation/emission wavelength with the optogenetic control also has to be taken into account. For example, optogenetic systems activated by blue light should not be combined with GFP: the excitation wavelength of GFP (∼488 nm) overlaps with the activation spectrum of most blue-light-activated systems (often a broad peak from 400–500 nm, e.g. for LOV2 (Salomon et al. 2000)), which could lead to cross-activation of the optogenetic system upon analysis of the fluorescent protein. Similarly, red fluorescent proteins such as mCherry (excitation and emission peak at ∼587 nm and ∼610 nm, respectively) may overlap with cyanobacteriochrome- and phytochrome-based optogenetic systems. Studies have therefore mostly used mCherry as a read-out for green light-controlled optogenetic systems (such as Magnets (Baumschlager, Aoki and Khammash 2017; Chen and a read-out for green light-controlled optogenetic systems (such as Magnets (Baumschlager, Aoki and Khammash 2017; Chen and a read-out for green light-controlled optogenetic systems (such as Magnets (Baumschlager, Aoki and Khammash 2017; Chen and a read-out for green light-controlled optogenetic systems (such as Magnets (Baumschlager, Aoki and Khammash 2017; Chen

**BEYOND THE HORIZON—FUTURE OPPORTUNITIES OF OPTOGENETIC APPLICATIONS IN BACTERIA**

After a slow start, the application of optogenetic principles to control bacterial functions is gaining speed rapidly. A broad PubMed search for ‘optogenetics bacteria’ already brings up 407 hits, more than half of which have been published in the last four years. Similarly, a search for ‘host bacteria’ in the manually curated OptoBase database yields 92 papers, 66 of which have been published since 2017.

While optogenetic control of gene expression and, increasingly, the direct control of protein function by light have been established in bacteria, other approaches that were put forward or already implemented in eukaryotes have not yet been applied in prokaryotes. In this last paragraph, we want to highlight some examples to demonstrate the widespread potential of optogenetic applications in bacteria and potentially inspire readers to think beyond the horizon.

Eukaryotic optogenetic applications that aim at light-dependent protein degradation by proteasome recruitment (Renicke et al. 2013; Baaske et al. 2018) have been established for years and could be considered for optogenetic applications in bacterial species like *Mycobacterium* or *Streptomyces*, which also carry proteasomes (Becker and Darwin 2017). Furthermore, the development of light-controlled proteases based on the bacterial SarA-SmpB system (Karzai, Roche and Sauer 2000) or the direct influence on degron systems, where a protein degradation sequence (degron) is caged by a photoactivatable domain (Usherenko et al. 2014; Mondal et al. 2019), could be considered. For the direct optogenetic control by sequestration of cytosolic proteins, the bacterial membrane was so far used as the main sequestration point, due to its large surface and relative ease of anchoring of proteins (Lindner et al. 2020; Tang et al. 2021). Applying phase separation (Huang et al. 2020) may provide an alternative, although so far, the dynamic range of activation/deactivation was relatively low. Future applications can also consider the light-dependent recruitment of a target protein to subcellular compartments such as polar protein structures like PopZ or HubF (Surovtsev and Jacobs-Wagner 2018). To overcome the limited range of blue light in tissues or large cultures, upconversion nanoparticles that can convert near-infrared to blue light (Chen et al. 2014) might be used for the application of blue light-controlled systems (which make up the majority of both optogenetic base systems and applications) by far-red light. There are still only few examples for engineered intramolecular optogenetic systems beyond the TCS used for transcriptional control (Lee et al. 2008; Wong, Mosabhir and Truong 2015; Jones, Mistry and Tavassoli 2016). Future applications could aim at controlling signaling cascades by modifying different kinases (Leopold, Chernov and Verkhusha 2018) or phosphatases (Hongdusit et al. 2020; Hongdusit and Fox 2021). Arroyo-Olarte and colleagues proposed to investigate host-pathogen interactions using optogenetic control, such as a modified inositol phosphatase which might alter the host phosphoinositide level by light during infection processes like induced phagocytosis caused by *Yersinia* or *Listeria* (Arroyo-Olarte et al. 2018). A review collection of optogenetic applications in eukaryotes that cover subcellular organization (Kichuk, Carrasco-López and Avalos 2021), signal transduction (Mühlhäuser et al. 2017) or widespread utilizations and design strategies (Brechun, Arndt and Woolley 2017; Endo and Ozawa 2017; Oh et al. 2021) can also be considered as an information and inspiration base for new developments in bacteria.

**Requirement and availability of cofactors**

Optogenetic switches that are based on cryptochromes, BLUF- or LOV-domains require flavin cofactors (FMN, FAD) that are ubiquitous and present in sufficient concentrations in most bacteria (Entsch and Ballou 2013). Other cofactors like phycocyanobilin (PCB) or biliverdin, which are needed for optogenetic switches based on phytochromes or cyanobacteriochromes are not necessarily present in bacteria and often have to be co-synthesized (Ma et al. 2020; Uda et al. 2020) (Fig. 1). In the case of phycocyanobilin (PCB), a cofactor for several systems in the red/far-red range, this is ensured by the integration of two synthesis enzymes Ho1 and PcyA, which catalyze the transformation of protoheme to biliverdin and its subsequent reduction to phycocyanobilin, respectively (Gambetta and Lagarias 2001; Ma et al. 2020). It was shown that PCB can affect the biomass of some bacteria at higher concentrations (Raghavan, Salim and Yadav 2020), suggesting toxicity, which should be considered for the respective systems.

**Preparatory experiments**

To establish an optogenetic system and estimate its parameters in a given host bacteria, it is recommendable to perform preliminary experiments. As an example, the binding of a (compatible) fluorescent bait protein to the membrane anchor can be used to test the function of the system for a given anchor construct, expressed at different levels. The dynamics of tethering and release events can then be directly visualized by fluorescence microscopy (Lindner et al. 2020). Preparatory experiments are especially useful prior to complex, costly, and/or long-term experiments, and may be skipped otherwise.

**Troubleshooting and optimization**

The main variables for troubleshooting and optimization, both for the preparatory and main experiment, are (i) illumination settings, (ii) the anchor/bait ratio, and, if possible, (iii) variants of the base system with different affinities for the anchor. While problems arising from incomplete binding of the interaction partners call for stronger illumination, higher anchor/bait ratio, and/or the application of variants with higher affinity, the opposite problem, detrimental binding under ‘off’ conditions, may be counteracted by lower (or pulsed) illumination, lower anchor/bait ratios, and/or less affine protein variants.

Fig. 3 summarizes a suggested workflow and main considerations for direct optogenetic control experiments.
Given the great potential of optogenetics, its obvious advantages for non-invasive, highly precise interventions, and the ever-expanding toolkit, it will be exciting to watch the development and use of optogenetic systems in prokaryotes to improve both our understanding of bacterial processes and their application in basic research, biotechnology, and medicine.

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Conflicts of interest. AD and FL are co-authors on a patent application relating to the use optogenetic switches to control bacterial functions, such as protein translocation into eukaryotic host cells.

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