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Optogenetics Illuminates Applications in Microbial Engineering

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Keywords

optogenetics, microbial engineering, dynamic control, metabolic engineering, cybergenetics

Abstract

Optogenetics has been used in a variety of microbial engineering applications, such as chemical and protein production, studies of cell physiology, and engineered microbe–host interactions. These diverse applications benefit from the precise spatiotemporal control that light affords, as well as its tunability, reversibility, and orthogonality. This combination of unique capabilities has enabled a surge of studies in recent years investigating complex biological systems with completely new approaches. We briefly describe the optogenetic tools that have been developed for microbial engineering, emphasizing the scientific advancements that they have enabled. In particular, we focus on the unique benefits and applications of implementing optogenetic control, from bacterial therapeutics to cybergenetics. Finally, we discuss future research directions, with special attention given to the development of orthogonal multichromatic controls. With an abundance of advantages offered by optogenetics, the future is bright in microbial engineering.

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1. INTRODUCTION

The applications of optogenetics have evolved over the last two decades from its early roots in neuroscience (1). Originally, optogenetics referred to the use of light to initiate neuronal perturbations using genetically encoded photoreceptors (2). These perturbations in tandem activate fluorescent proteins, enabling visualization of cellular activities such as gene expression, protein fate, and apoptosis (3). However, optogenetics has transcended neuroscience, and a variety of additional fields have benefited from its unique capabilities. Today, optogenetics more broadly uses genetically encoded photoresponsive proteins to facilitate light-activated control of cellular processes in general and has found applications in diverse fields ranging from developmental biology and cell signaling to microbial engineering.

The advantages of light-activated control explain why optogenetics has been adopted so rapidly in various fields. In addition to being reversible and having low toxicity, optogenetics offers tunable precise spatiotemporal control (4). Unlike chemical inducers, light is noninvasive, unaffected by diffusion, and orthogonal to endogenous cellular activity. It causes little to no unwanted side effects with minimal cross talk and is not subject to cellular uptake requirements, degradation, or secretion (5). In addition, light duty schedules can be used to modulate activation of the photoresponsive proteins and their downstream effects. As a result, optogenetics can potentially control cellular functions with minimal off-target effects (6).

Beyond neurons and mammalian cells, optogenetic controls have been established in both prokaryotic and eukaryotic microbes. Many systems have been developed in model organisms such as *Escherichia coli* (7), *Bacillus subtilis* (8), and *Saccharomyces cerevisiae* (9). Most systems are designed to establish light-activated gene expression and repression, which can then be applied for transcriptional control of desired cellular functions. Translational and posttranslational controls offer unique benefits of faster response times but remain comparatively less developed (10). Overall, optogenetic systems use many different photoresponsive proteins activated in wavelengths ranging from near-infrared to ultraviolet light (11). However, most systems that have been developed respond to blue light as the chromophores they require are flavin nucleotides, which are naturally ubiquitous in all cells and thus do not need to be produced synthetically by the cell or added to growth media. Nevertheless, the array of available wavelengths grants flexibility in designing optogenetic systems and opens the possibility of establishing orthogonal controls of multiple cellular functions using multichromatic circuits. This expansive repertoire offers vast potential in investigating and advancing microbial engineering.

This article provides a summary of the optogenetic tools developed for microbes, with an emphasis on applications. For more information on optogenetic systems in general, previous reviews have discussed near-infrared responsive systems (12), light-oxygen-voltage (LOV) domains (13), and additional photoresponsive proteins (6, 11, 14–17). Although systems to control microbes with light have been developed using photocaged molecules (18–20), they are not covered in this review, as their light-responsive components are not genetically encoded. We first briefly introduce the wide array of optogenetic tools available and then discuss the applications of these tools in microbial engineering in more depth. The applications have been organized by the following research areas: metabolic engineering and protein production, microbial growth and cooperative behavior, cell physiology, biomedical applications, and cybergenetics. Lastly, we examine the challenges photoresponsive microbial systems encounter and explore future research directions on the application of optogenetics to microbial engineering.

2. OPTOGENETIC SYSTEMS FOR MICROBIAL ENGINEERING

Several optogenetic tools have been developed to control microbes with a variety of light wavelengths (**Table 1**). Although different in their mechanisms and applications, all of these systems

Table 1 Summary of microbially applied optogenetic systems

Response wavelength	System	Mechanism	Microorganism	Level of control	Specific circuits/tools	Additional references
Ultraviolet-violet/green	UirS–UirR	Kinase and response regulator	*Escherichia coli*	Transcriptional		31
	UVR8/COP1	Heterodimerization	*Saccharomyces cerevisiae*	Transcriptional		142
Blue	EL222	Homodimerization	*E. coli*	Transcriptional	BLAT/BLRT (67)	
					opto-CRISPRi (63)	116
			Sinorhizobium meliloti			77
			S. cerevisiae		OptoEXP (59)	100, 101, 132
					OptoINVRT (59, 60)	
					OptoAMP (61) OptoQ-AMP/ INVRT (62)	
				Posttranslational		41
	YF1/FixJ	Kinase and response regulator	*E. coli*	Transcriptional	pDawn/pDusk (28)	66, 81, 114, 115
					OptoLAC (64)	
					OptoTA (91)	
			Pseudomonas aeruginosa			74
			Lactococcus lactis			114
	AsLOV2	Conformational change either activates or inactivates chimera	*S. cerevisiae*	Genome engineering	LiCre (47)	35, 99
				Posttranslational	LANS (34)	
					LOVTRAP (143)	
					CLASP (36)	
					LINX (97)	
					LINuS (33)	
					iLID (40, 144)	
					TULIPs (37)	
			P. aeruginosa			117
	CRY2/CIB1	Heterodimerization	*S. cerevisiae*	Transcriptional		25, 126, 131
	CRY2	Oligomerization	*S. cerevisiae*	Posttranslational	OptoDroplets/ OptoClusters (40)	
	Magnet	Heterodimerization	*E. coli*	Transcriptional	Opto-T7RNAP (145)	
				Posttranslational		79
	VVD	Homodimerization	*S. cerevisiae*	Transcriptional	yLightOn (107)	
			E. coli	Genome engineering		45
	VVD/WC-1	Heterodimerization	*S. cerevisiae*	Transcriptional	FUN-LOV (27)	
					HAP-LOV (146)	
	PixD/PixE	Oligomerization	*S. cerevisiae*	Posttranslational	PixELLs (38, 147)	
	PAL	RNA binding	*E. coli*	Translational		148
	bPAC	Photoactivated nucleotidyl cyclase	*E. coli*	Posttranslational	BlaC/BlgC (42)	119
			P. aeruginosa			118
			Toxoplasma gondii			149

(*Continued*)

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Table 1 (*Continued*)

Response wavelength	System	Mechanism	Microorganism	Level of control	Specific circuits/tools	Additional references
Green/red	CcaSR	Kinase and response regulator	*E. coli*	Transcriptional		30, 65, 66, 113, 128, 150–152
			Bacillus subtilis			8
Red	Cph-OmpR	Kinase and response regulator	*E. coli*	Transcriptional		7, 66, 87, 151, 152
Red/far red	PhyB/PIF3	Heterodimerization	*S. cerevisiae*	Genome engineering	L-SCRaMbLE (46)	
				Transcriptional	PhiReX (153)	127
				Posttranslational	DeLight (154)	32, 155, 156
Near-infrared	IsPadC	Homodimerization	*E. coli*	Transcriptional	iLight (23)	
	BphP1–PpsR2	BphP1 binds PpsR2	*E. coli*	Transcriptional		157
	BphS/BphO	Bacteriophytochome c-di-GMP synthase	*E. coli*	Transcriptional	NRAT (67)	75
				Posttranslational		43, 44
			P. aeruginosa	Posttranslational		80

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consist of at least one light-responsive protein that is activated when exposed to a specific range of wavelengths. The wide array of systems, combined with their diverse mechanisms, has set a strong foundation for the development of tools that operate at the transcriptional, posttranslational, and even genomic levels.

Transcriptional regulation is the most common level of optogenetic control used in engineered microbes. Transcriptional circuits can be divided into one- and two-component systems. One-component systems are composed of a single protein that is both the light receptor and transcriptional inducer (**Figure 1*a***). A key example is the photoresponsive EL222 protein from *Erythrobacter litoralis*, which forms homodimers when exposed to blue light (21). When activated, EL222 binds to a cognate DNA sequence, which can lead to either activation or repression of transcription depending on the location of the recognition site as well as whether or not the protein is fused to an activation domain (22). Because one-component circuits require expression of only one protein, they benefit from simplicity and small size. In addition, their activity does not depend on adequately balanced expression of a second component. Despite these advantages, one-component systems had been developed only for blue light until recently. A near-infrared one-component system evolved from a bacterial phytochrome, called iLight, was recently reported in *E. coli* with promising future capabilities (23).

Two-component systems have been developed for a wider array of wavelengths. Unlike their one-component counterparts, two-component systems require interaction of two different proteins to receive light and induce a response. These systems operate by forming heterodimers, such as PhyB/PIF3 (24), CRY2/CIB1 (25), Magnet (26), and VVD/WC-1 (27) (**Figure 1*b***). In some systems, each component is fused to either a DNA-binding or activation domain, which come together to form a functional transcription factor in the light, such as in the yeast FUN-LOV (fungal-LOV) system (**Figure 2*a***). Other two-component systems developed for microorganisms consist of a light-responsive kinase and a cognate transcriptional response regulator, as in the case of YF1/FixJ (28), CcaSR (29, 30), and UirS/UirR (31) (**Figure 1*c***). Changes in light conditions affect the activity of the kinase and thus the phosphorylation state of the transcriptional regulator, which determines its ability to activate transcription. Most systems use the transcriptional response regulator to directly induce expression of the gene of interest. However, the pDawn system

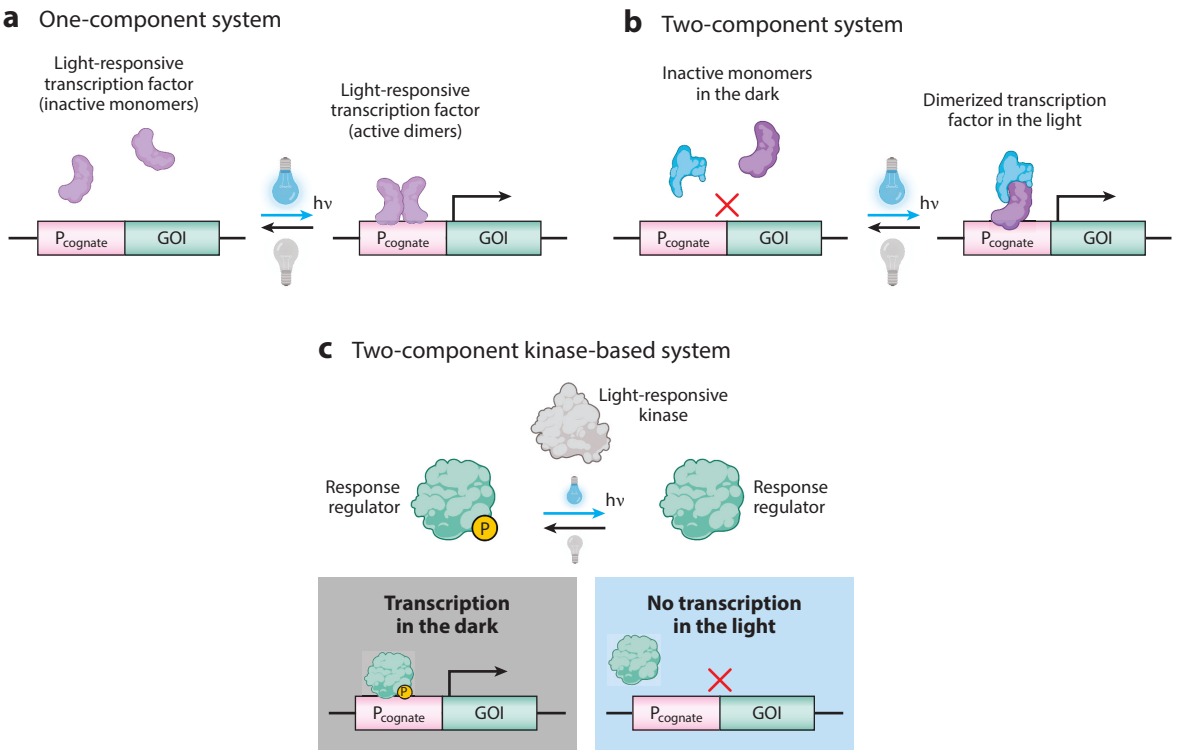

Figure 1

Schematic of one- and two-component transcriptional systems. (*a*) One-component systems use a single light-responsive protein to induce transcription. Examples include the EL222 and VVD systems, both of which operate in blue light. (*b*) Two-component dimeric systems form heterodimers in the light to create a functional transcription factor. The red/far-red PhyB/PIF3 and the blue-light Magnet proteins fit in this category. (*c*) Two-component kinase-based systems rely on a photosensitive kinase that phosphorylates a response regulator in the dark to induce transcription. Examples include the green/red CcaSR and the blue-light YF1/FixJ systems. Abbreviation: GOI, gene of interest. Figure adapted from images created with BioRender.com.

derived from YF1/FixJ uses it to express a second transcriptional regulator, the cI repressor, which controls expression of the gene of interest, thus inducing an inverted response to light (**Figure 2*b***). A major advantage of two-component systems is that they have been developed for a wide range of activation wavelengths. However, the need to express two proteins and the system sensitivity to their relative expression levels can complicate their use. Another important consideration is that systems activated by wavelengths longer than blue light require chromophores such as biliverdin and phycocyanobilin, which are not endogenous to many microorganisms and must be added to culture media or synthetically produced by the host. Despite these challenges, two-component systems are exceptionally valuable for their flexibility in architecture and choice of wavelength, which offer advantages in many applications.

Beyond transcriptional circuits, optogenetic systems have also been developed to control cells at the posttranslational level. A common strategy is to control the subcellular localization of proteins with light. Several systems use the blue light–responsive AsLOV2 domain from *Avena sativa* to either hide or expose a localization signal of a chimeric protein to direct them to the nucleus, cytosol, or membrane (32–36). Light-responsive proteins have also been used to form protein complexes via inducible protein binders (37) and dynamic enzyme clusters (38–40). Aside from

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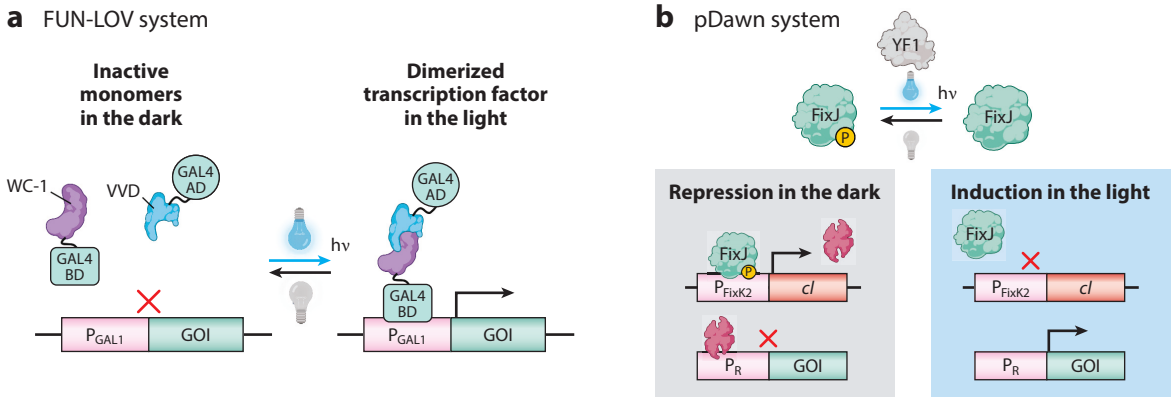

Figure 2

Two examples of two-component transcriptional systems. (*a*) The *Saccharomyces cerevisiae* FUN-LOV system is a dimerizing two-component system that uses a mechanism similar to a yeast two-hybrid assay. In this system, the photoresponsive VVD and WC-1 elements are linked to the GAL4 DNA binding and activation domains, respectively. These two units dimerize in blue light to form a functional transcriptional activator that induces the P_{GAL1} promoter. (*b*) pDawn is a commonly used two-component system consisting of a light-responsive kinase and its response regulator. This circuit uses the YF1/FixJ system to express another transcriptional regulator, which induces an inverted response that activates transcription in blue light. Abbreviation: GOI, gene of interest. Figure adapted from images created with BioRender.com.

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controlling localization, other posttranslational tools exist to induce protein degradation in the light and even regulate the intracellular concentrations of second messengers like cAMP and c-di-GMP (41–44). Thus, many different strategies exist for introducing optogenetic control at the posttranslational level.

Optogenetic tools have also been used for cellular regulation at the genomic level in yeast and bacteria. Several light-inducible recombinases have been developed, which excise a portion of the genome when exposed to light. Most of these systems use a split recombinase strategy, in which two pieces of the enzyme are fused to dimerizing optogenetic components such as VVD in *E. coli* (45) or PhyB/PIF3 in *S. cerevisiae* (46). When exposed to light, the dimerized components form a functional recombinase capable of genomic modifications. Another type of light-responsive recombinase developed in yeast, LiCre, is composed of a single protein chimera in which AsLOV2 is N-terminally fused to a truncated Cre recombinase (47). The light-induced conformational changes of AsLOV2 cause this recombinase to be active when exposed to blue light and inactive in the dark. Together with transcriptional and posttranslational tools, these systems provide a strong and versatile platform for microbial engineering for a variety of applications.

3. APPLICATIONS OF OPTOGENETIC SYSTEMS

3.1. Applications of Optogenetics in Metabolic Engineering and Protein Production

Microbial fermentations for chemical and protein production are commonly operated in two temporal phases to decouple cell growth from production. Frequently, biosynthetic pathways of interest compete with cellular growth for common resources or metabolites, leading to impaired biomass accumulation and product yields (48). To decouple these processes, genes controlling growth and production can be dynamically controlled by changing carbon sources (49–51), depleting nutrients (52, 53), or adding chemical inducers (54–57). These approaches have been

used successfully for decades to control microbial fermentations for improved yields and titers. However, these strategies rely by definition on media composition, which may be difficult and costly to adjust, especially when using real-world complex substrates. These methods also impede highly tunable and reversible induction, which would greatly improve the ability to balance metabolic pathways to improve production. Furthermore, chemical inducers can cause unintended side effects in cells, be metabolized, or be pumped out of the cell, reducing their efficacy (58). Therefore, the benefits derived from improved tunability, reversibility, and orthogonality require new methods of dynamic control that optogenetics can provide.

Optogenetic circuits have been developed to enhance light control of engineered metabolisms. To date, transcriptional optogenetic circuits for metabolic engineering have been developed for three main purposes: (*a*) to directly induce gene expression with light, (*b*) to invert the transcriptional response of the native system, and (*c*) to amplify the transcriptional response for higher levels of gene expression (**Figure 3**). The two circuit types of light induction and dark induction offer bidirectional control in which different sets of enzymes can be induced in opposite light conditions. This capability can be used to decouple growth and production by placing an essential enzyme under a light-activated circuit and a production enzyme under an inverter circuit (**Figure 4*a***). Optogenetic amplifier circuits aim to boost the transcriptional response and sensitivity to light to address challenges of light penetration in high-cell-density fermentations. With their diverse capabilities, optogenetic circuits have been developed with both one- and two-component systems for applications in metabolic engineering.

The first demonstrations of optogenetics in metabolic engineering used the VP16-EL222 one-component system in *S. cerevisiae*. The simplest application used VP16-EL222 to directly control expression of genes of interest with light using the OptoEXP circuit (59) (**Figure 3*a***). However, motivated by concerns of light penetration, a set of inverter circuits (OptoINVRT) were developed to exploit darkness as the inducing agent in high-cell-density fermentations (59, 60). Harnessing the GAL regulon, OptoINVRT circuits induce the P_{GAL1} promoter in the dark by directly controlling expression of its Gal80p repressor in blue light with VP16-EL222 (59, 60) (**Figure 3*b***). OptoEXP and OptoINVRT circuits were used simultaneously to dynamically balance growth and chemical production in two-phase fermentations operated with periodic light pulses. This was achieved by using OptoEXP to control *PDC1* (in a triple *pdc1/pdc5/pdc6* deletion strain), an essential gene that diverts most glucose toward ethanol production. In addition, genes in biosynthetic pathways of interest that compete with *PDC1* were placed under the control of OptoINVRT circuits, including the genes for lactate dehydrogenase (*LDH*) and acetolactate synthase (*ILV2*) for the production of lactic acid and isobutanol, respectively. These fermentations achieved high production of lactic acid and the highest titers and yields of isobutanol (in the peer-reviewed literature) and 2-methyl-1-butanol recorded in yeast (59, 60). To demonstrate the scalability of optogenetic controls in microbial fermentations, OptoEXP was used to robustly and homogeneously induce green fluorescent protein (GFP) in a 5-L bioreactor at cell densities of up to 50 OD_{600} (59). Using an amplifier circuit achieved even higher levels of GFP induction with only ~1% of the light dose applied to the bioreactor (61). These results demonstrate the value of using optogenetic circuits for bidirectional control of growth and production while exploiting the reversibility afforded by periodic light pulses in metabolic engineering applications.

Whereas the OptoEXP and OptoINVRT systems enable two-phase fermentations in which production occurs in the dark, the more recently developed light-activated amplifier circuits provide the new capability of light-induced production (61). The original amplifier circuits, known as OptoAMP, also harness the yeast GAL regulon to achieve at least 23-fold-higher light sensitivity than OptoEXP. These circuits use wild-type or light-hypersensitive mutants of EL222 to regulate the expression of the strong Gal4p transcriptional activator, which then

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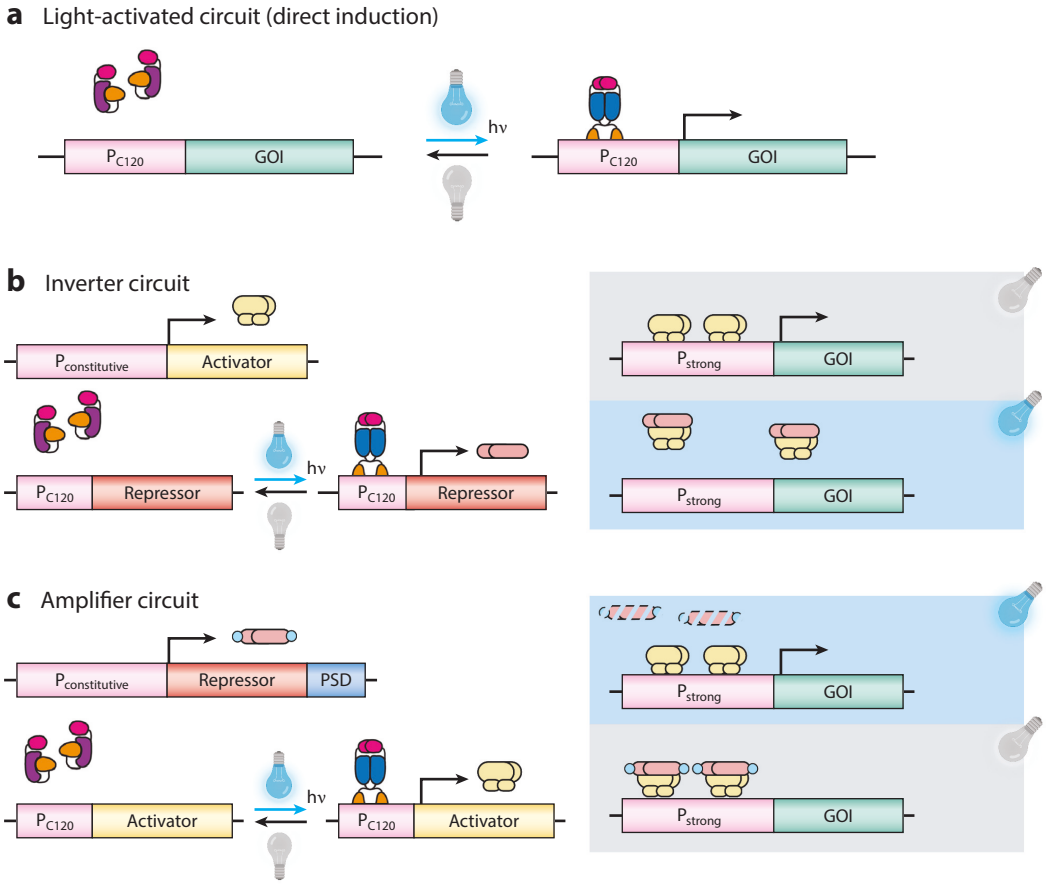

Figure 3

Three types of transcriptional circuits for metabolic engineering. The architecture of three circuit types is shown using the blue light–responsive EL222 system as an example. (*a*) The simplest implementation of this system directly controls the gene of interest (GOI) with the C120 promoter. VP16-EL222 binds to this promoter in blue light, inducing a relatively weak transcriptional response. Panel adapted from Zhao et al. (59). (*b*) Inverter circuits reverse the response to induce expression in the dark rather than in the light. The GOI is controlled by a strong promoter, whereas the C120 promoter drives transcription of its repressor. The repressor is produced in the light, causing the GOI to be induced only in the dark. (*c*) Amplifier circuits reverse the logic of the inverter circuits to provide strong activation in the light. The C120 promoter induces an activator in the light, whereas the repressor is expressed constitutively. Fusing this repressor to a photosensitive degron (PSD) maximizes expression in the light while minimizing leakiness in the dark. Panel adapted from Zhao et al. (61). Figure adapted from images created with BioRender.com.

induces expression of genes of interest from wild-type or engineered versions of the P_{GAL} promoters (**Figure 3*c***). The ability to induce metabolic pathways with light using OptoAMP circuits makes it possible to operate bioreactors in three or more temporal phases (instead of the traditional two-phase fermentations), each characterized by a unique light duty cycle (**Figure 4*b***). To demonstrate this new capability, three-phase light-controlled fermentations were used to control biosynthetic genes for the biosynthesis of isobutanol, lactic acid, and naringenin, boosting production. One disadvantage of the original OptoAMP circuits is that they cannot be used simultaneously with OptoINVRT in the same strain, because both systems exploit the GAL regulon. This challenge was addressed, however, by the development of OptoQ-AMP and

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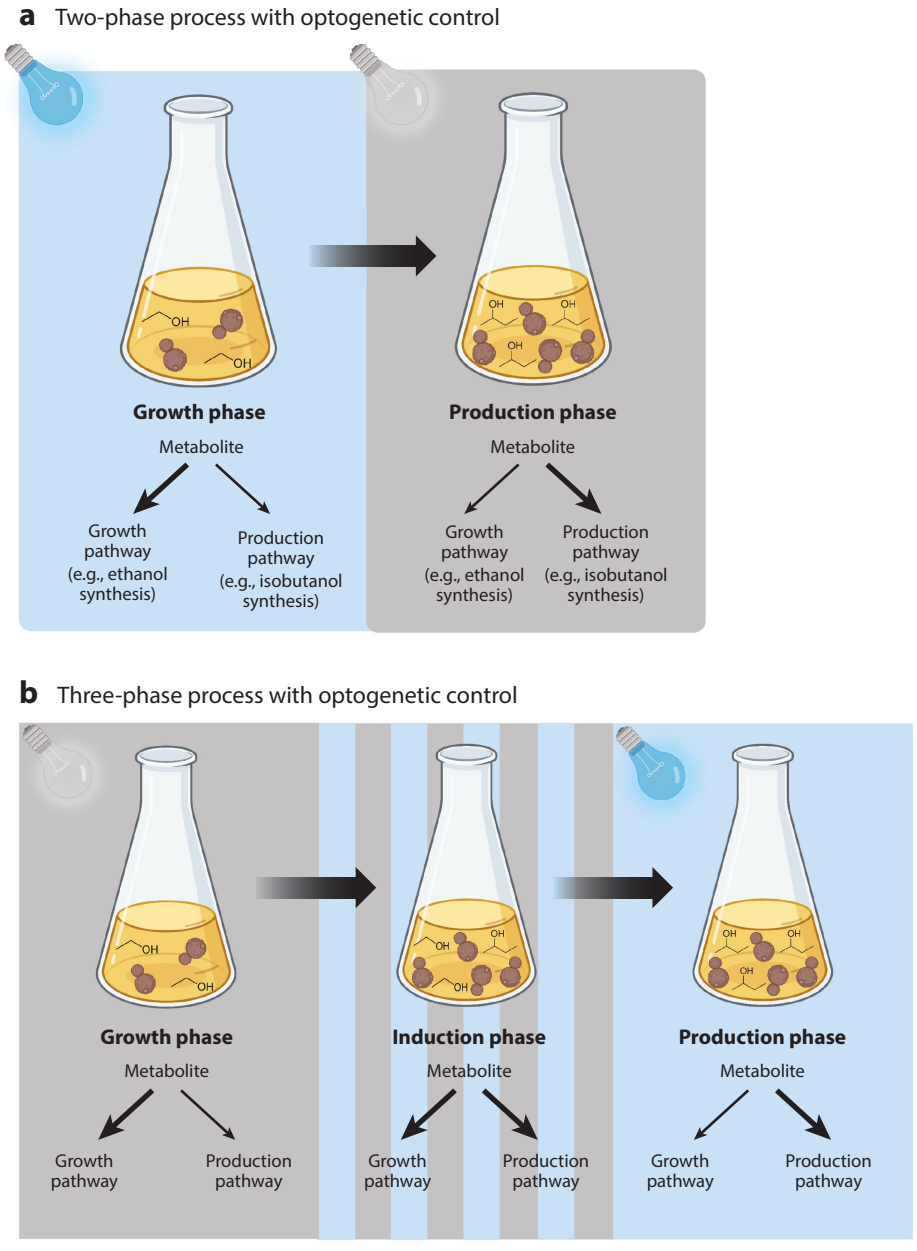

Figure 4

Optogenetic control of multiphase fermentations. (*a*) The current paradigm for dynamic control uses a two-phase process to decouple growth and production. Optogenetic circuits can be applied to fit this model in the form of a light-driven growth phase and darkness-induced production phase. Such a process can be achieved by controlling a gene essential for growth with a light-activated circuit and placing the gene(s) for production under an inverter circuit. (*b*) Amplifier circuits make it possible to induce production in the light, which opens potential to split fermentations into three or more phases. An example is shown in which cells are grown in the dark and induced in pulsed light before being shifted to the light-driven production phase. Figure adapted from images created with BioRender.com.

OptoQ-INVRT circuits, which harness the orthogonal quinic acid operon from *Neurospora crassa* to permit simultaneous optogenetic inversion and amplification of different sets of genes in *S. cerevisiae* (62). The combination of optogenetic inverters and amplifiers in the same strain opens a vast number of future applications in metabolic engineering.

Optogenetic circuits based on one-component systems have also been developed for metabolic engineering in bacteria. The optogenetic-CRISPR interference (opto-CRISPRi) platform uses EL222 to activate expression of a defective Cpf1 RNA-guided endonuclease in blue light. This protein is then directed to a specific DNA locus determined by the guide RNA sequence and represses transcription of downstream genes (63). A key advantage of opto-CRISPRi is its flexibility, because it can be applied to repress transcription at any locus simply by adjusting the guide RNA sequence. This system was used to enhance muconic acid titers in *E. coli* by restricting flux to the competing amino acid biosynthesis and glycolytic pathways in blue light. Together with the yeast optogenetic circuits, this platform solidified the value of EL222 for metabolic engineering applications in bacteria as well as yeast.

Bacterial two-component systems have also been applied to metabolic engineering. In *E. coli*, the blue light–activated pDawn system (**Figure 2*b***) was used to develop the light-repressed OptoLAC circuits (64). These circuits invert the response of pDawn by using it to express the LacI repressor in light, thus inducing genes of interest downstream of lacO-containing promoters in the dark. These circuits were used to induce production of isobutanol, mevalonate, and recombinant proteins in the dark at levels comparable or superior to those obtained with the chemical inducer isopropyl β-D-1-thiogalactopyranoside (IPTG). Another two-component system, CcaSR, has been used to make metabolic switches that prioritize different pathways in green or red light. One of these switches controls flux between glycolysis and the methylglyoxal pathway by inducing *tpiA* expression in green light and repressing it in red, which has potential applications in the synthesis of products derived from methylglyoxal or dihydroxyacetone phosphate (65). In a similar application, CcaSR was used to regulate the Embden–Meyerhof–Parnas and oxidative pentose phosphate pathways by controlling *pgi* expression, thus enabling modulation of total glycolytic flux through the former pathway from 0.5% in red light to 50% in green light, with the rest going through the latter pathway (30). A drawback of both switches is that they regulate only one enzyme at their metabolic branchpoints, so channeling of flux might be more exclusive if an inverted or orthogonal system was applied to the opposing branch. Nevertheless, the additional wavelengths these two-component systems offer grant flexibility when designing optogenetic systems and open opportunities for the development of bidirectional switches.

FUN-LOV is a different kind of two-component system that has been used for potential biotechnological applications in *S. cerevisiae* (27). This dimeric system induces transcription in blue light using a mechanism reminiscent of the yeast two-hybrid assay, in which the WC-1 and VVD LOV domains from *N. crassa* are fused to the DNA-binding and activation domains of Gal4p, respectively. The LOV domains dimerize in blue light, forming a functional transcription factor that activates the P_{GAL1} promoter (**Figure 2*a***). This system was applied to express the *FLO1* gene, inducing flocculation in light. It was also used to express a heterologous limonene synthase, achieving 2.5-times-higher gene induction with blue light than with traditional galactose induction. Although this system has not yet been used for chemical production, the promising results demonstrated thus far suggest it may have similar success in metabolic engineering applications.

Beyond these transcriptional circuits, optogenetics has also been applied to metabolic engineering using a single-chain chimeric photoresponsive recombinase. The chimeric light-inducible Cre recombinase, LiCre, was developed in *S. cerevisiae* by fusing the AsLOV2 photoreceptor domain to the N terminus of the Cre recombinase (47). This fusion causes the recombinase to be activated in the light and inactivated in the dark. This approach differs from other optogenetic recombinases,

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which use a split protein strategy to induce recombination by reconstituting the recombinase in light (45, 46). Compared to those systems, LiCre shows faster and stronger activation, as well as lower residual activity in the dark (47). This system was used to induce β-carotene production in a strain constitutively expressing the *crtE* and *crtI* genes, two of the three heterologous genes in the biosynthetic pathway, and having expression of the third gene, *crtYB*, which codes for a bifunctional phytoene synthase and lycopene cyclase, blocked by a terminator upstream of its coding region. By flanking this terminator with two LoxP sites, it could be excised by LiCre when activated by blue light, thus restoring the full biosynthetic pathway and inducing β-carotene production. In addition, a similar strategy was used to inhibit the competing ergosterol pathway by excising *ERG9* in the light. An important difference between this platform and other transcriptional circuits is that the effects of activating LiCre are not reversible, as they involve irreversible genomic modifications. However, the rapid activation and low leakiness of the LiCre system are valuable for precise dynamic control at the genome level.

Photoresponsive split proteins have also been used to control the production of valuable chemicals in *E. coli*. PhyB and PIF3 were separately fused to different domains of a split intein containing separate segments of T7 RNA polymerase (24). Red light–triggered dimerization of PhyB and PIF3 led to the formation of an active intein, which would then splice a functional T7 polymerase. This system was used to induce the lycopene biosynthetic pathway in *E. coli* (24). Although lycopene production increases with light stimulation, the system still requires chemical induction with IPTG to express the phycocyanobilin chromophore and the photosensitive intein chimera pairs. Additionally, because intein splicing results in a stable T7 polymerase, the process is difficult to reverse. Nevertheless, the red light activation of gene expression this system enables could be used orthogonally with other systems induced by blue, green, or infrared (IR) light or darkness to control different metabolic pathways.

In the future, optogenetic controls of metabolism will likely expand toward applications using multichromatic transcriptional induction of different enzymes and light-triggered posttranslational controls. A study using red-, green-, and blue-responsive circuits in *E. coli* to synthesize pigments from precursors in artistic two-dimensional patterns foreshadows the use of multichromatic controls (66). A more recent study applied the EL222 system and a near-infrared light activation tool (NRAT) to regulate the cell cycle in *E. coli*, which enhanced production of acetoin and poly(lactate-*co*-3-hydroxybutyrate), demonstrating a novel way in which multichromatic control can benefit bioproduction (67). Although this study highlights the potential of using multiple systems activated by orthogonal wavelengths to control metabolic pathways, this area remains largely unexplored.

Another direction with great potential in the future of metabolic engineering is the use of posttranslational controls like light-responsive synthetic organelles. Light-controlled assembly and disassembly of synthetic organelles to dynamically cluster enzymes can be used to divert flux through different branches of complex metabolic pathways. This has been demonstrated in the production of different derivatives of the multibranched violacein pathway, using synthetic liquid organelles reversibly assembled by blue light–induced oligomerization of Cry2-olig or darkness-induced formation of PixELL complexes (38). Optogenetic controls that act posttranslationally are not necessarily substitutes for optogenetic transcriptional controls but may complement each other in the future.

The combination of optogenetic transcriptional and posttranslational controls will enhance the ability to regulate microbial metabolism for bioproduction. Transcriptional optogenetic circuits are robust and reversible but can be slow to respond, as genes of interest must first be transcribed and translated to be activated, and their protein and messenger RNA (mRNA) must be degraded to be inactivated. Posttranslational tools, in contrast, are both fast and reversible; however, they

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can be costly to the cell if used for a prolonged period of time. For instance, if a light-inducible posttranslational regulator is being used to inactivate an enzyme, the cell will waste resources toward producing the targeted enzyme if not controlled transcriptionally as well. Therefore, the robustness and economy of transcriptional controls could complement the speed and reversibility of posttranslational controls to provide synergistic light control of microbial metabolism with rapid response at minimal cost to the cell.

3.2. Applications of Optogenetics in Microbial Growth and Cooperative Behavior

Microbial cells flourish in aggregate populations, which facilitate division of labor between cells and protection from environmental stressors. This cooperative behavior can lead to biofouling and resistance to antimicrobial agents; however, it can also be engineered for applications in bioremediation, biocatalysis, and even biofouling prevention (68). Formation of these microbial communities is linked to communications between cells, the tampering of which can affect the stability of populations, their spatial distribution, and overall function. This has been demonstrated by manipulating mechanisms such as quorum sensing (69); second messengers (70, 71); and, for microbes that form biofilms, the biopolymer matrix (72). Dynamic controls could assist in engineering more complex biological systems, such as to induce biofilm formation and control microbial consortia population dynamics. Although chemical inducers have been used successfully to control such complex behaviors (73), optogenetics provides unique advantages as a reversible and minimally invasive method of control.

Optogenetics is a powerful way to control and prevent biofilm formation. Most optogenetic systems that have been applied to control bacterial biofilms regulate the concentration of the second messenger c-di-GMP, which stimulates adhesin production and shifts the bacteria from a planktonic to a sedentary state. This signal can be degraded by a phosphodiesterase, reducing the likelihood of biofilm formation. Placing the phosphodiesterase gene under control of the pDawn system results in a strain of *Pseudomonas aeruginosa*, the biofilm formation of which is inhibited by blue light (74). Another study engineered a light-activated diguanylate cyclase in *E. coli* by fusing a near-infrared (NIR) light–activated bacteriophytochrome from *Rhodobacter sphaeroides* to the N terminus of a diguanylate cyclase from the cyanobacteria *Synechocystis* sp. PCC 6803 (75). NIR light activates the chimera, catalyzing guanosine triphosphate into c-di-GMP. These two optogenetic systems have also been combined into a dual-wavelength system in which NIR light synthesizes c-di-GMP and blue light degrades it, thus reversibly controlling biofilm formation (**Figure 5*a***). This dichromatic system was paired in *E. coli* with a quorum-quenching gene circuit, thereby disrupting the biofilms of other bacteria and mitigating biofouling (43). Other genes can be introduced into the microbial system to make catalytic biofilms, which have a greater tolerance to environmental stress compared to planktonic cells and therefore potential for improved bioproduction (76). A study used light to optimize biofilm formation in a strain expressing tryptophan synthase genes, which led to increased tryptophan yields (44). These studies demonstrate that optogenetically regulating c-di-GMP level is a robust approach for controlling biofilm formation.

Other mechanisms have also been exploited to control biofilm formation with light. The biopolymer matrix components of biofilms can also be the target of optogenetic control. The EL222 system has been used to control the expression of exopolysaccharide biosynthetic genes in *Sinorhizobium meliloti*, enabling optogenetic control over matrix synthesis and therefore biofilm formation (77). Another strategy used photoswitchable cell adhesion to control bacterial biofilms with light. Magnet proteins, which heterodimerize in blue light (26), can be displayed on cell surfaces and be used as dynamic light-responsive adhesin mimetics to control cell recruitment to

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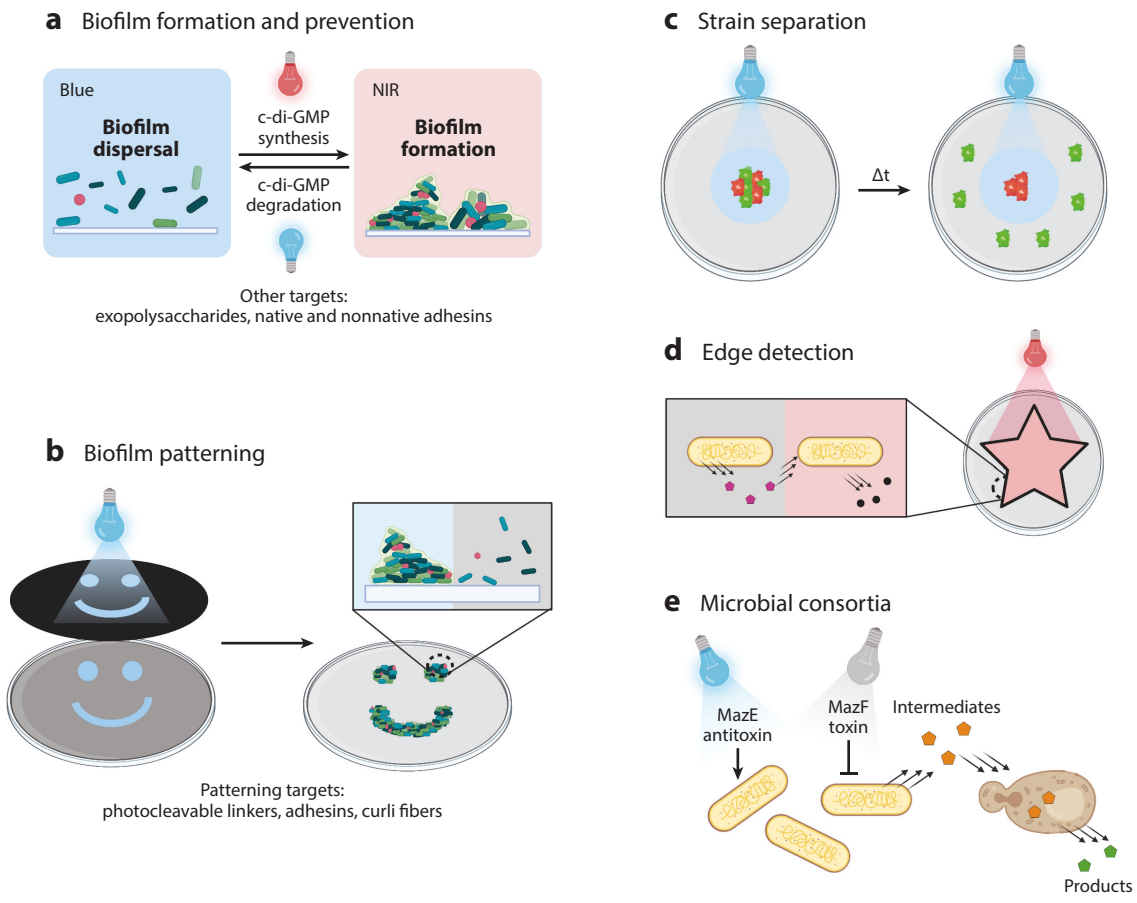

Figure 5

Optogenetic applications in growth and cooperative behavior. (*a*) Optogenetic controls can be established on key components of microbial biofilms, thereby establishing light-sensitive biofilm formation and prevention. For example, NIR- and blue-light circuits have been developed for the synthesis and degradation of the second messenger c-di-GMP, respectively. (*b*) The high spatial precision lent by optogenetics provides a noninvasive approach to high-resolution biofilm patterning. When surface pretreatment is not needed, biofilms can be patterned via light on a variety of surfaces, from plastics to textiles to ceramics. (*c*) Strains can be physically separated via optogenetic control of CheZ, an enzyme that inhibits bacterial tumbling. In blue light, cells with CheZ under the EL222 system exhibit greater displacement than wild type, an effect that can be visualized with a GFP-expressing CheZ strain and an RFP-expressing WT strain. (*d*) Optogenetics can also be used to encode algorithms in cells, such as in bacterial edge detection. In the light, bacteria produce β-galactosidase in the presence of 3-oxohexanoyl-homoserine lactone, which only bacteria in the dark produce and transport. Therefore, the black pigment from β-galactosidase is seen only at the edge of blue light and darkness. (*e*) Optogenetic control of *Escherichia coli* growth in *E. coli–Saccharomyces cerevisiae* consortia allows real-time manipulation of population distribution and therefore the metabolic division of labor. In the dark, the toxin MazF is expressed, inhibiting *E. coli* growth, and in blue light, the antitoxin MazE is produced, inhibiting MazF. Abbreviations: GFP, green fluorescent protein; NIR, near-infrared; RFP, red fluorescent protein; WT, wild type. Figure adapted from Lalwani et al. (91) and from images created with BioRender.com.

biofilms with light (78). Each Magnet monomer was expressed in different *E. coli* strains, which, when mixed in a 1:1 ratio, produced a biofilm in blue light but not in darkness. This system was then paired with a bioluminescence biosensor for Hg^{2+} such that in the presence of mercury the blue light emitted from the biosensor triggered bacterial aggregation for enhanced sensitivity

(79). Optogenetics has thus been proven effective for dynamic control of biofilm formation and its prevention, with potential applications in industry, medicine, and the environment.

Optogenetics also allows for precise spatial control of engineered functions, which can be useful in biofilm patterning. Traditionally, biofilm patterning methods involve cell printing or pretreatment of the application surface to obtain desired spatial cellular distributions. In contrast, optogenetics provides high spatial resolution without requiring direct contact with the application surface (**Figure 5*b***). The optogenetic controls of c-di-GMP synthesis described above (43) have been used for bioprinting in *P. aeruginosa* (80). By linking the circuit to a c-di-GMP fluorescent reporter, biofilm formation can be visualized. Biofilm formation can thus be controlled to adhere to the shape of an NIR and blue light pattern (80). Another method to achieve high-resolution cell patterning with optogenetics is to control native adhesins with light-responsive circuits. In *E. coli*, expressing the adhesin Ag43 with pDawn has been shown to decrease the rate of biofilm desorption, allowing spatial patterning to a resolution of 25 μm (81). Another type of biofilm component, curli fibers, is secreted by *Enterobacteria* and assembled extracellularly into strong amyloid fibers. Multichromatic systems, composed of the red light–activated Cph8 kinase, green light–activated CcaSR, and blue light–activated YF1, have been used to control the expression of different variants of the CsgA structural component of curli fibers. This made it possible to induce cell adhesion onto polystyrene, textiles, and ceramics in biofilm arrangements determined by light patterns (82). This so-called optogenetic lithography may allow future integration of living microorganisms with different materials for a wide range of research, medical, and commercial applications.

Optogenetics can also be applied to control cell motility and bacterial programming (**Figure 5*c***). Bacterial motility is powered by a protonmotive force (83), which can be controlled with light. *E. coli* has been engineered to express proteorhodopsin, a photon-driven proton pump, resulting in cells that swim only in green light (84). By controlling cell motility instead of adhesion, the time delay to establish a pattern is reduced from the order of hours to minutes (85). Other methods target the protein phosphatase CheZ, which inhibits cell tumbling when overexpressed. Using the EL222 system to control CheZ expression enables further cell displacement with increasing blue light intensity (86). Moreover, a mixture of red fluorescent protein (RFP)-expressing cells with CheZ under EL222 control and green fluorescent protein (GFP)-expressing wild-type cells can be physically separated from each other with blue light and visualized through their respective fluorophores (**Figure 5*c***). Photoresponsive bacteria have also been engineered with logic gates for edge detection (87). By using the phytochrome Cph8, *E. coli* was engineered to secrete 3-oxohexanoyl-homoserine lactone (AHL) as a chemical signal and express the cI repressor only in the dark. Additionally, the *lacZ* gene used to produce a black pigment was engineered such that it was repressed by cI but transcribed by transcription factor LuxR bound to AHL. With this arrangement, only cells at the edge of a red light region (which did not induce cI repressor) and within the range of AHL diffusion would activate *lacZ* transcription and produce pigment (**Figure 5*d***). Therefore, a black pigment is present along the light–dark interface, demonstrating bacterial edge detection through relative spatial awareness. These studies show how optogenetics can be applied in programming algorithms for complex microbial behaviors.

Population dynamics of microbial consortia can also be controlled with light by regulating the relative growth rates or cellular differentiation of their microbial constituents using optogenetic circuits. Coculture fermentations can significantly improve chemical production by dividing the metabolic labor and optimizing different biosynthetic modules across multiple strains (88). A major challenge of this approach, however, is maintaining a stable population, as the fastest-growing strain typically outcompetes the rest of the consortium members. Current strategies to address this challenge involve adjusting initial inoculation sizes (89) or engineering the strains to

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be codependent (90). However, once the fermentations are inoculated with the different strains, the population distributions cannot be easily tuned with these methods to find and maintain optimal compositions for chemical production. In contrast, by using light to control the relative growth rates of coculture members throughout fermentations, optogenetics offers a solution for population stability and optimization in microbial consortia. The first proof of concept of this approach was demonstrated recently in a consortium of *E. coli* and *S. cerevisiae*, in which the growth of *E. coli* was regulated with OptoTA, a blue light–responsive circuit that uses the pDawn/pDusk system to control the relative expression of the MazFE toxin–antitoxin pair (91) (**Figure 5*e***). In the dark, the toxin MazF is expressed, which degrades mRNA and inhibits growth, but blue light induces the antitoxin MazE, which neutralizes MazF to enable growth. This system was applied in a consortium that split the production of isobutylacetate and naringenin across *E. coli* and *S. cerevisiae*. By exposing the cocultures to different light duty schedules, their population composition could be controlled to improve production. This demonstration required only optogenetic control of *E. coli* because it grows much faster than yeast. However, cocultures containing two or more dominant strains will require additional optogenetic controls to individually regulate their growth rates. A second strategy used the EL222 system to induce expression of Cre recombinase in *S. cerevisiae*, which would excise the gene for a fluorescent protein flanked by LoxP sites and replace it with a gene for a different fluorescent protein or a transcription factor that would induce additional fluorescent proteins (92). This unidirectional light-induced cellular differentiation scheme was used to establish stable microbial consortia in liquid and solid media with spatiotemporal control. The use of optogenetics to control the population dynamics of microbial consortia is poised to make significant breakthroughs in microbiome research and engineering, bioremediation, and coculture fermentations for products derived from complex metabolic pathways.

3.3. Applications in Fundamental Research on Cell Physiology

Relative to traditional approaches, the tunability, reversibility, and orthogonality of optogenetics can reduce bias and artifacts when investigating cell physiology. Although traditional methods of perturbing cells, like mutating genes or applying stresses, have proven valuable for fundamental research (93, 94), these techniques can be slow, nonspecific, and subjective. These qualities can blur the causal link between the perturbation and response, making it difficult to elucidate sensitive mechanisms (95). The unique capabilities of optogenetics can resolve these challenges by permitting small perturbations with minimal off-target disruptions. In addition to its tunability and orthogonality, optogenetics also benefits from rapid kinetics, as the application of light is instantaneous and is not subject to delays caused by diffusion, cellular uptake, or degradation of chemical inducers. Therefore, the noninvasive, nontoxic, and orthogonal nature of optogenetics has enabled new strategies to study elusive fundamental questions in biological processes that have been studied for decades, such as cell signaling, transcription, translation, and the cell cycle.

Optogenetics has helped elucidate cell signaling pathways in yeast. The most common strategy to exert light control in signaling pathways has been to use photoresponsive enzymes that are active in the light and inactive in the dark (14). For example, an adenylate cyclase linked to a blue light sensor–responsive FAD domain known as bPAC (96) was used to study the dynamics of the protein kinase A (PKA) signaling pathway in yeast. This light-responsive cyclase converts ATP to cAMP in blue light, which activates the signaling pathway. This approach was used to elucidate the dynamics by which the PKA pathway localizes the Msn2 regulator to the nucleus and captured the change from an activated to an inactivated PKA pathway. These insights revealed an underdamped oscillation in PKA levels and uncovered a negative feedback loop from Ras1/2-mediated cAMP production (41). Optogenetics has also been used to interrogate epigenetic signaling by

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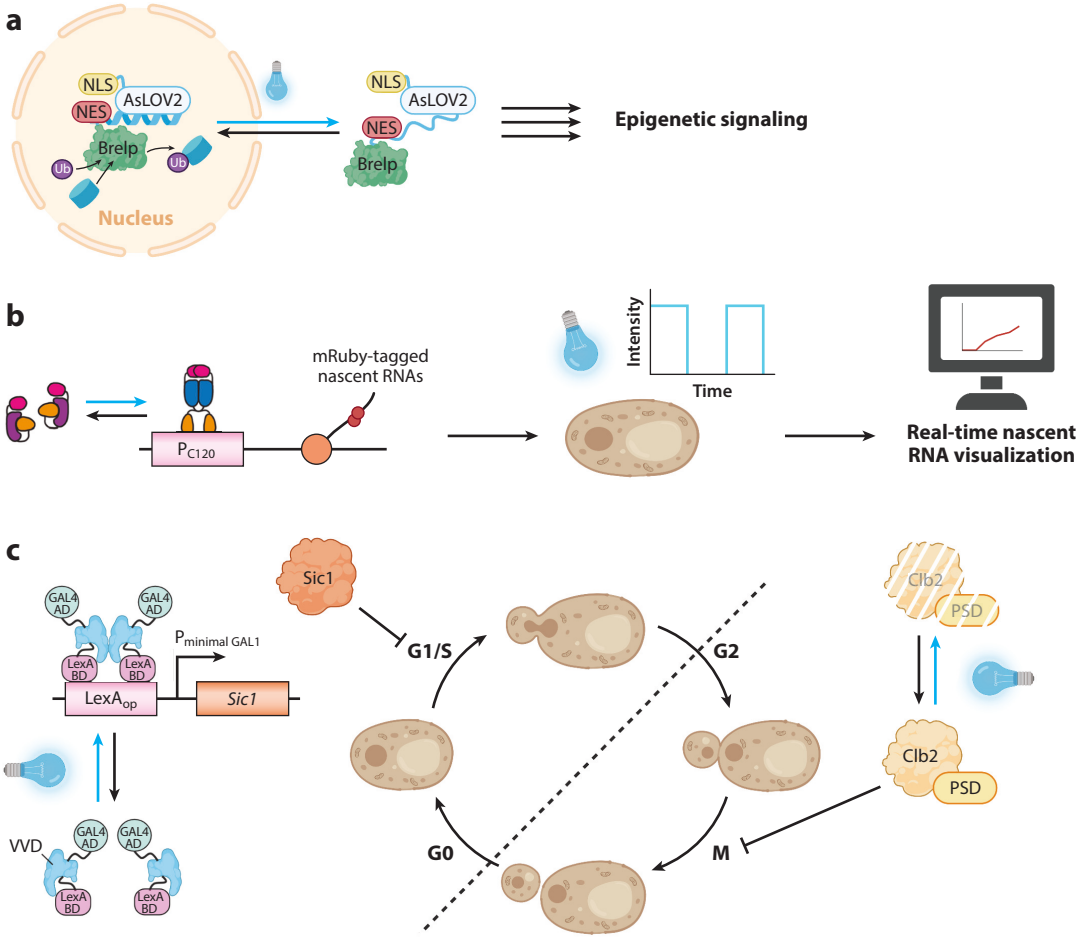

Figure 6

Optogenetics applied to fundamental research in cell physiology. (*a*) Optogenetic nuclear export and localization can be used to probe epigenetic signaling, in this case with the BreI ubiquitin ligase. In blue light, the LINX system shuttles BreIp out of the nucleus, allowing the enzyme to ubiquitylate its target histone. With the precision of the optogenetic control, the half-life of this reaction could be determined. (*b*) The EL222 system can be applied to produce nascent RNAs that can be fluorescently tagged. The yeast are exposed to different light cycles to visualize and measure transcription in real time. (*c*) Optogenetics has been applied to control two different stages of the cell cycle. In blue light, the LVAD system produces a truncated form of Sic1p, which inhibits the G1/S transition state. Similarly, the cyclin Clb2p arrests mitosis. To establish optogenetic control, Clb2p can be fused to a photosensitive degron, where the complex is degraded in blue light. Abbreviation: PSD, photosensitive degron. Figure adapted from images created with BioRender.com.

controlling histone H2B ubiquitylation with light. This study used the LINX system, involving a nuclear export sequence embedded in the Jα helix of AsLOV2, which is also fused to a nuclear localization signal (97). Light activation of AsLOV2 exposes the nuclear export sequence, whereas darkness leaves only the nuclear localization signal exposed, thereby targeting the protein to the cytosol in the light or to the nucleus in the dark. Fusing LINX to Bre1p, an E3 ubiquitin ligase, allowed for dynamic control of H2B ubiquitylation, which provided greater resolution of ubiquitylation and deubiquitylation timescales compared to previous studies using chemical induction (98) (**Figure 6*a***). Optogenetics was also applied to study membrane phospholipid asymmetry, which

is essential in many cell functions, including cell signaling (99). The asymmetry is maintained by lipid transporters like P4-ATPases, which are activated by protein kinases. In *S. cerevisiae*, these kinases were substituted with a phototropin native to *Chlamydomonas reinhardtii* (*Cr*PHOT), which was found to complement the kinases in blue light. This tool was the first to replace the function of P4-ATPase activity with an optogenetic system, as well as the first to optically control the distribution of non-phosphatidylinositol phospholipids. Although the mechanism of P4-ATPase regulation remains unknown, *Cr*PHOT could be a valuable tool in future studies of membrane asymmetry. Centered on pathways conserved across eukaryotes, these discoveries made in yeast models provide valuable insights into mammalian mechanisms as well.

Optogenetics has also been used to study transcription. The stochastic and highly regulated nature of transcription has traditionally made it challenging to perturb and study in real time without influencing other biological processes. However, the unique capabilities of optogenetics offer many opportunities to overcome this challenge. The VP16-EL222 transcription factor has been used in yeast to dynamically induce expression of mRNA containing several stem-loops, which bind to a fluorescent reporter to enable rapid readout of transcriptional dynamics and hysteresis (100). This system's high spatiotemporal control allowed for single-cell light-tunable measurements (**Figure 6*b***), which revealed that transcriptional responses do not change after subsequent light pulses, suggesting that transcription is memoryless. Another study used pulsatile inputs with this VP16-EL222/RNA readout system, modulating the duration of the light pulses while maintaining the period (101). This pulse-width modulation reduced cell-to-cell variability while providing another dimension outside of amplitude for tuning light dosage, revealing that fixed expression ratios can be generated through pulsatile regulation without needing to modify the genetic circuit. The effects of pulsatile and continuous light inputs on gene expression have also been studied using transcription factors shuttled between the nucleus and plasma membrane with the CLASP (controllable light-activated shuttling and plasma membrane sequestration) system (36). This study focused on transcriptional activation by Crz1p, which is a key regulator in the calcium stress response in yeast. Controlling Crz1p activity with varying light pulses revealed that its transcriptional dynamics depend not only on light pulse length but also on target gene identity. Additionally, it was found that pulsatile input can sometimes induce higher expression levels than continuous light over time. New optogenetics-enabled interrogations in microorganisms, such as these, will continue to give new insights on the basic nature of transcription, as well as assist in the modeling and optimization of gene expression levels for practical applications.

The high spatiotemporal precision of optogenetics has also enabled new studies on translation in microorganisms. Reversible light-dependent inhibition of the human eukaryotic initiation factor 4E (eIF4E) has been achieved in yeast by a chimera of a LOV domain and the eIF4E binding protein 4EBP2, which inhibits eIF4E (102). The refined cLIPS (circularly permuted LOV inhibitor of protein synthesis) construct inhibits growth in blue light of a strain that depends on eIF4E activity for growth. This study demonstrates the feasibility of using optogenetics to study protein translation in microorganisms by interrogating the dynamic interactions between a specific pair of proteins.

Optogenetics has also been applied to probe posttranslational regulation of cell function by controlling protein degradation with light in microorganisms. Photosensitive degrons (PSD) have been developed by combining the AsLOV2 domain with a murine ornithine decarboxylase degron (103). In blue light, the PSD module targets any fused proteins for degradation by the proteasome. Interestingly, when the PSD module is fused to membrane proteins in the endoplasmic reticulum of yeast, the targets are degraded approximately 10 times faster than soluble proteins tagged with the same PSD (104). This endoplasmic reticulum–associated degradation pathway was investigated by systematically interrogating the effect of different components of the ubiquitylation

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machinery on this light-sensitive degradation, revealing the involvement of Uba1p, Ubc6p, Ubc7p, and Doa10p. This study not only demonstrates the feasibility of using the PSD to study protein degradation pathways in yeast but also suggests the possibility of using it to study other cellular processes by manipulating the stability of key specific proteins with light.

Optogenetics also shows promise in studying and engineering the progression through the cell cycle. In *S. cerevisiae*, the G1 phase in the cell cycle is moderated by Sic1p, which inhibits cyclin complexes. Consistent with this role, the start of the S phase is marked by high levels of Sic1p proteolysis, and yeast strains with truncated *SIC1* lacking a degradation sequence undergo a prolonged G1 phase (105). This truncated *SIC1* gene was placed under optogenetic control to modulate the yeast cell cycle using yLightOn (106). This one-component system consists of a protein fusion called LVAD, named because it contains the LexA DNA-binding domain, the Vivid LOV domain, and the *GAL4* activation domain. In blue light, LVAD binds to a LexA operator upstream of a *GAL1* minimal promoter, thus inducing transcription of genes downstream of this synthetic promoter. By controlling the expression of a truncated *SIC1* gene with yLightOn, cells could be arrested at the G1/S phase when exposed to blue light (**Figure 6*c***). In a separate study, the cyclin Clb2p, involved in cell cycle progression, was fused to a PSD to allow optogenetic control of mitosis (107). Clb2p was degraded in blue light, allowing growth and division, whereas darkness caused Clb2p accumulation and cell cycle arrest at the metaphase/anaphase transition (**Figure 6*c***). These examples demonstrate the enormous potential of using optogenetics to control, engineer, and study the cell cycle in yeast.

3.4. Optogenetics in Biomedical Research Applications

Although optogenetics in biomedical research typically is associated with applications in neuroscience, it has been employed more recently to study and engineer microbe–host interactions. These relationships may be beneficial, such as the mutualism of the gut microbiome, or detrimental, such as the virulence of microbial infections. In any case, precise control over microbial behavior in situ is an empowering capability to study these interactions. Chemical inducers have been used for this purpose; however, these agents are subject to slow and uncontrolled diffusion and degradation and can cause undesirable side effects in both the microorganism and its host (108–111). These qualities can impose limits in control precision, lead to complex pharmacokinetic effects, and pose challenges in establishing causal links. In contrast, the high tunability, reversibility, and orthogonality of optogenetics offer new strategies to study and engineer microbe–host relationships without the limitations caused by degradation, diffusion, or side effects of chemical agents.

There is vast potential in using optogenetics to study microbiomes. Although many correlations exist between the different microbiomes in the human body and physical well-being, the complexity of the microbiome makes it difficult to elucidate the individual role of any one microbial member (112). Optogenetics can help navigate this complexity by reducing the system down to its simplest components and interrogating specific interactions. A recent pioneering study used optogenetics to investigate the effect of colanic acid (CA) microbial production in the gut of *Caenorhabditis elegans* on its longevity (113). A strain of *E. coli* was engineered with the CcaSR system to control the expression of RcsA, the activator of CA biosynthesis, and fed to *C. elegans* (**Figure 7*a***). The worms were then paralyzed with levamisole, which causes mitochondrial stress resembling that of aging and neurodegenerative disease. When CA biosynthesis was activated with green light, *C. elegans* exhibited reduced mitochondrial stress and enhanced longevity, demonstrating the local health effects of CA on surrounding tissue and the entire worm. Although this study exploited the optical transparency of *C. elegans* to control the biosynthesis of only one compound

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with light, it has laid the foundation for future applications of optogenetics to study microbiomes and their host interactions.

Optogenetics has also been used to control engineered microbe–host interactions, in which the microorganism acts as a live therapeutic to aid in healing. This concept has been demonstrated

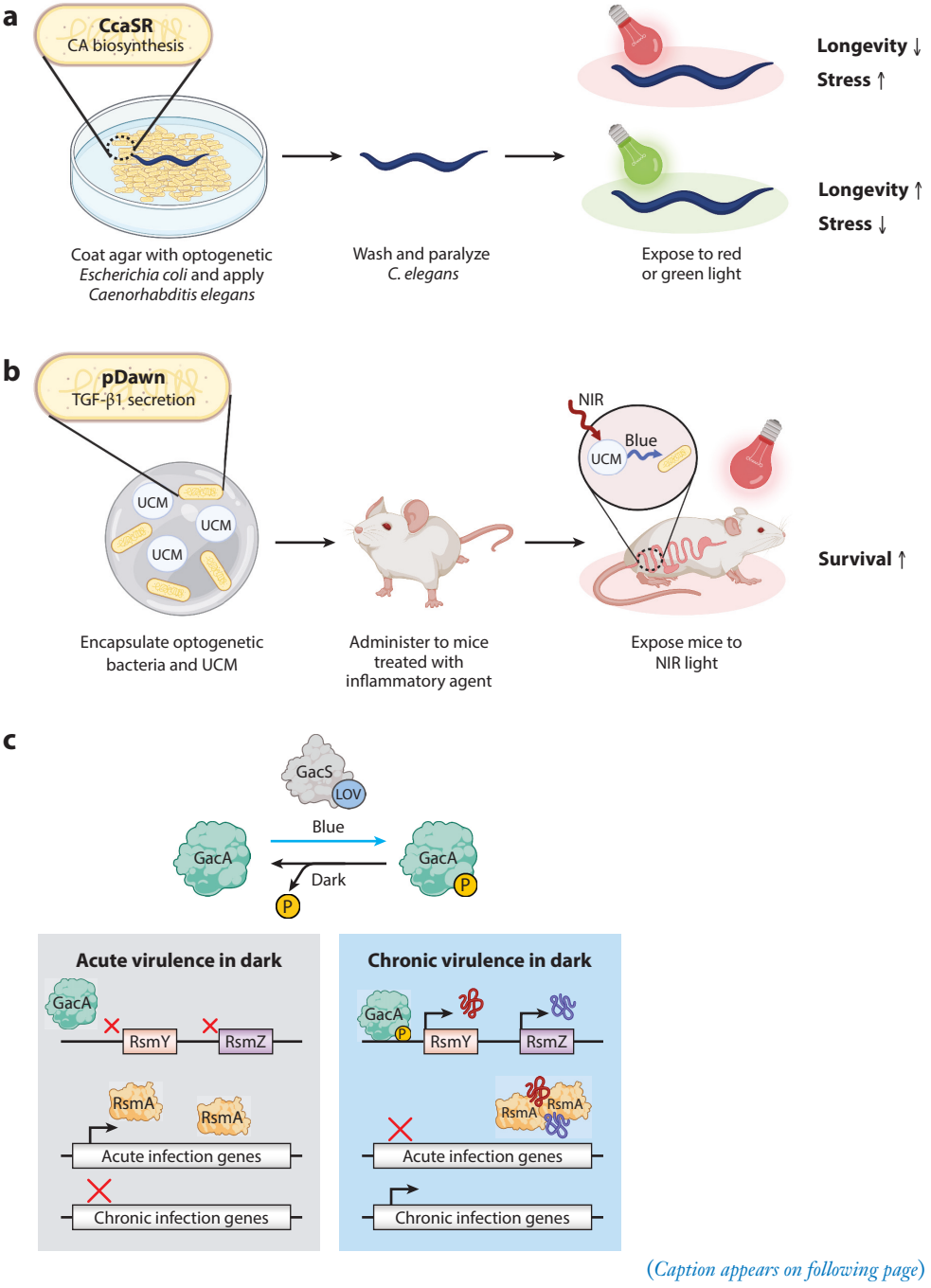

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Figure 7 (*Figure appears on preceding page*)

Biomedical applications of microbial optogenetics. Optogenetics has been used to examine the impact of individual microbiome species, develop new therapies, and study virulence. (*a*) The effect of CA in the gut was studied using optogenetically controlled *Escherichia coli* in *Caenorhabditis elegans*. The bacteria were fed to the worms, which were then washed and paralyzed to ensure retention in the gut. Red or green light, respectively, repressed or induced CA production, affecting mitochondrial stress and longevity. (*b*) A living therapeutic for ulcerative colitis uses the pDawn system to regulate production of TGF-β1 from *E. coli*. Encapsulation with an upconversion material aids in activation by converting NIR to blue light. Exposure to NIR light activates expression of the therapeutic gene, enhancing survival of treated mice. (*c*) Virulence of *Pseudomonas aeruginosa* can be controlled by regulating the Gac/Rsm signaling cascade. A chimeric GacS kinase phosphorylates GacA in blue light, which activates transcription of the RsmY and RsmZ small RNAs. These RNAs sequester the RsmA regulatory protein, which leads to activation of genes involved in chronic infection and repression of those associated with acute virulence. In the dark, when GacA is not phosphorylated, RsmY and RsmZ are repressed, which allows RsmA to be active. The active RsmA represses genes associated with chronic virulence and induces those involved in acute infection. Abbreviations: CA, colanic acid; NIR, near-infrared; UCM, upconversion material. Figure adapted from images created with BioRender.com.

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for the treatment of ulcerative colitis using optogenetically controlled *E. coli* in a mouse model (**Figure 7*b***). These engineered living therapies used pDawn to control secretion of either transforming growth factor-β1 or immunosuppressive cytokine interleukin-10 (114, 115). To address the challenge of limited light penetration across animal tissues, the *E. coli* was administered in a hydrogel doped with an upconversion material that emits blue light when exposed to NIR light. Because NIR radiation is more penetrating than blue light, this strategy allowed effective activation of pDawn in bacteria within the mouse gut. When these treatments were used, mice exposed to NIR light experienced less severe symptoms and had a higher survival rate compared to their untreated counterparts. A similar approach was used to treat malignant tumors. A *Lactococcus lactis* strain was equipped again with pDawn to control the secretion of the cytokine interferon-γ in blue light (114). When orally administering the engineered strain along with upconversion material, mice exposed to NIR light experienced reduced tumor growth. In a separate study, a similar strategy was used to improve drug delivery using a tumor-targeting *E. coli* strain engineered with the EL222 system to control the expression of tumor necrosis factor-α only when activated with light (116). As done previously, an upconversion material was used to enhance photoactivation through tissues; however, the material in this study was modified with folic acid to specifically target it to the tumor. When mice injected with this treatment were exposed to NIR light at tumor location, the tumor-localized bacteria could inhibit tumor growth. These early studies demonstrate the potential of using optogenetically controlled microorganisms therapeutically, including those activated by blue wavelengths with enabling upconversion materials.

Optogenetics has also been applied to study pathogen–host interactions. The Gac/Rsm regulatory cascade in *P. aeruginosa*, involved in determining whether an infection will be acute or chronic, was regulated optogenetically to improve our understanding of the virulence and lifestyle of this pathogen (117) (**Figure 7*c***). GacS is a sensor kinase that responds to an unknown environmental stimulus by phosphorylating the GacA response regulator, resulting in the inhibition of RsmA, a protein that represses genes for chronic infections and induces acute infection. Thus, GacA phosphorylation is associated with chronic infection, whereas dephosphorylation leads to acute infection. This cascade was engineered with optogenetic controls by replacing the GacS sensor domain with a LOV domain, which led to light-dependent pathogenic behavior in infected *C. elegans*. Optogenetics has also been used to regulate the second messenger cAMP in *P. aeruginosa*, which controls several processes including virulence (118). By replacing the native adenylate cyclases with a photoactivated cyclase native to a *Beggiatoa* sp., cAMP levels

could be controlled with blue light. When the engineered *P. aeruginosa* was injected into mice, animals exposed to blue light developed larger skin lesions than those kept in the dark, demonstrating the connection between cAMP and infection. Virulence in *E. coli* has also been studied using similar optogenetic tools. A photosensitive switch to control cAMP levels in *E. coli* was developed using the same blue light–activated cyclase from the *Beggiatoa* sp. (119). Although not directly used to manipulate infection, this switch helped analyze how cAMP affects expression of virulence genes, revealing new proteomic insights that present avenues for further study into infection and new targets for antibacterial drugs. These studies demonstrate how optogenetics can be used to better understand virulence, host sensitivity, and mechanisms of infection.

Many opportunities remain to develop live microbial therapeutics and elucidate the relationships between microbes and their hosts using optogenetics. To date, these optogenetic studies have relied on blue light–responsive systems, which suffer from limited light penetration. Although upconversion materials address this challenge, these materials have a low luminescence efficiency and thus require intense illumination, which can cause overheating and thus off-target effects (120). Furthermore, questions remain regarding their long-term toxicity and possible accumulation in the body (121). Therefore, it would be beneficial to engineer the bacteria to respond directly to NIR light to eliminate the dependence on upconversion materials. Though less explored than the blue light–responsive systems, some NIR-activated systems have been developed in *E. coli* that could be valuable for these applications (67, 75). Elucidating the role of more components in the microbiome is another area of opportunity. In the same way that the effect of colanic acid on longevity was revealed (113), similar approaches could be used to clarify the role of other compounds found in the gastrointestinal tract. Furthermore, although early studies have focused on using optogenetics to better understand ulcerative colitis, malignant tumors, and bacterial infections, similar strategies could be applied to study other diseases. Ultimately, optogenetics has proven to be a valuable biomedical tool with vast potential for basic research and future therapies.

3.5. Cybergenetic Applications of Optogenetics

The emerging field of cybergenetics aims to control biological systems in real time using control theory and computer interfaces. These sophisticated control systems represent a new paradigm to study and engineer dynamical biological processes to better understand cellular regulatory networks and potentially bring cellular process control to biotechnology (10). In these systems, biological readouts such as fluorescence or optical density are monitored in real time to provide feedback to a controller, which adjusts the output of actuators to maintain a desired setpoint or reference trajectory (**Figure 8**). Several studies have used chemical agents to control chemostat or microfluidic systems (122–125), but this approach can pose challenges, as chemicals cannot be easily removed to reverse inputs, especially in non-microfluidic devices. Additionally, chemical inducers suffer from time delays caused by diffusion and cellular uptake, can cause off-target effects, and can be metabolized or pumped out of the cell, which can hamper feedback control. Light inputs, however, can be applied instantaneously and reversibly with minimal undesired effects and cannot be metabolized or transported out of the cell (10). Therefore, light is in many ways the ideal agent to interface biological systems with computers to control their dynamic behavior.

Thus far, applications of optogenetics to cybergenetics have focused on controlling fluorescent protein expression and cell growth. Robust real-time control of these cellular processes has been established using the two-component systems CRY2/CIB1 and PhyB/PIF3 in *S. cerevisiae* (126, 127) and CcaSR in *E. coli* (128, 129). These studies demonstrated the advantage of closed-loop over open-loop controls for achieving desired set points, particularly to recover from perturbations or in cases with varying set points (127–129). Furthermore, these studies have explored several

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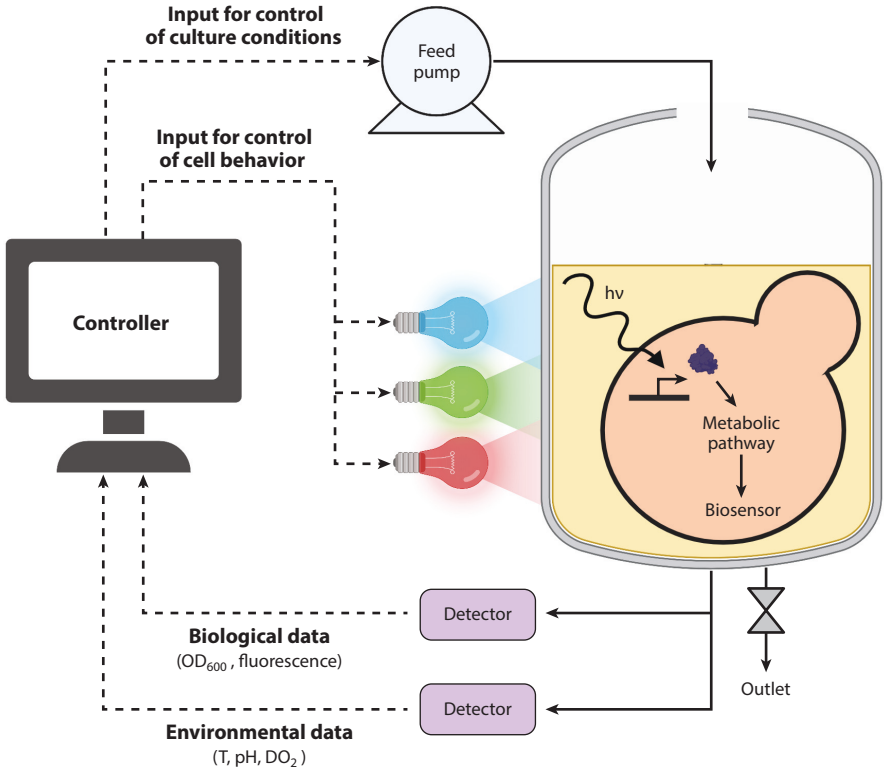

Figure 8

Feedback control of a microbial culture using optogenetics. Cybergenetics can be applied to control cellular behavior in a similar approach to how environmental parameters are commonly maintained. Light serves as an actuator that induces a genetically encoded response in the cells. This response influences the biosensor output, which is detected and fed to a controller. The controller converts the biosensor data to an appropriate light dose in real time to achieve the desired cellular behavior. Figure adapted from images created with BioRender.com.

controller types (128) and introduced new microfluidic (129) or turbidostat (128) devices for real-time optogenetic control of microbial systems. To illustrate the feasibility of light-based feedback control, fluorescent reporters have been used to model the dynamics of the yeast optogenetic circuits OptoEXP and OptoINVRT (130). In a landmark study, closed-loop control of protein production and cell growth in *E. coli* was achieved in a non-microfluidic device, using 25-mL turbidostats in a customized light delivery system (128). *E. coli* was engineered with CcaSR to control the expression of a fluorescent protein to study protein production, or to control expression of MetE, required for methionine biosynthesis, to control cell growth. The superiority of model predictive control over proportional-integral controllers was demonstrated for protein production in several scenarios. However, the difficulty of modeling optogenetically regulated cell division prevented the use of model predictive controls for cell growth. Nevertheless, a simple proportional-integral controller could achieve precise regulation of growth rate (128). These studies not only demonstrate that light is an effective agent to establish closed-loop controls using optogenetic actuators but also pave the way toward using optogenetics to develop feedback cellular process controls for biotechnological applications such as microbial protein or chemical production in bioreactors.

Optogenetic feedback control has also been applied to study microbial physiology and population behavior. A closed-loop optogenetic compensation (CLOC) strategy was used to study network dynamics in the yeast pheromone response pathway (131). In this study, expression of the *STE12* mating regulator was controlled optogenetically by exploiting the blue light–activated CRY2/CIB1 interaction, in which each component is fused to a DNA binding or activation domain. By combining genetic compensation with control theory and real-time in silico feedback, CLOC shed light on the natural transcriptional feedback control of the yeast mating pathway, a strategy that may be applicable to study other signaling pathways. Feedback control has also been used to investigate and model the stochastics of transcription at the single-cell level using VP16-EL222 in yeast (100). In this approach, nascent RNA levels were quantified and converted to appropriate light inputs in real time using an integral controller. Finally, cell-in-the-loop approaches use feedback control to emulate cell signaling with light inputs calculated from expression measurements, which has helped improve our understanding of microbial population behavior (132). In all of these cases, optogenetic activators were the enabling tools that facilitated the establishment of closed-loop controls, which were essential to study these fundamental questions.

Optogenetics holds great potential for further advancing the field of cybergenetics. Because biological systems are highly complex with inevitable time delays, further development of modeling tools will improve upon current capabilities. Whereas simple models are effective for some systems, others with longer time delays may benefit from implementing systems such as gray-box controllers that are guided by both mechanistic knowledge and neural networks (133). Optogenetic actuators with more rapid responses will also benefit the field. Optogenetic transcriptional circuits can be designed with improved response rates (60); however, optogenetic activators that operate posttranslationally are inherently faster because they bypass transcription and translation and have enormous potential as the field matures (37, 38, 134–137). Finally, multichromatic controls raise the possibility of developing simultaneous regulatory systems for multiple cellular functions, which would unlock unprecedented levels of computer-assisted feedback control of biological processes. Ultimately, cybergenetics empowered by optogenetics will enable new strategies to answer fundamental questions about cellular function dynamics and potentially develop new control systems for biotechnological processes.

4. CONCLUSIONS

Optogenetics has proven invaluable for microbial engineering in many fields. The ability of optogenetics to exert noninvasive, precise, and reversible perturbations is unparalleled among traditional methods of control. In addition, the vast repertoire of different photosensitive proteins grants flexibility in choosing systems with different capabilities for orthogonal spatiotemporal control. These characteristics are advantageous for controlling microbial processes and are reflected in the broad collection of optogenetic applications reviewed in this article. With applications ranging from chemical production to bacterial therapeutics, optogenetics has cemented its impact on microbial engineering.

Nevertheless, important challenges must be overcome to realize the full potential of optogenetics in microbial engineering. For example, a majority of the optogenetic circuits deployed thus far respond to blue light, as the corresponding flavin chromophores are ubiquitous in all prokaryotes and eukaryotes. However, a greater diversity of optogenetic tools activated by red-shifted wavelengths, especially for posttranslational controls, would be also valuable to benefit from enhanced light penetration and more fully harness the capabilities of optogenetics with multichromatic controls (11). An additional challenge is light penetration, which introduces complexity for large-scale fermentation processes and therapeutic systems. Although optogenetic

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inverter and amplifier circuits and upconversion materials help to address this challenge, they have yet to be tested in large-scale systems like industrial bioreactors or large animals. A wide variety of photobioreactor designs could address some of these concerns in fermentation processes, but implementing optogenetic systems that respond to longer, deeper-penetrating wavelengths would further help deploy optogenetic controls in large bioreactors.

In the future, additional microbial species will likely be engineered with optogenetic controls to take advantage of their unique capabilities. Organisms such as *Pseudomonas putida* or *Yarrowia lipolytica* are particularly interesting for chemical production, whereas *B. subtilis* (8) and *Komagataella phaffii* (138) are better suited for protein production. Additional candidates include *Bacillus megaterium*, which can secrete larger proteins than *E. coli* can (139), as well as the thermotolerant and methylotrophic yeast *Ogataea polymorpha* (139). Developing optogenetic systems for these organisms will enable light-controlled fermentation and cybergenetic technologies that exploit the special attributes of these organisms.

Future implementations of optogenetics could also expand the use of genome-level controls like inducible recombinases and endonucleases to broader applications. Whereas microbial optogenetic controls operating at the genomic level have focused on metabolic engineering (47) and microbial consortia (92), light-responsive recombinases might be valuable for microbial genome engineering in other contexts, such as microbiome or biomedical research. Additionally, applications of photoactivated endonucleases in microorganisms have thus far centered on employing catalytically defective mutants to repress transcription (63). These photoactivated endonucleases could potentially be turned into transcriptional activators by fusing defective mutants to an activation domain (140). Furthermore, fully functional endonucleases could also be used to offer light-controlled genome engineering. Such tools have been applied for genomic engineering in mammalian cells (141) but have yet to be applied in microorganisms for basic research or biotechnology.

Overall, the application of optogenetics in microbial engineering has blossomed, with much promise looking forward. As the field matures, the number of microbial species engineered with photoresponsive systems of ever-increasing diversity will continue to expand. Therefore, with optogenetics, the future of microbial engineering for basic and applied research is very bright.

DISCLOSURE STATEMENT

The authors have applied for several patents for some of the optogenetic circuits described in this article.

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