Optogenetic control of *Bacillus subtilis* gene expression

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The Gram-positive bacterium *Bacillus subtilis* exhibits complex spatial and temporal gene expression signals. Although optogenetic tools are ideal for studying such processes, none has been engineered for this organism. Here, we port a cyanobacterial light sensor pathway comprising the green/red photoreversible two-component system CcaSR, two metabolic enzymes for production of the chromophore phycocyanobilin (PCB), and an output promoter to control transcription of a gene of interest into *B. subtilis*. Following an initial non-functional design, we optimize expression of pathway genes, enhance PCB production via a translational fusion of the biosynthetic enzymes, engineer a strong chimeric output promoter, and increase dynamic range with a miniaturized photosensor kinase. Our final design exhibits over 70-fold activation and rapid response dynamics, making it well-suited to studying a wide range of gene regulatory processes. In addition, the synthetic biology methods we develop to port this pathway should make *B. subtilis* easier to engineer in the future.
**B. subtilis** is a model organism for studying how time-varying (dynamic), heterogeneous, and spatially-coordinated gene expression signals control single- and multicellular behaviors. For instance, in response to short-term energy stress (i.e., glucose/phosphate limitation), transcription of the general stress response regulon is activated in a pulsatile manner with a frequency proportional to the stress intensity. In contrast, environmental (e.g., osmotic) stress induces a single transcriptional pulse of this same regulon with an amplitude proportional to the rate of onset of the stress. In a separate transcriptional pulse of this same regulon with an amplitude contrast, environmental (e.g., osmotic) stress induces a single transcriptional output module (TOM) of the stress-responsive CcaSR v0.1, to demonstrate that it enables precision control of the expression of one or multiple genes, including at the single-cell level. In one of the studies, we engineered a synthetic CcaSR v0.1 with very weak output in red light and similar or greater *Ppcg* output in green. We introduced the corresponding CcaS PAS deletions in the context of our optimized (v2.0) system, resulting in an *E. coli* CcaSR v3.0 system with very low leakiness and nearly 600-fold dynamic range. Various versions of the CcaSR system have been used alone and in combination with additional optogenetic tools with different wavelength specificities to achieve precise spatial and temporal control of the expression of one or multiple genes, including at the single-cell level. In one of the studies, we programmed linear ramps and sine waves of expression of a transcriptional response regulator in order to characterize the input/output (I/O) dynamics of a widely-used synthetic gene circuit.

Here, we combine lessons learned in our previous work with several novel synthetic biology approaches to port CcaSR into *B. subtilis*. Our initial design, which is based on *E. coli* CcaSR v2.0, does not respond to light. We utilize fluorescent protein fusions to reveal that *ho1*, *pcyA*, and *ccsA* are poorly expressed. Recoding of the initial ORF sequences and several modifications of the gene expression cassettes substantially improve expression. Despite these optimizations, we find that PCB levels remain low. Inspired by previous work on enzyme fusion and scaffolding, we engineer a *ho1*-pcyA translational fusion, which results in high PCB levels. Next, we demonstrate that *Ppcg* is derived from *Synechocystis* PCC6803, is weak relative to other *B. subtilis* promoters. To increase transcriptional output, we chimerize *Ppcg* with a strong constitutive *B. subtilis* promoter. Then, we increase CcaSR dynamic range by screening the best-performing miniaturized CcaS variants in the context of our system. Finally, we characterize the steady state and dynamic I/O of the optimized system, named *B. subtilis* CcaSR v1.0, to demonstrate that it should enable characterization of a wide range of *B. subtilis* gene regulatory processes. The principles elucidated during this debugging and optimization process should be of great utility to future *B. subtilis* synthetic biology applications, and more generally, to any situation where porting genetic circuits between bacterial species is required.

### Results

**B. subtilis** CcaSR v0.1 does not respond to light. Our first implementation, namely CcaSR v0.1, comprised three independent modules integrated into distinct genomic loci: the PCB production module (PPM v0.1, Fig. 1a), light-sensing module (LSM v0.1, Fig. 1b), and transcriptional output module (TOM v0.1, Fig. 1c). This design was based on the insights obtained from our previously engineered *E. coli* CcaSR circuit, where dynamic range increased with higher expression of *ho1* and *pcyA* and was optimal at intermediate *ccsA* and *ccrR* expression levels. Thus, we engineered a synthetic *ho1*-pcyA operon under control of the strong constitutive *B. subtilis* promoter PppA and released the copy of the synthetic ribosome binding site (RBS) MF001 (see “Methods” section) in LSM v0.1 and TOM v0.1, respectively. Finally, to measure the output of the
system, we encoded superfolder green fluorescent protein (sfGFP) under P_cpcG2_172 and RBS MF001.

To characterize B. subtilis CcaSR v0.1, we induced expression of ccaS and ccaR by adding different combinations of xylose and IPTG concentrations under both red and green light, resulting in a total of 96 different conditions. Then, we assayed the output of the system by measuring the resulting sfGFP fluorescence in calibrated Molecules of Equivalent Fluorescent (MEFL) units via flow cytometry (see “Methods” section). We observed that even in the absence of xylose, sfGFP levels increased up to 40-fold in response to IPTG addition (Supplementary Fig. 1B, C), likely due to CcaR phosphorylation from a small molecule donor such as acetyl phosphate37, while reporter activity was not detectable with a control TOM where the DNA binding domain of CcaR was removed (Supplementary Fig. 2). These results indicated that CcaR was properly expressed and capable of binding to and activating transcription from P_cpcG2_172 in B. subtilis. However, B. subtilis CcaSR v0.1 did not show any meaningful response to light under any of the conditions tested (Supplementary Fig. 1D). Furthermore, in contrast to E. coli CcaSR v2.028, addition of the ccaS inducer had little effect on transcription from P_cpcG2_172 (Supplementary Fig. 1B, C). Taken together, these results suggested that PCB was poorly expressed, ccaS was poorly expressed, and/or CcaS was non-functional.

### Debugging and optimizing PCB production.

Cph1(Y176H) is an engineered Synechocystis PCC6803 phytochrome protein that becomes weakly red fluorescent upon PCB-binding3,39. We set out to utilize this protein to examine PCB levels produced from PPM v0.1 (Fig. 1a). To ensure that we could detect the fluorescent signal, we expressed cph1(Y176H) from our recent LacI-T7 promoter system, which results in very strong IPTG-inducible protein expression40. As a positive control, we added purified PCB (see “Methods” section) to B. subtilis cells overexpressing cph1(Y176H) with saturating IPTG. Surprisingly, the bacteria were very weakly red fluorescent (15.7 ± 1.9 Molecules of Equivalent Allophycocyanin, MEAP) (Supplementary Fig. 3A, B) (see “Methods” section). We suspected that cph1(Y176H) was poorly expressed. To examine this possibility, we constructed a cph1(Y176H)-sfGFP translational fusion and measured green fluorescence as before. Indeed, saturating IPTG yielded only 113 ± 27 MEFL of green fluorescence (Supplementary Fig. 3C, D) compared to 432,000 ± 20,000 MEFL when sfGFP is expressed directly from the same LacI-T7 system40.

Highly expressed bacterial genes tend to exhibit little mRNA secondary structure near the RBS41-44. Computational analysis revealed that our synthetic RBS MF001 formed stable secondary structure with the initial cph1(Y176H) ORF sequence (Supplementary Fig. 3E). To increase expression, we replaced several of the first 15 codons with synonymous versions (resulting in cph1(Y176H)*) to predict the reduction in the problematic structure (Supplementary Fig. 3F). Indeed, this recoding process yielded a nearly 200-fold increase in IPTG-induced Cph1(Y176H)-sfGFP levels (20,100 ± 1,600 MEFL, Supplementary Fig. 3D). As expected, induction of cph1(Y176H)* resulted in much stronger red fluorescence (848 ± 52 MEAP) in the presence of exogenously supplied PCB (Supplementary Fig. 3B).

We next integrated PPM v0.1 into our cph1(Y176H)* strain, and measured red fluorescence to assay PCB production (Fig. 2, see “Methods” section). Unexpectedly, fluorescence remained below our limit of detection (Fig. 2). As with cph1(Y176H), we suspected that ho1 and/or cyaA may be poorly expressed leading to low PCB levels. Indeed, sfGFP translational fusions to these two enzymes resulted in low fluorescence values (246 ± 14 MEFL; Supplementary Figs. 4, 5) compared to a control strain where only sfGFP was expressed from P_rpsL (1580 ± 190 MEFL) (Supplementary Fig. 6). Thus, we redesigned their expression cassette by separating both genes into independent translational units, codon-optimizing the initial 15 codons of ho1 (resulting in ho1*) and the complete sequence of cyaA (resulting in cyaA**), and introducing a transcriptional terminator downstream of cyaA** to eliminate potential mRNA instability45. These changes resulted in a dramatic increase in expression of both enzymes (21,400 ± 1500 MEFL; Ho1-sfGFP, 8350 ± 400 MEFL for PcyaA-sfGFP) (Supplementary Figs. 4, 5). Thus, a new PPM v0.2 design was constructed based on these modifications (Fig. 2). However, PPM v0.2 produced very little red fluorescence (25.7 ± 6.2 MEAP) via the cph1(Y176H)* PCB probe. This result suggested that high levels of Ho1 and PcyaA were not sufficient for robust PCB production in B. subtilis.

Enzyme colocalization has been shown to increase yield in synthetic metabolic pathways, likely by generating high local concentrations of intermediate metabolites that overcome thermodynamically unfavorable steps34,35. To test whether colocalizing Ho1 and PcyaA could increase PCB yields, we constructed PPM v0.3, wherein ho1* is translationally fused to cyaA** via a flexible linker, and the fusion is again transcribed from P_mSig (see “Methods” section). Strikingly, fusing these enzymes resulted in an 80-fold increase in red fluorescence (2070 ± 130 MEAP, Fig. 2),
which was more than double the fluorescence that occurred with exogenous PCB addition. Interestingly, levels of Ho1-PcyA-sfGFP from PPM v0.3 (19,900 ± 1100 MEFL, Supplementary Fig. 8) were comparable to those of the non-fused enzymes in PPM v0.2. Thus, the dramatically enhanced PCB levels were not due to higher enzyme expression, but likely to more efficient transfer of BV from Ho1 to PcyA. We hypothesized that PPM v0.3 would support robust CcaSR function in B. subtilis.

![Fig. 2 Debugging and optimizing the PCB production module. B. subtilis expressing cph1(Y176H) from LacI-T7 were grown in the absence or presence of IPTG, and in the presence of 10 μM exogenous PCB, or in combination with PPM v0.1, v0.2, or v0.3. The resulting red fluorescence of the Cph1(Y176H)-PCB conjugate was measured via flow cytometry. Bars show the mean of three experiments run on separate days. Black dots show values of individual experimental replicates. N.D.: not detected (see “Methods” section). Source data is available in the Source Data file.]

![Fig. 3 Debugging and optimization of the light-sensing module. a Annotated sequence of the legacy xylose-inducible promoter PxyA in LSM v0.1, obtained from integration plasmid pAX018,81. The –35, –10, and +1 sites82 and xylR operators xylOL and xylOR83 have been previously identified. The operators are followed by an unwanted untranslated region including the xylA RBS, and a vestigial truncated xylA ORF (xylA'). A second, truncated xylA RBS (xylA†) is present in pAX01 to enable translation of a gene of interest placed downstream. Brackets indicate the end of the legacy PxyA promoter, as well as sequential truncations PxyA(+66) and PxyA(+47), where problematic parts of PxyA are eliminated. The start codon of ccaS is shown at the end in bold. b Measurement and optimization of ccaS expression. We first inserted ccaS-sfGFP as in (a). Next, we truncated PxyA to remove the vestigial elements (resulting in PxyA(+66)) and placed ccaS-sfGFP directly after the xylA RBS. Then, we switched the xylA RBS with synthetic RBS MF001 (see “Methods” section) and recoded the initial 15 codons of ccaS. Finally, we replaced the vestigial antisense promoter P3 with synthetic terminators L3S1P52 and L3S2P5684, shortened the PxyA(+66) 5′UTR (resulting in PxyA(+47)), and moved the entire cassette to the xylA chromosomal locus. c Expression of CcaS-sfGFP from each engineered module shown in (b). Bars show the mean of three experiments run on separate days. Dots show values of individual experimental replicates. N.D.: not detected (see “Methods” section). Source data is available in the Source Data file.]

Debugging and optimizing CcaS expression. We next utilized an sfGFP fusion to analyze ccaS expression from LSM v0.1. We observed no detectable fluorescence (Fig. 3), suggesting that ccaS is also very poorly expressed. A detailed analysis of the corresponding DNA sequence revealed several potentially disruptive elements downstream of PxyA that were inherited from our source material, the widely used plasmid pAX0118. In particular, the +1 site and xylR operators were followed by a 59-bp untranslated region (UTR) containing the xylA RBS, the first five codons of the xylA ORF, a stop codon that prematurely terminates this ORF, a second truncated copy of the xylA RBS, and an antisense promoter (Fig. 3). To eliminate any expression problems that could arise from these elements, we removed the vestigial ORF, the truncated xylA RBS, and the UTR after the +1 site. We then swapped the RBS MF001, the initial ccaS ORF as before, and replaced the antisense promoter with terminators. Additionally, to eliminate the possibility of unwanted xylose consumption, we replaced the genomic xylose utilization operon with this new construct (LSM v0.2; Supplementary Fig. 9). As hoped, these changes resulted in a relatively large increase in CcaS expression (520 ± 74 MEFL) (Fig. 3, Supplementary Fig. 10). We hypothesized that LSM v0.2 would be sufficient for B. subtilis CcaSR function.

Optimizing output promoter activity. Strong maximal transcription is desirable for any engineered promoter system, including our optogenetic system. We next examined the activity of P_{cpcG2-172} by comparing it to five B. subtilis constitutive promoters of different strengths under control of the house-keeping σA-RNA polymerase36,46,47 (see “Methods” section). To measure promoter strength in a standardized manner, we designed a promoter characterization module wherein a self-cleaving ribozyme is expressed from different promoters48, thus ensuring that sfGFP fluorescence is a function of promoter strength alone (Supplementary Figs. 11, 12). While expression from the five reference promoters ranged from 1076 ± 100 MEFL to 133,000 ± 12,000 MEFL (Supplementary Fig. 11), maximal CcaR induction resulted in only 555 ± 65 MEFL sfGFP from P_{cpcG2-172} (Fig. 4, Supplementary Fig. 12). Thus, P_{cpcG2-172} is relatively weak in B. subtilis.

To increase P_{cpcG2-172} activity, we first swapped the original −10 hexamer with the consensus B. subtilis sequence to enhance σA-RNA polymerase recruitment. Indeed, this modification increased output expression in the presence of fully induced CcaR nearly 3-fold (1470 ± 110 MEFL; Fig. 4). To further enhance activity, we then swapped the proximal region, or sequence following the −10 hexamer, with those from the two strongest promoters in our constitutive library, P_{minD} and P_{veg}. Indeed, when ccaR is induced, both resulting chimeras (P_{cpcG2-minD} and P_{cpcG2-veg}) exhibited very strong output (32,400 ± 2800 MEFL and 64,000 ± 7600 MEFL, respectively). As expected, we observed low reporter levels when ccaR is not induced (174 ± 23 MEFL and 378 ± 38 MEFL, respectively) (Fig. 4). Fold activation values of both promoters in response to IPTG (186.8 ± 9.3 and 169.1 ± 3.6) are not significantly different (p = 0.10, see “Methods” section). Because output gene expression from a strong promoter can always be brought down via many RBS tuning techniques50,51, we chose to replace P_{cpcG2-172} in TOM v0.1 with P_{cpcG2-veg} (TOM v0.2) due to its superior activity.

B. subtilis CcaSR v0.2 responds to light. Next, we integrated PPM v0.3, LSM v0.2, and TOM v0.2 into the chromosome of a single B. subtilis strain, resulting in B. subtilis CcaSR v0.2. As before, we measured green fluorescence from cultures grown at...
CcaS miniaturization increases dynamic range. To increase the dynamic range of \textit{B. subtilis} CcaSR v0.2, we separately replaced ccaS in Lsm v0.2 with mini-ccaS3 and mini-ccaS10 (hereafter ccaSm3 and ccaSm10), two miniaturized variants that exhibit enhanced dynamic range in \textit{E. coli} \cite{29,30}. Indeed, both of the variants increased dynamic range approximately 10-fold (ccaSm3: 71 ± 12-fold, ccaSm10: 62 ± 12-fold) (Fig. 5, Supplementary Figs. 19, 20). In contrast to \textit{E. coli}, where ccaSm10 resulted in a much larger dynamic range, both variants give rise to statistically identical values in \textit{B. subtilis} (p = 0.49, see “Methods” section). In both organisms, however, ccaSm10 results in overall much lower output expression levels compared to ccaSm3. The reason why this only results in a larger fold-change in \textit{E. coli} is currently unknown. Due to its superior performance, we named the system containing ccaSm3 \textit{B. subtilis} CcaSR v1.0, and carried it forward for further characterization.

Characterization of \textit{B. subtilis} CcaSR v1.0 Light Response. To compare \textit{B. subtilis} CcaSR v1.0 activity to that of our \textit{E. coli} systems, we measured the steady-state transfer function (Fig. 6b) by growing the engineered strain under varying intensities of green light (see “Methods” section). With increasing green light intensity, sfGFP gradually increased from 2000 ± 190 to 108,700 ± 9200 MEFL in a manner well approximated by a Hill function. Fits to the experimental data revealed a Hill coefficient of 1.88 ± 0.16 and a 50% activation intensity of 4.66 ± 0.63 μmol m⁻² s⁻¹ (Fig. 6b). Thus, \textit{B. subtilis} CcaSR v1.0 exhibits analog green light-intensity dependent transcriptional output, as our \textit{E. coli} systems do. Interestingly, this transfer function was more gradual and the 50% activation intensity was more four times higher than in the \textit{E. coli} system (Hill coefficient: 2.737 ± 0.044, 50% activation intensity: 1.075 ± 0.025 μmol m⁻² s⁻¹)\textsuperscript{30}. The origins of these differences are not immediately clear and warrant further investigation.

Finally, we characterized the response dynamics of CcaSR after an instantaneous switch from saturating red light to green light (Fig. 6c) or vice-versa (Fig. 6d). Both responses showed a short delay followed by an exponential-like increase or decrease in sfGFP fluorescence until saturation, with half-maximum activation \((t_{1/2}^{on})\) and deactivation \((t_{1/2}^{off})\) times of 105.1 ± 1.5 and 74.97 ± 0.39 min, respectively (see “Methods” section). These response times are similar to those observed in \textit{E. coli}, albeit slightly slower \((t_{1/2}^{on} = 77 \text{ min}, t_{1/2}^{off} = 62 \text{ min})\textsuperscript{30}. To explain this difference, we note that in our previous \textit{E. coli} work we have used an antibiotic protocol to arrest cell growth and protein synthesis and allow maturation of all intracellular sfGFP\textsuperscript{20}. Because all sfGFP was mature at the time of measurement, the effect of sfGFP maturation kinetics in the observed dynamics was eliminated. In contrast, we have been unable to develop a similar \textit{B. subtilis} maturation protocol. Therefore, sfGFP maturation kinetics slow the observed response dynamics\textsuperscript{52}, especially as expression increases and more immature sfGFP is produced. Thus, these results show that CcaSR exhibits similar dynamic I/O properties in \textit{B. subtilis} and \textit{E. coli}.

Discussion

We have engineered CcaSR as the first optogenetic tool for \textit{B. subtilis}, the leading model Gram-positive bacterium. CBCRs, and their bilin-binding relatives the phytochromes, tend to respond to lower intensities of light than other commonly used optogenetic tools, such as the flavin-binding LOV domain or Cryptochrome 253. Although slightly greater light intensities are required to activate CcaSR in \textit{B. subtilis} as compared to \textit{E. coli}, they are still within the range of intensities used to modulate most other bacterial optogenetic tools\textsuperscript{34-37}. Furthermore, these do not lead to phototoxic effects in \textit{B. subtilis} (Supplementary Fig. 21).

One of the challenges when porting a bilin-binding photoreceptor into a heterologous host organism is chromophore availability. PcyA has evolved to utilize cyanobacterial or plant ferredoxins to produce PCB, and it has been shown to work poorly with Gram-positive ferredoxins\textsuperscript{58}. Recently, two groups achieved efficient PCB production in mammalian cells by expressing a plant ferredoxin and a ferredoxin-NADPH reductase in addition to ho1 and pcyA\textsuperscript{59,60}. In contrast, here we showed that a translational ho1- pcyA fusion can dramatically increase PCB production without additional enzymes. Theoretical models suggest that colocalization alone should not improve yield unless multiple copies of each enzyme are clustered\textsuperscript{35,61}, or the enzymes naturally engage in substrate channeling, the transfer of intermediate metabolites via protein-protein interaction\textsuperscript{62}. Interestingly, channeling may naturally occur between human heme oxygenase and biliverdin reductase in the human heme catabolic pathway\textsuperscript{63}. Our results are consistent with Ho1 and PcyA engaging in substrate channeling, where the flexible linker increases the rate of BV transfer and therefore the metabolic flux. Translational fusions may be a general method to increase biosynthetic pathway yields in cellular environments where some accessory proteins are lacking.

As in \textit{E. coli}, CcaSR should enable unrivaled temporal and spatial control of gene expression in \textit{B. subtilis}. In particular, by
conducting additional step ON and step OFF kinetic measurements at different initial and final light intensities, we should be able to construct a general mathematical model of how CcaSR output gene expression changes over time in response to any time-varying light input signal, following our established methods. Then, we can utilize our biological function generator method to design light signals capable of driving sophisticated gene expression signals with quantitative accuracy.

By encoding regulatory proteins as the output of CcaSR, such signals can be used as a tool to interrogate the dynamical signal processing properties of important cellular decision-making networks such as those involved in stress response, sporulation, and biofilm formation. For example, while the mechanism by which pulses of the master sporulation regulator Spo0A arise has been elucidated, their impact on downstream processes remains unclear. Interestingly, the use of a chemically-inducible promoter to express the constitutively-active mutant Spo0A-sad67 does not trigger sporulation. It has been proposed that fast Spo0A activation leads to early repression of genes that are essential for sporulation, thus leading to non-viable spore formation.

A computational tool that modifies the constitutively-active mutant Spo0A-sad67 could also be ported into B. subtilis and combined with CcaSR to achieve multiplexed control of gene expression dynamics. Such a technology would be particularly useful in examining whether B. subtilis gene circuits require specific combinations of dynamical gene expression signals to function properly.

This work also advances B. subtilis synthetic biology in several ways. First, our results suggest that genes ported into B. subtilis from other organisms may frequently be inefficiently translated due to mRNA secondary structure between the RBS and initial ORF sequence. Since strongly expressed bacterial genes have an initial codon bias that minimizes secondary structure and biofilm formation. For example, while the mechanism by which pulses of the master sporulation regulator Spo0A arise has been elucidated, their impact on downstream processes remains unclear. Interestingly, the use of a chemically-inducible promoter to express the constitutively-active mutant Spo0A-sad67 does not trigger sporulation. It has been proposed that fast Spo0A activation leads to early repression of genes that are essential for sporulation, thus leading to non-viable spore formation. CcaSR could be used to create different dynamical expression patterns of Spo0A-sad67, such as ramps of different slopes and pulses of different amplitudes, frequencies, or phases, in order to understand how the downstream sporulation circuit responds to Spo0A activity dynamics. Moreover, the ability to use light to control gene expression in individual cells could be exploited when studying processes involving noisy or spatially-delimited gene expression patterns.

We have recently characterized the full spectral response properties of CcaSR and the related red/far-red photoreversible sensor Cph8-OmpR to overcome inherent spectral overlap between the systems and achieve simultaneous and independent control of the expression dynamics of two separate genes in E. coli. We believe Cph8-OmpR or other bacterial optogenetic tools could also be ported into B. subtilis and combined with CcaSR to achieve multiplexed control of gene expression dynamics. Such a technology would be particularly useful in examining whether B. subtilis gene circuits require specific combinations of dynamical gene expression signals to function properly.

Fig. 6 Characterization of B. subtilis CcaSR v1.0 input/output properties. a Schematic of the final B. subtilis CcaSR v1.0 design. b Steady-state transfer function. c Step ON response. d Step OFF response. Dots and error bars show the mean and standard deviation, respectively, of three experiments run on separate days. Black lines represent model fits (see “Methods” section). Source data is available in the Source Data file.
recognizes the need for a well-insulated expression cassette in *B. subtilis*\(^{46}\), plasmids for C-terminal fusions to lacZ or other reporter genes offered by the Bacillus Genetic Stock Center are not properly insulated. It is possible that incorporation of a single terminator could greatly improve the limit of detection of future assays based on these plasmids.

The sequence determinants of the strength of promoters controlled by the housekeeping sigma factor \(\sigma^\text{B}\) are classically thought to be the \(-35\) and \(-10\) hexamers, and to a lesser extent a \(-16\) region and a A/T rich sequence upstream of the \(-35\) hexamer\(^{38-39}\). Here we show that the sequence downstream of the \(-10\) hexamer can greatly affect transcription as well. We found that \(P_{\text{PDEFZ-172}}\) output was low compared to a small library of constitutive promoters. To engineer a stronger promoter while keeping the CcaR operators intact, we swapped the sequence after \(P_{\text{PDEFZ}}\). Genomic homology fragments required for chromosomal integration were amplified using standard transformation methods. The transformants were plated on a strain PY79. A list of all strains constructed in this study can be found in Supplementary Data 3, and their complete sequences can be found on Genbank via the specified accession numbers. All other strains are available from the corresponding author on reasonable request.

In conclusion, we have engineered the first, to our knowledge, *B. subtilis* optogenetic system. *B. subtilis* CcaSR v1.0 should enable precise temporal and spatial control of gene expression in *B. subtilis*, and thus be widely used to study complex cellular processes such as sporulation, stress response, and biofilm formation, among others. We have also unveiled several design principles that should be of great utility in future *B. subtilis* synthetic biology applications.

**Methods**

**DNA and strain construction.** All cloning and experiments were performed in *B. subtilis* strain PY79. A list of all strains constructed in this study can be found in Supplementary Data 1. Strains with each of the final CcaSR modules (PPM v0.3, LSM v0.3a, TOM v0.2), the complete CcaSR v1.0 system, and the optimized cph1(Y176H)* v0.3, LSM v0.3a, TOM v0.2), the complete CcaSR v1.0 system, and the optimized cph1(Y176H)* were generated. A list of all strains constructed in this study can be found in Supplementary Data 3, and their complete sequences can be found on Genbank via the specified accession numbers. All other strains are available from the corresponding author on reasonable request.

Primers were ordered from Integrated DNA Technologies, Inc. All TCS genes were amplified from previous *E. coli* CcaSR plasmids\(^{38}\), Cph1(Y176H) was amplified from the PAM2 chimera Cph8 in pSR33.428. pAM2 Cph8 was designed using GeneOptimizer\(^{71}\) and ordered as a gBlock from Integrated DNA Technologies, Inc. The xylene and IPTG-inducible cassettes were amplified from integration plasmids pX0318 and pDR1119. Constitutive promoters were constructed via oligo annealing and extension. Sequences of \(P_{\text{PAM2}}, P_{\text{PAM4}}, P_{\text{PEG}}\) comprise bases from the \(-66\) to the +10 (\(P_{\text{PAM2}}, P_{\text{PEG}}\)) or +12 (\(P_{\text{PAM4}}\)) position of the corresponding promoters of the BioBricks library\(^{27}\). The sequence of \(P_{\text{PAM3D}}\) comprises bases from the \(-58\) to the +16 position of the corresponding chromosomal promoter\(^{40}\). \(P_{\text{PEG}}\) is identical to \(P_{\text{PAM4}}\)-derived \(P_{\text{PEG}}\) but with the unmodified \(P_{\text{AM3}}\)-derived 5′UTR. Synthetic RBS MF01 was obtained from integration plasmids pMFP352.\(^{2}\) Genomic homology fragments required for chromosomal integration were amplified from the purified genome of *B. subtilis* PY79. A list of genetic parts, along with their sequences, can be found in Supplementary Data 2. All systems were built as linear double-stranded integration modules (IMs)\(^{73}\). The IMs contain the DNA of interest and a selection marker flanked by 1,5-kb-long sequences homologous to a region of the *B. subtilis* genome (integration locus) where chromosomal integration via double crossover occurs. IMs were assembled from PCR-amplified parts using GoldenGate\(^{44}\). The resulting Golden Gate products were amplified using NEB Phusion DNA Polymerase and gel purified to obtain the IM. 500 ng or more of each IM was transformed into competent *B. subtilis* cells using the BioBricks transformation method depending on initial density of colonies. Colonies were picked the next day and grown in LB media at 37 °C and 250 RPM for ~2 h until turbidity is visible. Finally, freezer stocks were prepared with 700 μL culture and 300 μL 60% glycerol, and stored at ~80 °C. This method avoids subcloning of integration plasmids in *E. coli*, as long as each PCR amplified DNA is obtained. A list of all IMs constructed in this study can be found in Supplementary Data 3, and their complete sequences can be found on Genbank via the specified accession numbers.

For sequence verification, an overnight LB culture was grown from a freezer stock, and 2 μL saturated culture was used as template for a 50 μL PCR reaction, either with Taq or Phusion DNA Polymerase. PCR products obtained in this fashion were gel-purified and sent for sequence verification to Genewiz, Inc. To construct strains with two IMs, two separate strains containing each IM were cloned and sequence-verified independently, as described above. Next, one of these strains was made competent, and the genomic DNA of the second was extracted from an overnight culture using the Promega Wizard Genomic DNA Purification Kit. Next, 500–1000 ng of purified genomic DNA was transformed into the competent strain as described above to produce both IMs. Correct integration of both modules was verified via PCR. If necessary, a strain with two IMs was made competent and a third IM was integrated via the same procedure.

**Codon optimization.** For each of the first 15 codons, a synonymous codon was chosen to reduce GC and increase AU content, with A preferred over U, with no four consecutive codon triplets. The free energy of the resulting RNA strand was calculated via Nupack\(^{53}\) using the sequence from the transcription start site up to the 90th nucleotide residue of the ORF.

**Media and experimental protocols.** We used a modified M9 media for experiments. 1 L 5× M9 salts at pH ~6.8 were prepared with 64 g Na2HPO4, 7H2O, 15 g KH2PO4, 2.5 g NaCl, 5 g NH4Cl, 9.2 mL 6 M H2G, and up to 1 L D2O. For 1 L M9, we used 200 mL 5× M9 salts, 20 mL 10% casamino acids, 6.67 mL 60% glycerol, 1 mL 50 mM FeCl3/100 mM C2H6O2 solution, 2 mL 50 mM MnSO4, 2 mL 1 M MgSO4, 100 μL 1 M CaCl2, and D2O up to 1 L. Glycerol was used as a carbon source since glucose strongly reduces expression from \(P_{\text{PAM2}}\). The FeCl3 solution appears necessary to support robust growth of PCB-producing strains. For every experiment, an overnight LB culture was grown from a freezer stock of each relevant strain. The next day, saturated cultures (OD600 ~3) were diluted 104-105-fold in M9. For non-optogenetic experiments, media was distributed in culture tubes (3 mL per tube), inoculated with the appropriate inducers, and incubated in a shaker operating at 250 RPM and 37 °C until the OD600 reached between 0.08 and 0.15 (6-9 h depending on the strain). When indicated, saturating concentrations of xylene (0.5%) and IPTG (500 μM) were used. Inducer concentrations used in Fig. 6 were 0.00917% xylene and 10.0 μM IPTG. In optogenetic experiments, media was distributed (500 μL per well) in 24-well dark-walled clear-bottomed plates (ArcticWhite AWLS303008). Next, the appropriate inducers were added, and plates were sealed with adhesive foil (VWR 60941-126). Plates were mounted onto LPAs running the appropriate light exposure programs, and incubated until the OD600 reached between 0.08 and 0.15. Culture tubes or plates were then transferred to ice. 100 μL of each sample was transferred to a flow cytometer tube containing 1 mL PBS for measurement. PCB was purified from Spirulina powder using methanolysis, as previously described\(^{76}\). Samples, where purified PCB was added, were incubated in culture tubes at 250 RPM and 37 °C for 5 h. Next, 10 μM purified PCB was added under a green safelight, and tubes were returned to the incubator for 1 h. At the end of the experiment, tubes were placed on ice and Cph1(Y176H) fluorescence was measured via flow cytometry.

**Optical hardware.** Eight 24-well Light-Plate Apparatuses (LPAs)\(^{77}\) equipped with green (520-2 KB, WP7083ZGD/C, Kingbright, CA, USA) and red (660-LS, L2-0- RST50-1, LEDSupply, VT, USA) LEDs were used for all optogenetic experiments. These were mounted in a shaking incubator operating at 250 RPM and 37 °C. Total LED power output in μmol s\(^{-1}\) was measured using a spectrometer (StellarNet UVN-SR-25 LT16) attached to a six-channel integrating sphere (StellarNet IS6). The average light intensity was calculated by dividing the total power output by the area of a circular plate well with a radius of 7.5 mm, as previously\(^{32}\). To calibrate the power output of each LED, we adjusted the current using the LPA Dot Correction setting to achieve saturating intensities of 20 μmol m\(^{-2}\) s\(^{-1}\) or more for red LEDs and 50 μmol m\(^{-2}\) s\(^{-1}\) or more for green LEDs. Each LED was measured while powered from the same LPA socket used in experiments. The precisely measured intensities and dot correction values were recorded. Custom Python scripts were written to use these recordings to achieve light intensities necessary in each experiment.

**Flow cytometry analysis.** The sfGFP fluorescence distribution of each *B. subtilis* culture sample was measured using a BD FACScan flow cytometer with an excitation source of 488 nm and an emission window of 520–522 nm. 10,000–30,000 events were collected per sample. A suspension of calibration beads (Spherotech RCP-30-5A) in PBS was measured with each experiment. After data acquisition, raw.fcs flow cytometry files were processed using FlowCal\(^{36}\). Cell populations were gated by forward scatter/size scatter density (Supplementary Fig. 24), retaining 50% of the total number of events. Next, fluorescence of each gated event in arbitrary units was converted into standardized MEFL (Molecules of Equivalent Fluorescence) values using the calibration bead data. The cell fluorescence of each culture sample was then obtained from the median MEFL fluorescence of all gated events in that sample. Finally, the sfGFP fluorescence was obtained by subtracting the fluorescence of each sample from the cell fluorescence of a control sample lacking any fluorescent reporter gene measured the same day (autofluorescence control). The strain used as an autofluorescence control for any strain containing
PPM v0.3 was a strain containing the PPM v0.3 only. The autofluorescence strain for any other strain was a wild type PT79. The single cell Cph1(Y176H) fluorescence distribution was measured using a BD FACSCanto II. The fluorescent channel used (APC-A) was used a 633 nm red laser as the excitation source, and an emission window of 670/50 nm. Calibration beads were measured as above. FlowCal was used to gate cell populations and calibrate fluorescence data to MEAP (Molecules of Equivalent Allophycocyanin). The cell fluorescence of each culture sample was then obtained from the median MEAP fluorescence of all gated events in that sample. Finally, the Cph1(Y176H) fluorescence was obtained by subtracting the cell fluorescence of each sample from the cell fluorescence of a control wild type PT79 sample.

Statistical analysis. Each experiment was replicated three times over different days. Fluorescence of each sample is reported as the mean ± std dev. of the sfGFP fluorescence of all gated events in that sample. Supplementary Figs. 14D and 15D, Kd2 is the inducer concentration for half-maximum activation, and k is the Hill coefficient. Fitting was performed using the LmFit python package79 with the Levenberg-Marquardt algorithm. Experimental data from three replicates were combined and fitted simultaneously. To adequately fit low and high fluorescence values, the error to minimize was defined as the difference between the logarithm of a fluorescence data point and the logarithm of the model prediction. Fitted parameter values and their uncertainties can be found in Supplementary Data 4.

Kinetic response modeling and fitting. The response of the CcaSR system to an instantaneous change in light intensity was modeled as a differential equation system of the form:

\[
\frac{dp}{dt} = k_p \cdot (p(t) - \bar{p}(t))
\]

\[
\frac{dc}{dt} = k_p \cdot (c(t) - \bar{c}(t))
\]

\[
\frac{dG}{dt} = k_G \cdot (G(t) - \bar{G}(t))
\]

where, \( p(t) \) represents the sfGFP production rate, \( \bar{p}(t) \) represents immature sfGFP, and \( G(t) \) is the fully mature, observed sfGFP. Their dynamics are determined by rate constants \( k_p \), \( k_c \), and \( k_G \). \( \tau \) represents the delay before a change in sfGFP production actually occurs. Finally, \( c \) is the system input, and is a function of light intensities \( I_p \) and \( I_c \). Units for \( c, p(t), \) and \( G(t) \) have been chosen such that, in steady state, \( c = p = G = \bar{c} = \bar{p} = \bar{G} = \text{const} \) and thus \( c \) determines the steady state output fluorescence. To simulate an instantaneous change from red to green light (Fig. 6c), the model was solved with initial conditions \( p(0) = \bar{p}(0) = G(0) = \bar{G} = c = \bar{c} = \bar{G} = \text{const} \) and \( c = \bar{c} \), \( G \), and \( \bar{G} \) obtained from the Hill function fit. Similarly, an instantaneous change from green to red light (Fig. 5d) was simulated with \( p(0) = \bar{p}(0) = G(0) = \bar{G} = c = \bar{c} = \bar{G} = \text{const} \). Fitting was performed using LmFit as described above.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Sequences of all integration modules used in this study are available from Genbank via the accession numbers listed in Supplementary Data 3. Flow cytometry data and scripts used to generate all figures are available from figshare (https://doi.org/10.1088/m4989/8198995899). Parameters of Hill function fits can be found in Supplementary Data 4. Source data for Figs. 2–6 and Supplementary Figs. 1, 8, 10–22 can be found in the Source Data file. Any other relevant data can be obtained from the authors upon reasonable request.

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Author contributions
S.M.C., M.F., O.A.I., and J.J.T. conceived of the project. S.M.C. designed and built DNA constructs. S.M.C. and E.A.B. collected data. S.M.C. performed all data analyses. O.A.I. and J.J.T. supervised the project. S.M.C., M.F., O.A.I., and J.J.T. wrote the manuscript.

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