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# An Engineered Constitutive Promoter Set with Broad Activity Range for *Cupriavidus necator* H16

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S *Supporting Information*

ABSTRACT: Well-characterized promoters with variable strength form the foundation of heterologous pathway optimization. It is also a key element that bolsters the success of microbial engineering and facilitates the development of biological tools like biosensors. In comparison to microbial hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*, the promoter repertoire of *Cupriavidus necator* H16 is highly limited. This limited number of characterized promoters poses a significant challenge during the engineering of *C. necator* H16 for biomanufacturing and biotechnological applications. In this article, we first examined the architecture and genetic elements of the four most widely used constitutive promoters

of *C. necator* H16 (i.e., P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25}) and established a narrow 6-fold difference in their promoter activities. Next, using these four promoters as starting points and applying a range of genetic modifications (including point mutation, length alteration, incorporation of regulatory genetic element, promoter hybridization, and configuration alteration), we created a library of 42 constitutive promoters, all of which are functional in *C. necator* H16. Although these promoters are also functional in *E. coli*, they show different promoter strength and hierarchical rank of promoter activity. Subsequently, the activity of each promoter was individually characterized, using L-arabinose-inducible P_{BAD} promoter as a benchmark. This study has extended the range of constitutive promoter activities to 137-fold, with some promoter variants exceeding the L-arabinose-inducible range of P_{BAD} promoter. Not only has the work enhanced our flexibility in engineering *C. necator* H16, it presented novel strategies in adjusting promoter activity in *C. necator* H16 and highlighted similarities and differences in transcriptional activity between this organism and *E. coli*.

KEYWORDS: *Cupriavidus necator H16, Ralstonia eutropha H16, gene expression, constitutive promoter, synthetic biology, metabolic engineering*
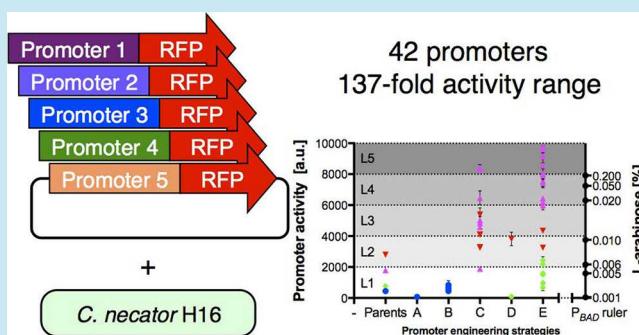
Cupriavidus necator H16 (or *Ralstonia eutropha* H16) is a chemolithoautotrophic soil bacterium, most widely known for its ability to accumulate polyhydroxyalkanoates (PHA).[1] This metabolically versatile organism is capable of utilizing a wide range of energy and carbon sources (including H_2 and CO_2) to support growth and achieving high cell density.[2] These intrinsic properties have cemented its potential applications in biological CO_2 capture and utilization[3,4] as well as commercial-scale production of diverse bioproducts[5] including polymers,[6−9] hydrocarbons,[10−14] and amino acids.[15] Its potential will rapidly come into fruition, aided by the development of molecular tools. These include genome engineering methods to permanently alter its metabolic phenotype,[16] expression vectors to assemble heterologous pathways,[8,17] transposon-based random mutagenesis,[18] and transformation method to introduce recombinant plasmids.[19]

Maximal product yield and titer are key requirements in biomanufacturing. To this end, metabolic pathway optimization is vital in eliminating metabolic bottlenecks that compromise cellular productivity and metabolic phenotypes

that are detrimental to cell viability. Proven strategies of tuning gene expression of a metabolic pathway include varying plasmid copy number, gene dosage, and promoter strength, among others. Leveraging on promoter strength for pathway optimization is the most straightforward strategy, which involves tuning promoter activity at both transcriptional and translational levels.

L-Arabinose-inducible P_{BAD} promoter and anhydrotetracycline-inducible P_{tet} promoter are most widely applied to tune expression of genes, gene clusters, or operons in *C. necator* H16.[20−23] With a P_{BAD} promoter, high inducer concentration (up to 1 g/L) is required to achieve high expression yield. The leaky P_{tet} promoter, on the other hand, is induced by a weak antibiotic that is undesirable and its promoter strength is comparatively weaker. These factors greatly limit the use of these two promoters for large-scale fermentation. The more recently developed 3-hydroxypropionic acid-inducible sys-
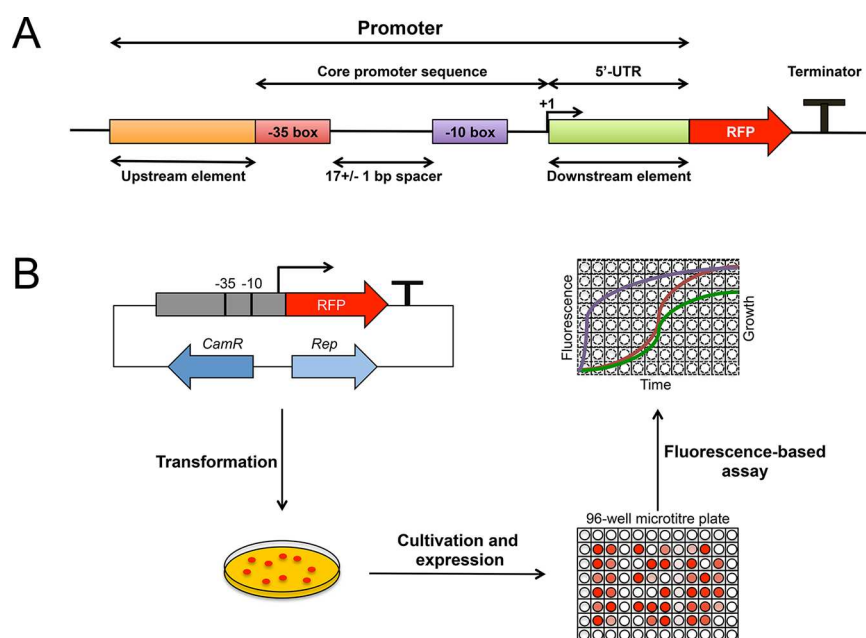
Figure 1. (A) Promoter definition used in this study. (B) High-throughput characterization of engineered promoters using a fluorescence-based assay (5′-UTR, 5′-untranslated region; bp, base pair; *CamR*, chloramphenicol resistance gene; *Rep*, replication gene; RFP, red fluorescent protein).

tems[24] and the *p*-cumate- and IPTG-inducible P_{j5} promoters[25] are promising alternatives. Nonetheless, achieving scalable and tunable gene expression of a multigene pathway by solely relying on inducible expression systems is severely limited by the poor modularity of inducer-based systems (or potential risk of unwanted inducer crosstalk) and a limited number of *C. necator* H16-compatible inducible promoters. Further, the use of inducers such as L-arabinose or anhydrotetracycline on a large scale is commercially uneconomical.

In this regard, engineering constitutive promoters with a broad range of activities is a more facile means to modularly adjust gene expressions of a multigene pathway to the desired levels or ratios. In addition to facilitating static metabolic control, constitutive promoters are used to engineer more efficient inducible promoters[23] and to construct metabolite-sensing genetic circuits that in turn facilitate dynamic metabolic control in microorganisms.[26] Examples of constitutive promoters for use in *C. necator* H16 include P_{lac} and P_{tac} promoters, native *C. necator* H16 promoters such as P_{phaC1} promoter, and coliphage T5 promoter and its variants such as P_{j5}, P_{g25}, P_{n25}, and P_{n26} promoters.[17,21,27] Despite these precedent studies, there are knowledge gaps that hinder constitutive promoter utilization and engineering for *C. necator* H16, which are: (1) lack of a universal definition of promoter architecture, (2) lack of a universal reference scale for hierarchical ranking of constitutive promoter activities, and (3) limited examples of rational promoter engineering. The latter is in stark contrast to promoter engineering reported for *E. coli* and yeast.

In this study, we first examined the architecture of four notable *C. necator* H16-compatible constitutive promoters: the native P_{phaC1} promoter, a semisynthetic P_{rrsC} promoter, and two coliphage T5 promoters, P_{j5} and P_{g25}. We then evaluated their activities using *in vivo* fluorescence measurement of red fluorescent protein (RFP) expression to establish an understanding of the relationship between promoter architecture and activity. Guided by these structure−function relationships, we next proceeded to rational engineering of these four parental

promoters. Our engineering strategies include combinations of point mutation, length alteration, incorporation of regulatory genetic element, promoter hybridization, and configuration alteration. This resulted in a collection of 42 promoters displaying a range of promoter activities. Of these, there are composite promoter variants that are stronger than the P_{j5} promoter; the latter has previously been acclaimed to be the strongest known constitutive promoter for gene expression in *C. necator* H16.[17] This new promoter library is envisaged to further propel the biotechnological applications of *C. necator* H16.

■ RESULTS AND DISCUSSION

Defining a Promoter and Quantifying Its Activity. Standardizing the definition of a promoter is deemed necessary and particularly relevant to this study for four obvious reasons: (1) to objectively benchmark the activities of wildtype and engineered promoters, (2) to critically assess promoter structure−function relationships, (3) to measure the effectiveness of various promoter engineering strategies, and (4) to compare promoters reported by various research groups. In this study, we describe a promoter as a constellation of three distinct genetic elements as shown in Figure 1A: (part 1) a core promoter sequence comprising −35 box, −10 box (or the Pribnow box), +1 transcriptional start, spacer of 16−18 bp between the −35, and the −10 boxes as well as the spacer between the −10 box and +1 site, (part 2) an upstream element (UP) that refers to the entire DNA sequence upstream of the core promoter sequence, and (part 3) a downstream element spanning the nucleotide after the +1 transcriptional start and the nucleotide before the translation initiation codon. Therefore, the 5′-untranslated region (5′-UTR) consists of the +1 transcriptional start and the downstream element, with the latter typically containing *cis*-acting regulatory elements such as the ribosome binding site (RBS). Putting it simply, a promoter is a defined stretch of sequence upstream of the translational start. This definition is not uncommon in genome annotation, particularly when the
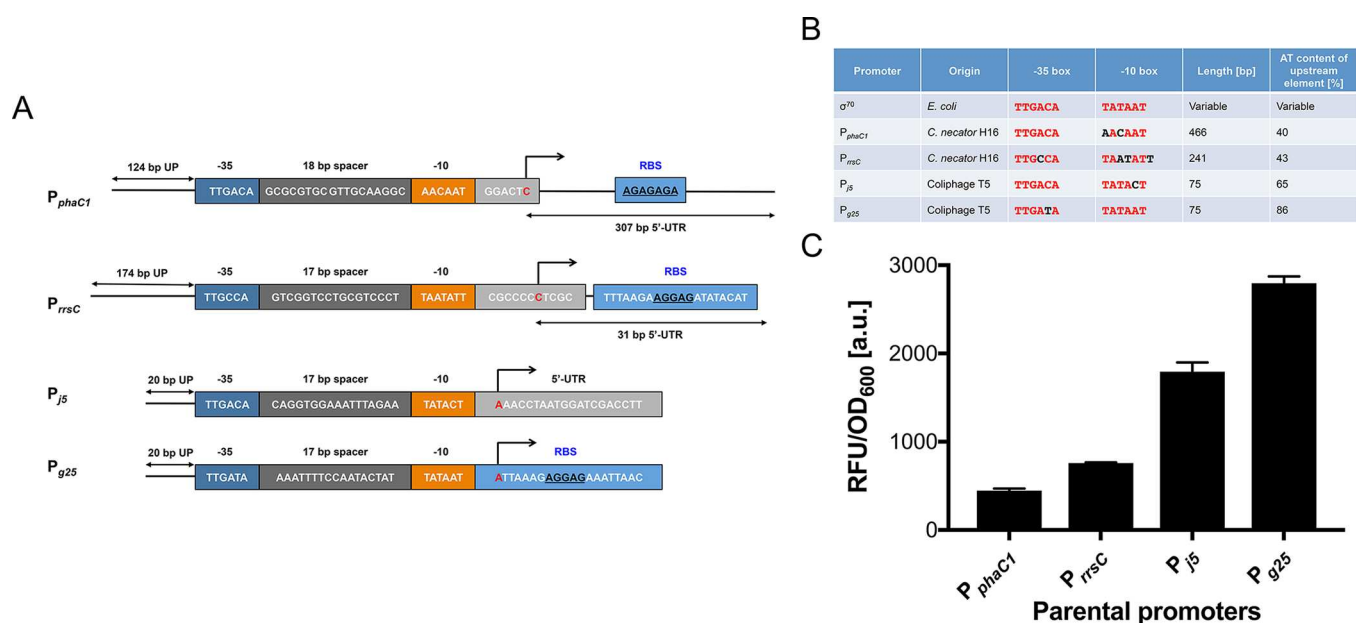
Figure 2. (A) Boundaries and architectures of the four parental promoters, P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25}, used in this study. (B) Comparison of the four parental promoters to an *Escherichia coli* σ^{70} promoter. (C) Activities of the four parental promoters.

promoter boundary is unclear or ambiguous. This definition also presupposes that the functional characteristics of a given promoter are composite effect of all genetic elements within the predefined promoter architecture. To quantify promoter activity in *C. necator* H16, RFP was used as a reporter protein (Figure 1B). Briefly, DNA fragments corresponding to a promoter and *rfp* gene were cloned, in tandem, into a broad host range pBBR1MCS plasmid backbone harboring a chloramphenicol resistance gene (Figure 1B and Supporting Information, Figure S1).

Defining the Boundaries and Architectures of Four Parental Promoters. Because the four parental promoters, P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25}, form the basis of this entire study, clearly defining their boundaries and examining their architectures are essential such that all subsequently engineered promoters can be compared against these four "standards". All four parental promoters contain −35 box and −10 box that are almost identical to the hexameric promoter consensus sequences recognized by the *E. coli* housekeeping sigma factor σ^{70} (Figure 2A,B).[28] P_{phaC1} is known to be a relatively strong native promoter.[21,23,27] On the basis of the data reported by Fukui et al., the strength of P_{phaC1} is ~58% that of P_{tac}.[21] It has been widely studied and applied for improved PHA-based biopolymer production in *C. necator* H16.[21,27] However, P_{phaC1} promoters of different lengths are used in various studies, making objective comparison impossible. In this study, P_{phaC1} promoter is defined as a 466-bp DNA sequence upstream of the translation start of the *phaC1* gene. Previous studies of this promoter affirmed the presence of a 7-bp "AGAGAGA" Shine−Dalgarno (SD) sequence within its 5′-UTR. This native RBS is located 11 bp upstream of the translational start.[27] The P_{rrsC} promoter used in this study is a combination of a 210-bp DNA sequence upstream of the +1 transcriptional start of the native *rrsC* gene, the first 5-bp of the native 5′-UTR and a 26-bp synthetic genetic element. The latter comprises a 20-bp RBS found in the pBBR1c-RFP P_{BAD} promoter (see Supporting Information) flanked by an upstream 6-bp *Bgl*II restriction site. The

synthetic RBS contains a purine-rich 5-bp "AGGAG" SD sequence known to markedly improve translation efficiency. This P_{rrsC} is almost identical to the one used in Li and Liao,[23] with only minor difference in the downstream element that contains an RBS. Finally, the P_{g25} and P_{j5} promoters used in this study are both 75-bp DNA sequences, identical to those previously reported by Gentz and Bujard.[29]

Narrow Range of Promoter Activities between the Four Parental Promoters. Fluorescence measurement of RFP expression revealed a narrow range of promoter activities (defined as relative fluorescence unit normalized by optical density; see Methods and Materials) between the four parental promoters (Figure 2C). The ratio of promoter activities between the strongest (P_{g25}) and the weakest (P_{phaC1}) is only 6.3. The promoter activity of P_{rrsC} is 1.7-fold higher than that of P_{phaC1}. This difference may be attributed to the synthetic RBS in P_{rrsC} being more effective in promoting translation compared to the native RBS in P_{phaC1}. The coliphage T5 promoters, P_{j5} (75 bp) and P_{g25} (75 bp), are much shorter in length compared to P_{phaC1} (466 bp) and P_{rrsC} (241 bp). They have previously been reported as some of the strongest constitutive promoters in *E. coli*.[29] The strong transcriptional activity was also verified in *C. necator* H16.[17] The A/T rich sequence of many coliphage T5 promoters, particularly in their upstream elements, has been implicated in accounting for their high transcriptional activity.[29] Indeed, the upstream elements of both P_{j5} and P_{g25} promoters show high A/T contents (65% for P_{j5} and 85% for P_{g25}), and the difference in the A/T content may partly be responsible for the higher activity of P_{g25} relative to P_{j5} (Figure 2B). In addition, P_{g25} possesses within its 5′-UTR the purine-rich 5-bp SD sequence used in most commercially available pET and pBAD vectors, which is lacking in P_{j5} (Figure 2A). Important to point out, Gruber et al. had an opposite observation that the strength of P_{j5} was stronger than that of P_{g25}.[17] The promoter sequences used by Gruber et al. were different compared to those used in this study, although the core promoter sequences remain identical (Supporting Information, Figure S2). Promoters in Gruber et

Table 1. Summary of 42 Parental Promoters and Their Variants Engineered Using a Combination of Promoter Engineering Strategies (A = Point Mutation, B = Length Alteration, C = Incorporation of Regulatory Genetic Element, D = Promoter Hybridization, and E = Configuration Alteration)[a]

parental promoter	A (point mutation)	B (length alteration)	C (incorporation of regulatory genetic element)	D (promoter hybridization)	E (configuration alteration) transcriptional amplifier (secondary promoter) P_{phaC1}	P_{g25}	$P_{j5[A1C1C2]}$
P_{phaC1} [1-6-466]	$P_{phaC1[A1]}$ [1-1-466]	$P_{phaC1[B1a]}$ [1-8-441] $P_{phaC1[B1b]}$ [1-10-416] $P_{phaC1[B1c]}$ [1-6-366] $P_{phaC1[B1d]}$ [1-12-342]					
P_{rrsC} [1-11-241]				$P_{rrsC[D6]}$ [1-1-258] $P_{rrsC[D4]}$ [1-1-241]	$P_{rrsC[E1]}$ [2-34-713] $P_{rrsC[E1D6]}$ [2-31-730] $P_{rrsC[E1D4]}$ [1-14-713]		$P_{rrsC[E3]}$ [1-22-434] $P_{rrsC[E3D6]}$ [1-21-451] $P_{rrsC[E3D4]}$ [1-9-434]
P_{j5} [1-25-75]			$P_{j5[C1]}$ [1-26-136] $P_{j5[C1C2]}$ [4-91-162] $P_{j5[A1C1C2]}$ [3-64-162] $P_{j5[A1D6aC1C2]}$ [3-70-179] $P_{j5[A1D6bC1C2]}$ [3-68-196] $P_{j5[C2]}$ [5-117-101] $P_{j5[A3C2]}$ [5-116-100]		$P_{j5[E1C1C2]}$ [5-120-634] $P_{j5[E1A1C1C2]}$ [4-104-634] $P_{j5[E1A1D6aC1C2]}$ [4-112-651] $P_{j5[E1A1D6bC1C2]}$ [4-111-668] $P_{j5[E1C2]}$ [5-134-573] $P_{j5[E1A3C2]}$ [5-137-572]	$P_{j5[E2C1C2]}$ [4-106-268] $P_{j5[E2A1C1C2]}$ [4-84-268] $P_{j5[E2A1D6aC1C2]}$ [4-90-285] $P_{j5[E2A1D6bC1C2]}$ [4-87-302] $P_{j5[E2C2]}$ [5-125-207] $P_{j5[E2A3C2]}$ [5-128-206]	
P_{g25} [2-39-75]			$P_{g25[C2]}$ [2-57-101] $P_{g25[D1C2]}$ [3-75-156]	$P_{g25[D1]}$ [2-53-130]	$P_{g25[E1]}$ [3-61-547]	$P_{g25[E2]}$ [2-46-181]	$P_{g25[E3]}$ [2-45-268]

[a]Numbers in bracket represent promoter digital identifier, in the format of [activity level − relative activity to $P_{phaC1[A1]}$ − promoter length].

al. were essentially a core promoter sequence flanked by NotI and EcoRI restriction sites. Further, they both contain an RBS of T7 gene 10, downstream to the core promoter sequence. These differences could explain the discrepancy between the two studies. It further emphasizes the necessity to define a promoter for objective comparison of promoters used, created or engineered by various researchers.

Our initial examination of the four parental promoters highlighted two critical points which guided our subsequent promoter engineering: (1) conservation of −35 and −10 boxes is important for maintaining high transcriptional activity in *C. necator* H16, and (2) promoter length or A/T content or *cis*-acting genetic regulatory element (such as a synthetic RBS) could potentially influence transcriptional activity in *C. necator* H16 significantly.

Expanding the Range of Promoter Activities through Rational Engineering. Informed by our understanding of the four "standard" promoters, we proceed to increase the range of promoter activities beyond the existing 6.3-folds between P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25}. Our objectives are threefold: (1) creating both weaker ("tuning down") and stronger ("tuning up") promoter variants to further expand the promoter activity range, (2) generating promoter variants that exceed or at least cover the entire L-arabinose inducible range of P_{BAD} promoter,

and (3) developing a set of promoters with gradual increase in activity (i.e., having promoters with activities evenly distributed across the entire promoter activity scale).

To this end, we applied a range of rational engineering approaches. These strategies, summarized in Supporting Information, Table S1 and Figure S3, can be loosely classified into five categories: (A) point mutation, (B) length alteration, (C) incorporation of regulatory genetic element, (D) promoter hybridization, and (E) configuration alteration. Category A, point mutation, includes base substitution, single-base insertion, and single-base deletion. Category B, length alteration, refers to truncation or extension of a promoter from either terminus and insertion or deletion of a stretch of random DNA sequence. Incorporating *cis*-acting translational regulatory elements such as T7 stem-loop and RBS are grouped within category C. Category D, promoter hybridization, encompasses both creating hybrid promoters and incorporating *cis*-acting transcriptional regulatory element such as an operator. Category E, configuration alteration, involves transcriptional amplification using a secondary promoter that is placed in divergent configuration to a primary promoter. In other words, composite promoters are placed in category E. Each category is further divided into subcategories (e.g., C1 for T7 stem-loop and C2 for RBS in category C) and subsub-
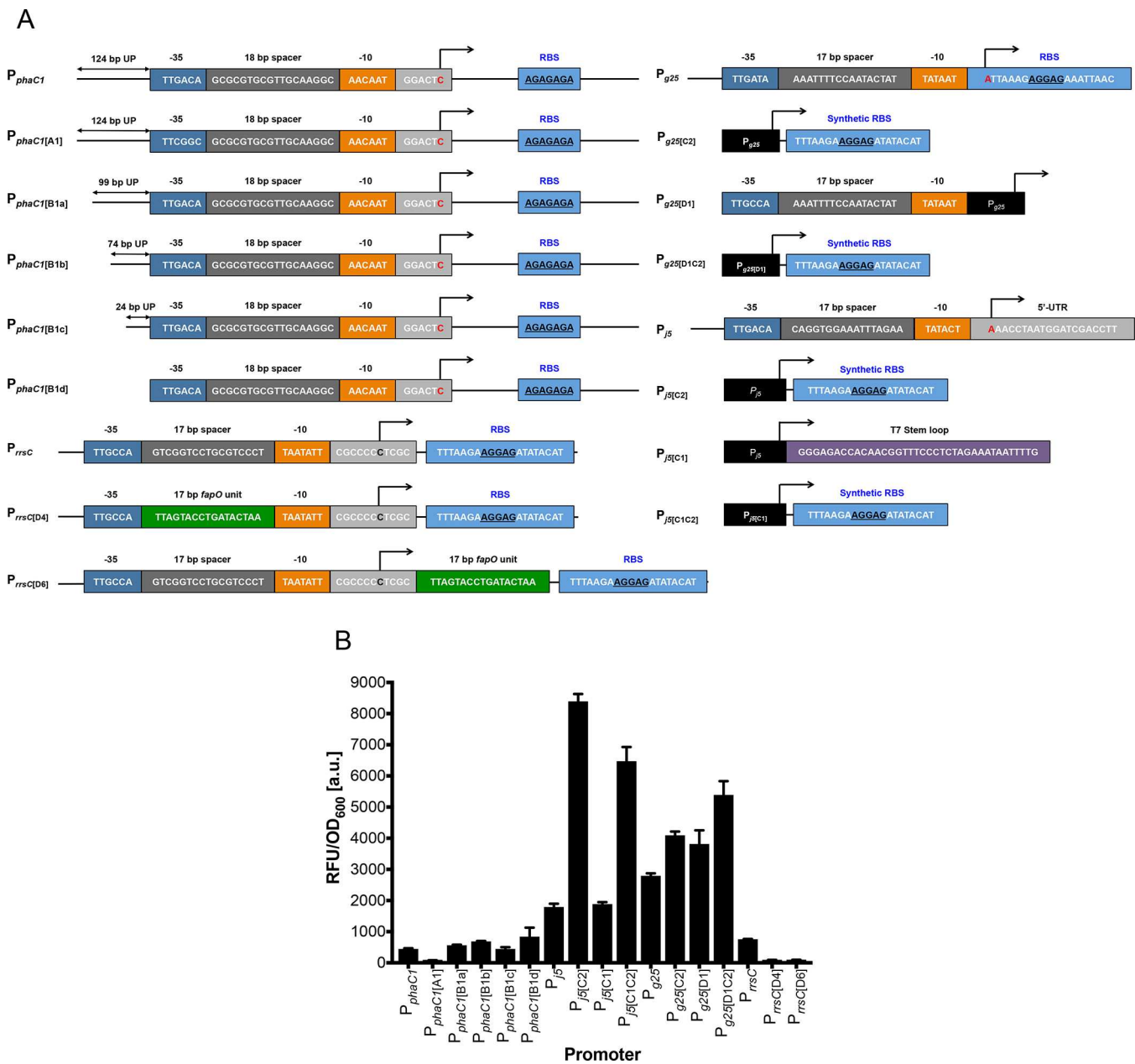
Figure 3. (A) Architectures of parental promoters and their engineered variants. (B) Activities of parental promoters and their engineered variants.

categories (e.g., B1a for truncation of 25 bp upstream of −35 box and B1b for truncation of 50 bp upstream of −35 box in category B) to pinpoint specific modification made. Using a combination of the aforementioned strategies, we created in total 38 promoter variants as summarized in Table 1.

Promoter Nomenclature. On the basis of the classification of our promoter engineering strategies, we devised a promoter nomenclature system to systematically name all 38 engineered promoters (Table 1). The system is designed to provide three pieces of key information: parental promoter, modifications made, and promoter architecture, using the standard format of $P_{parent[M1M2M3\cdots]}$. In this nomenclature system, capital P signifies a promoter. Italic subscript "*parent*" indicates the parental promoter from which the engineered promoter is derived. All modifications made are summarized in bracketed subscript "[M1M2M3···]," with the modifications arranged in sequence of appearance to reflect the engineered

promoter architecture. As an example, $P_{j5[C1C2]}$ is a promoter variant engineered from the parental promoter P_{j5} by inserting a T7-stem loop (denoted by $_{C1}$) as well as an RBS (denoted by $_{C2}$) in its 5′-UTR. The T7-stem loop was placed upstream of the RBS, as indicated by $_{C1}$ that comes before $_{C2}$.

Mutations in −35 Box Tuned Transcriptional Activity down. Li and Liao previously reported $P_{phaC1-G3}$ promoter, in which its −35 box was mutated from "TTGACA" to "TTCGGC" (Figure 3A).[23] This promoter variant was shown to retain 15% of the activity of its P_{phaC1} parent when characterized using enhanced green fluorescent protein (eGFP) as a reporter.[23] To validate our initial hypothesis that conserved −35 and −10 boxes are necessary in maintaining high transcriptional activity in *C. necator* H16, we recreated $P_{phaC1-G3}$, and this variant was named $P_{phaC1[A1]}$ in our nomenclature system. RFP fluorescence measurement ascertained that $P_{phaC1[A1]}$ retains 16% of the activity of P_{phaC1}

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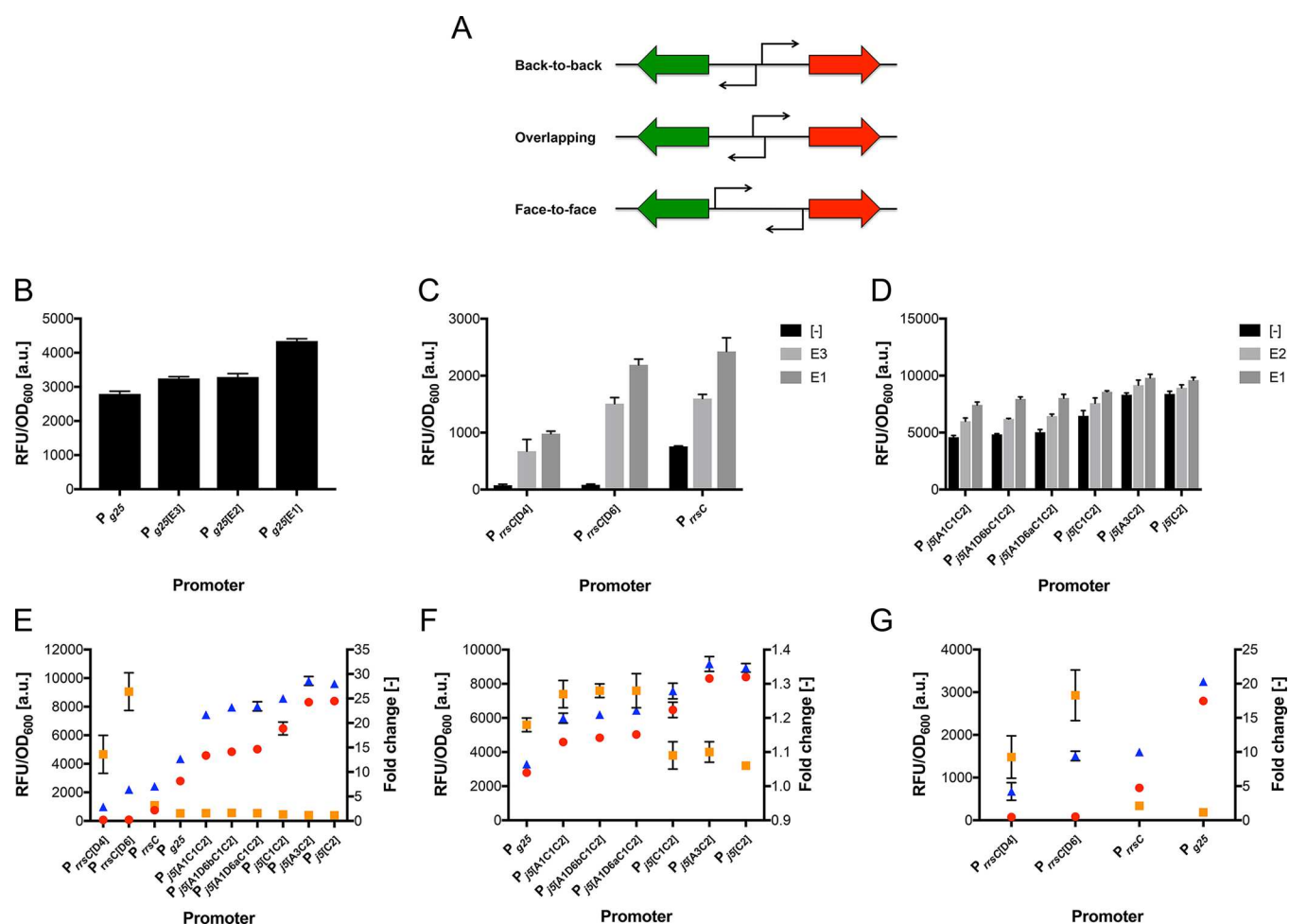
Figure 4. (A) For gene pairs in HH arrangement, promoters that effect divergent transcription can be organized in three possible ways: back-to-back, overlapping, or face-to-face. (B) Composite promoters engineered using P_{g25} as parental promoter. (C) Composite promoters engineered using P_{rrsC} as parental promoter. (D) Composite promoters engineered using P_{j5} as parental promoter. (E) Composite promoters with P_{phaC1} as secondary promoter. (F) Composite promoters with P_{g25} as secondary promoter. (G) Composite promoters with $P_{j5[A1C1C2]}$ as secondary promoter. In graphs E and F, red, blue and orange symbols represent primary promoter activity, composite promoter activity, and fold change, respectively.

(Figure 3B). This data also indicated that both reporter proteins, RFP and eGFP, give similar outcome in transcriptional activity quantification. RFP and eGFP are commonly used in synthetic biology for characterization of biological parts (e.g., promoter, terminator).[30,31] Li and Liao also created a promoter library with mutated −35 box (TTNNNN). All promoter variants screened showed lower activity in comparison to the wildtype promoter.[23] Therefore, mutations in −35 box likely tune the transcriptional activity down.

A Minimal P_{phaC1} Promoter with Enhanced Activity. P_{phaC1} (466 bp) is the longest promoter among the four parental promoters. To find the minimal functional sequence, we created $5' \rightarrow 3'$ truncated variants of P_{phaC1}: 25 bp truncation in $P_{phaC1[B1a]}$, 50 bp in $P_{phaC1[B1b]}$, 100 bp in $P_{phaC1[B1c]}$, and 124 bp in $P_{phaC1[B1d]}$ (Figure 3A). Interestingly, removing the entire upstream element (124 bp) in $P_{phaC1[B1d]}$ resulted in highest promoter activity, which is 2-fold higher compared to that of its P_{phaC1} parent (Figure 3B). This suggested that cis-acting elements exist within the upstream element of P_{phaC1} and contribute to transcriptional suppression.

Synthetic RBS and RBS Repeat Increased Transcriptional Activity. Our initial study with the four parental promoters, along with a previous study conducted by Bi et

al.,[20] motivated us to further investigate the effects of cis-acting translational regulatory elements on promoter activity. We focused specifically on the 26-bp synthetic genetic element derived from P_{rrsC} (containing a 20-bp RBS found in the pBBR1c-RFP P_{BAD} promoter flanked by an upstream 6-bp BglII restriction site, herein denoted as synthetic RBS) and the 37-bp T7 stem-loop reported by Bi et al.[20] We observed a 4.7-fold increase in promoter activity when a synthetic RBS was added to P_{j5} (the $P_{j5[C2]}$ variant) (Figure 3B). On the contrary, there was no significant change in promoter activity when a T7 stem-loop was added to P_{j5} (the $P_{j5[C1]}$ variant). A drop in promoter activity was observed when a T7 stem-loop was added to $P_{j5[C2]}$ (the $P_{j5[C1C2]}$ variant). As such, synthetic RBS is an effective means to amplify promoter activity. We then added the same synthetic RBS to P_{g25} and created a variant $P_{g25[C2]}$ that also showed ∼50% increase in promoter activity. A smaller promoter activity increase in $P_{g25[C2]}$ could be attributed to a pre-existing effective SD sequence ("AGGAG") in P_{g25}.

Repeat of −35 and −10 Boxes Increased Transcriptional Activity. The −35 and −10 boxes are highly conserved regions in prokaryotic promoters, essential for the binding of RNA polymerases. To test if creating more binding sites for

RNA polymerase would further increase transcriptional activity, we created the $P_{g25[D1]}$ variant. In this variant, we duplicated the DNA sequence spanning −35 box and −10 box. In essence, this hybrid promoter is a tandem repeat of two P_{g25} promoters, and we observed ∼40% increase in promoter activity (Figure 3). Again, incorporating a synthetic RBS ($P_{g25[D1C2]}$) gave an additive effect, resulting in further increase in promoter activity.

Operator Insertion Reduced Transcriptional Activity Drastically. Hybrid promoters are crucial genetic elements in the construction of biosensors. Using a malonyl-CoA biosensor[26] as an example, we inserted the *fapO* operator sequence "TTAGTACCTGATACTAA" in P_{rrsC} promoter to create two variants: $P_{rrsC[D4]}$ (*fapO* inserted between −35 and −10 boxes) and $P_{rrsC[D6]}$ (*fapO* inserted within the 5′-UTR) (Figure 3A). *fapO* operator is conserved in Gram-positive bacteria such as *Bacillus subtilis* and acts as a *cis*-regulatory unit for transcriptional regulation of fatty acid biosynthesis. For both promoter variants, we observed a drastic activity reduction to ∼10% of their P_{rrsC} parent (Figure 3B). The position of operator and the copy number of operator could therefore significantly change the transcriptional activity of the resultant hybrid promoters. Our data corroborated a previous study by Li and Liao, where *tetO* operators were inserted to create P_{rrsC} hybrid promoters.[23]

Divergent Promoters, Arranged in Back-to-Back, Increased Transcriptional Activity. The distance between and the relative transcriptional directions of adjacent genes are known to be important in some organisms. Neighboring genes arranged in head-to-head (HH) orientation, for instance, could be coregulated and this has been proven experimentally.[32] For gene pairs in HH arrangement, promoters that effect divergent transcription can be organized in three possible ways: back-to-back, overlapping, or face-to-face (Figure 4A).[33] In a recent attempt to construct a malonyl-CoA biosensor for *C. necator* H16 (publication in preparation), we discovered the significance of divergent transcription in this organism. Therefore, we created 21 composite promoters to systematically investigate divergent transcription in *C. necator* H16. Each composite promoter is made up of two promoters arranged in back-to-back (or divergent to each other). The promoter driving the RFP expression is termed the primary promoter, while the counterpart is called the secondary promoter.

When P_{phaC1} (denoted as modification subscript E1), P_{g25} (modification subscript E2), and $P_{j5[A1C1C2]}$ (modification subscript E3) was applied individually as secondary promoters, they generally served as transcriptional amplifiers, increasing the transcriptional activity of the primary promoters (Figure 4B−G). The promoter activities of these three secondary promoters follow the order of $P_{j5[A1C1C2]}$ > P_{g25} > P_{phaC1} (eq E3 > E2 > E1). Interestingly, transcriptional amplification depends on the transcriptional activities of both the primary and the secondary promoters. For the same primary promoter, activity enhancement typically decreases with the increased activity of the secondary promoter (Figure 4B−D, herein described as secondary promoter effect). Also, for the same secondary promoter, activity enhancement decreases with the increased activity of the primary promoter (Figure 4E−G, herein described as primary promoter effect). We postulate that secondary promoter effect is attributed to the fact that weaker secondary promoter competes less with the primary promoter for transcriptional machinery or factors. If a secondary promoter of very high activity is used, the competition is so

strong that it diminishes the activity of the primary promoter (data not shown). The primary promoter effect observed is perhaps more intuitive and easier to comprehend. If the primary promoter displays high activity, it is more difficult to further improve its activity using an amplifier. It is important to point out that transcriptional enhancement resulted from divergent promoters is not universal to all prokaryotic systems. Generally, we did not observe such behaviors when we tested our composite promoters in *E. coli* (data not shown).

Promoter Characterization using P$_{BAD}$ as Reference Scale. This study resulted in a set of 42 constitutive promoters, including the four parental promoters. The ratio of promoter activities between the strongest ($P_{j5[E1A3C2]}$) and the weakest ($P_{phaC1[A1]}$, eq $P_{phaC1-G3}$ reported by Li and Liao[23]) is 137. These promoters showed incremental increase in activity across the entire scale (Figure 5A). To promote the
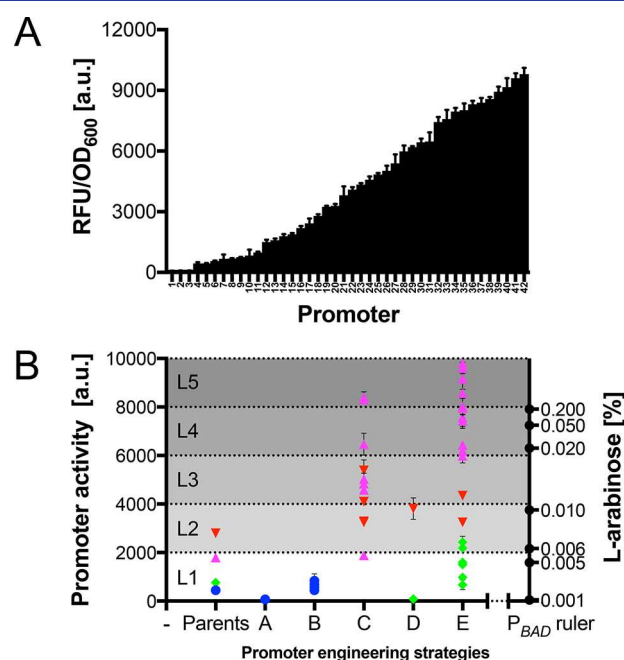

Figure 5. (A) Hierarchical ranking of all 42 constitutive promoters reported in this study. (B) The range of promoter activity was expanded from 6-fold to 137-fold after applying combination of promoter engineering strategies (A = point mutation, B = length alteration, C = incorporation of regulatory genetic element, D = promoter hybridization and E = configuration alteration). Promoters derived from P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25} were colored in blue, green, pink, and red, respectively. Promoters were categorized into five activity levels: Level 1 (with promoter activity between 0−2000 au), level 2 (2000−4000 au), level 3 (4000−6000 au), level 4 (6000−8000 au) and level 5 (>8000 au). Each promoter was benchmarked against P_{BAD} promoter, induced using various concentrations of L-arabinose, from 0.001% (w/v) to 0.200% (w/v).

widespread use of these promoters, we benchmarked each of them using L-arabinose inducible P_{BAD} promoter as a reference scale. Supporting Information, Figure S4, illustrates the dose-dependent induction of P_{BAD} promoter, using L-arabinose concentration from 0.001% (w/v) to 0.200% (w/v). Expression maxima was reached at 0.200% (w/v) L-arabinose. As depicted in Figure 5B, our engineered promoters covered the entire L-arabinose inducible range (indicated by scattered data points). We categorized all promoters into five activity levels to aid promoter selection: Level 1 (with promote

activity between 0−2000 au), level 2 (2000−4000 au), level 3 (4000−6000 au), level 4 (6000−8000 au), and level 5 (>8000 au). With a P_{BAD} promoter, one could only achieve expression levels between 1 and 4. Through promoter engineering, we obtained seven level 5 variants ($P_{j5[A3C2]}$, $P_{j5[C2]}$, $P_{j5[E1C1C2]}$, $P_{j5[E2C2]}$, $P_{j5[E2A3C2]}$, $P_{j5[E1C2]}$, and $P_{j5[E1A3C2]}$) with promoter activities exceeding that of P_{BAD} (Supporting Information, Table S2). For easy classification of all engineered promoters, we developed a numerical coding system (Table 1) and assigned a digital identifier to each promoter. This will allow us to develop a *C. necator* H16-specific promoter database (work in progress). Each promoter code is in the format of [X-Y-Z], with X representing activity level, Y representing relative activity to $P_{phaC1[A1]}$, and Z representing promoter length. While conceptualizing our engineered promoter nomenclature and coding systems, we have endeavored to make them universal such that they can be applied to other promoters yet to be developed.

Summary of Rational Promoter Engineering for *C. necator* H16.

Figure 6 provides an overview of the rational
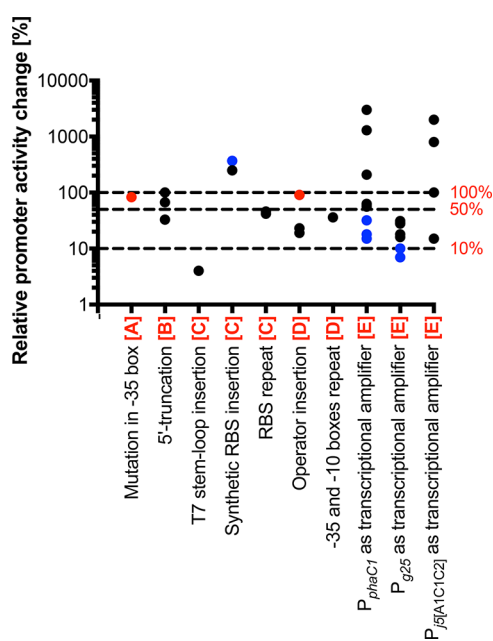
Figure 6. Relative promoter activity change upon application of promoter engineering strategies (A = point mutation, B = length alteration, C = incorporation of regulatory genetic element, D = promoter hybridization, and E = configuration alteration). Modifications that resulted in loss of promoter activity were indicated as red data points. Promoters with activities higher than P_{BAD} promoter were indicated as blue data points.

promoter engineering strategies discussed in this article. It shows the effect of a specific modification by looking at the promoter activity difference before and after that particular modification. Creating mutation(s) within −35 box and inserting operator sequence(s) resulted in drastic reduction in promoter activity (represented by red data points), while inserting a T7 stem-loop caused almost no change in promoter activity. On the contrary, inserting a synthetic RBS and applying a transcriptional amplifier (specifically P_{phaC1} or $P_{j5[A1C1C2]}$) gave the highest increase in promoter activity (>100%). In fact, those promoters that are stronger than P_{BAD} promoter (indicated as blue data points) were mostly created

using either one of these strategies or combination of them. All the other strategies provided marginal promoter activity increase (from 10% to 50%). Also clearly reflected in Figure 5B, creating divergent promoters is the most effective way of broadening the range of promoter activity. It is worthy of note that relative promoter activity change is dependent on the parental promoter, judging on the work presented in this article.

The Use of Engineered Constitutive Promoters in *C. necator* H16.

The use of strong constitutive promoters could potentially result in (a) bacterial growth impairment due to high metabolic burden and/or (b) protein excretion/leakage due to high protein expression level. To study these effects, we selected representative promoters from each activity level (Supporting Information, Table S3) and conducted further characterization. We observed similar growth for most of the strains (Figure 7), with growth rates (μ_{max}) falling between
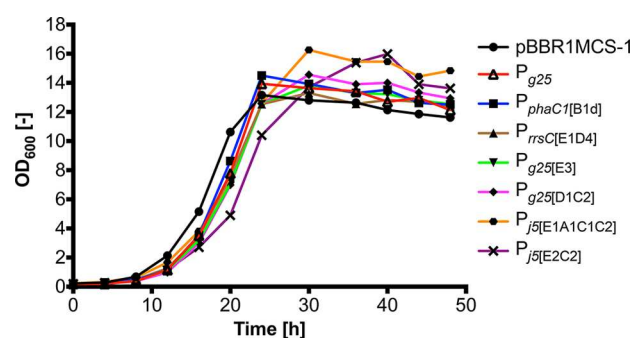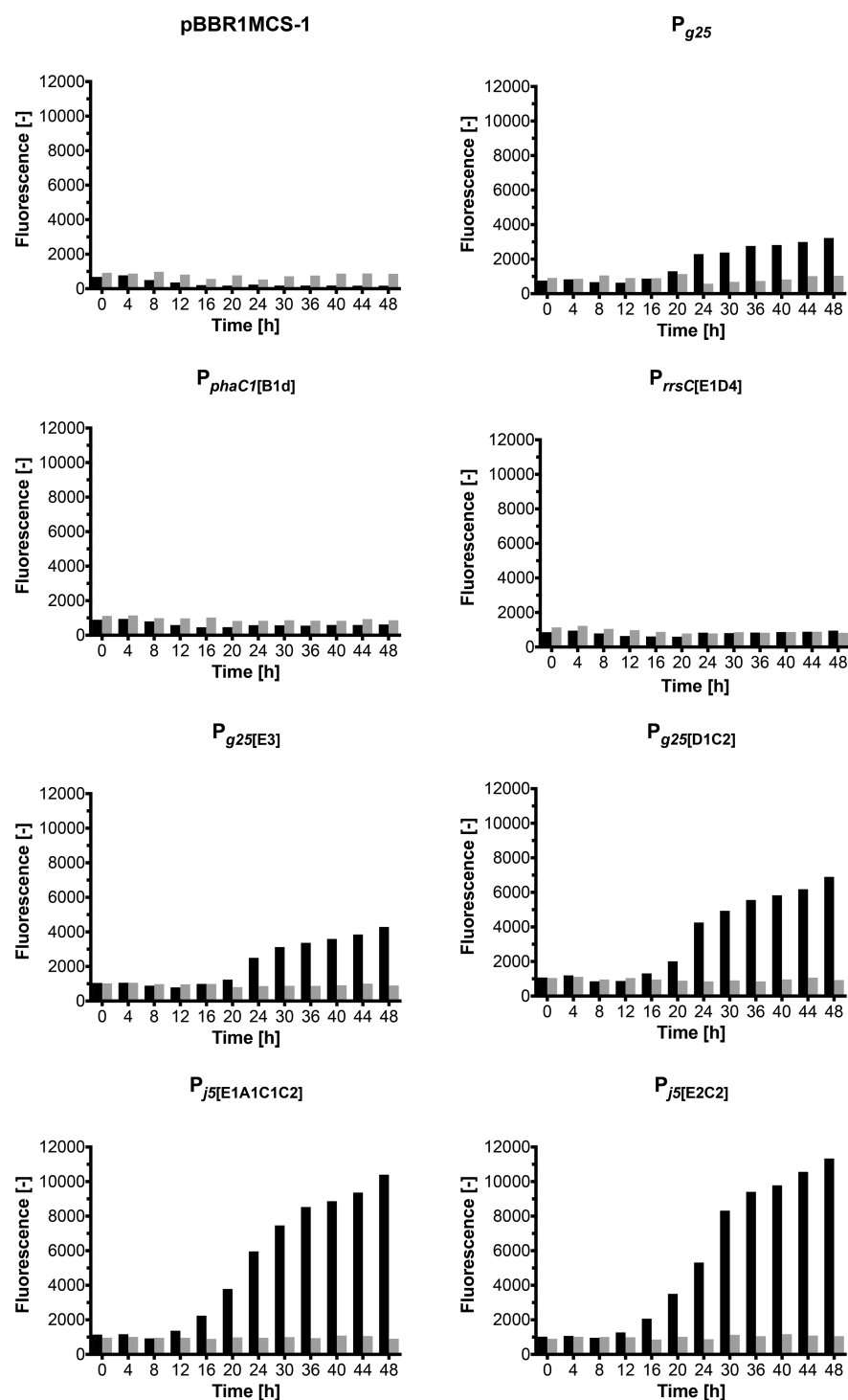

Figure 7. Growth curves of *C. necator* H16 harboring either pBBR1MCS-1 (control; black line) or plasmids containing various engineered constitutive promoters [P_{g25} (red line), $P_{phaC1[B\ 1d]}$ (blue line), $P_{rrsC[E1D4]}$ (brown line), $P_{g25[E3]}$ (green line), $P_{g25[D1C2]}$ (pink line), $P_{j5[E1A1C1C2]}$ (orange line), and $P_{j5[E2C2]}$ (purple line)].

0.21 and 0.24 h^{-1} (Supporting Information, Table S3). For promoter $P_{j5[E2C2]}$, which is a level 5 promoter, we noticed a slight drop in growth rate with μ_{max} of 0.18 h^{-1} (Supporting Information, Table S3). Comparing fluorescence of cell culture and of spent medium (Figure 8) confirmed that there was no protein excretion/leakage. Fluorescence of spent medium was maintained at the level of ∼1000 au throughout the bacterial cultivation. This value was almost identical to that of the control (*C. necator* H16 harboring pBBR1MCS-1). To study the time-dependent increase in fluorescence signal, we fitted the cell culture fluorescence vs time data to a four-parameter dose−response curve (Supporting Information, Figure S5 and Table S4) for all promoters from level 2 and above. The fluorescence increase was mainly caused by bacterial growth. If we divided the fluorescence measured (Figure 8) by the OD_{600} (Figure 7), the ratio was kept almost constant at cultivation times above 12 h (Figure S6), further verifying our approach in promoter activity quantification in 96-well plate by taking the RFU/OD_{600} at t = 48 h.

■ CONCLUSION

This article (1) reported and characterized a set of 42 constitutive promoters with a broad range of promoter activity, which are derived from the four most widely used constitutive promoters for *C. necator* H16 (P_{phaC1}, P_{rrsC}, P_{j5}, and P_{g25}), (2) introduced a nomenclature system and a coding system for engineered promoters, (3) sketched out the relationship

Figure 8. Fluorescence of cell culture (black columns) and of spent medium (gray columns) of *C. necator* H16 harboring either pBBR1MCS-1 (control) or plasmids containing various engineered constitutive promoters (P_{g25}, $P_{phaC1[B1d]}$, $P_{rrsC[E1D4]}$, $P_{g25[E3]}$, $P_{g25[D1C2]}$, $P_{j5[E1A1C1C2]}$, and $P_{j5[E2C2]}$).

between promoter architecture and its resultant activity, (4) highlighted similarities (conservation of −35 and −10 boxes) and differences (composite promoters) in transcriptional activity between *C. necator* H16 and *E. coli*, and (5) provided guidelines for rational promoter engineering. We strongly believe our constitutive promoter toolbox that exceeds the activity range of the inducible P_{BAD} promoter will serve the biotechnology community working on *C. necator* H16, be it

strain engineering for industrial biomanufacturing or developing advanced molecular biology tools for this organism.

■ MATERIALS AND METHODS

Materials. All DNA modifying enzymes were purchased from either New England Biolabs (Hitchin, UK) or Agilent (Craven Arms, UK). Nucleic acid purification kits were purchased from Qiagen (Manchester, UK). All oligonucleotides were synthesized by Eurofins (Ebersberg, Germany).

Strains. *Escherichia coli* DH5α was used for all molecular cloning, plasmid propagation, and maintenance. *Cupriavidus necator* H16 (DSM-428, purchased from DSMZ, Braunschweig, Germany) was used for all experiments described in this article.

Promoter Engineering and Sequences. All plasmids were derived from pBBR1c-RFP (see Supporting Information) and constructed using standard molecular biology techniques. All engineered promoters were verified by restrictive analysis and/or DNA sequencing, and their sequences were provided in the Supporting Information.

Bacterial Cultivation and Transformation. *C. necator* H16 was cultivated at 30 °C in nutrient broth (NB: 5 g/L peptone, 1 g/L beef extract, 2 g/L yeast extract, 5 g/L NaCl; pH 7.0 ± 0.2 @ 25 °C) supplemented with 10 μg/mL of gentamicin. Cells were transformed with plasmids using the electroporation protocol described by Tee et al.,[19] plated on NB agar supplemented with 10 μg/mL of gentamicin and 25 μg/mL of chloramphenicol, and incubated at 30 °C for 40−60 h. *E. coli* DH5α was transformed with plasmids using the standard CaCl$_2$ method, plated on TYE agar (10 g/L tryptone, 5 g/L yeast extract, 8 g/L NaCl, 15 g/L agar) supplemented with 25 μg/mL of chloramphenicol, and incubated overnight at 37 °C.

Promoter Activity Quantification Using Fluorescence Assay. Transformants of *C. necator* H16, carrying either an RFP-null or an RFP-expressing vector, were precultured in 96-well microtiter plate containing 200 μL/well of NB supplemented with 10 μg/mL of gentamicin and 25 μg/mL of chloramphenicol at 30 °C for 40 h. This preculture was used to inoculate a fresh clear-bottom 96-well microtiter plate [Greiner Bio-One (Stonehouse, UK)] containing 200 μL/well of NB supplemented with 10 μg/mL of gentamicin, 25 μg/mL of chloramphenicol as well as 0−0.2% (w/v) L-arabinose (when required) to induce RFP expression. The plate was cultivated at 30 °C for a total of 48 h. OD$_{600}$ and fluorescence (E_x 584 nm, E_m 607 nm; bottom read) were measured using SpectraMax M2e microplate/cuvette reader [Molecular Devices (Wokingham, UK)] after 12 h of cultivation and repeated at 6 h intervals. Relative fluorescence unit (RFU) was calculated by normalizing fluorescence value with the fluorescence value of *C. necator* H16 carrying an RFP-null vector (negative control). The RFU value therefore represents the fluorescence fold increase owing to RFP expression. RFU/OD$_{600}$ value was then calculated as the ratio of RFU and OD$_{600}$ value of the respective strain. The ratio was used to account for potential metabolic burden due to high protein expression level, affecting bacterial growth. Promoter activity (PA) was defined as the RFU/OD$_{600}$ value after 48 h of cultivation. The ratio of RFU/OD$_{600}$ was more or less a constant at cultivation time more than 12 h. All experiments were done in triplicate.

Fold Change and Relative Promoter Activity Change. Fold change and relative promoter activity change were calculated by using the formulas:

$$\text{fold change} = \frac{PA_2}{PA_1}$$

relative promoter activity change

$$= \frac{|PA_{after} - PA_{before}|}{PA_{before}} \times 100\%$$

Effects of Engineered Constitutive Promoters on Bacterial Growth and Protein Excretion. Selected plasmids were freshly transformed into *C. necator* H16, and single colonies were picked to prepare overnight cultures. Falcon tubes, containing 6 mL of fresh mineral salts medium (MSM)[34] supplemented with 10 g/L sodium gluconate (carbon source), 10 μg/mL gentamicin, and 25 μg/mL chloramphenicol, were inoculated at a starting OD$_{600}$ of 0.2. Cells were cultivated at 30 °C and sampled at regular time intervals. OD$_{600}$ of each sample was measured using BioPhotometer Plus [Eppendorf (Stevenage, UK)]. For all samples collected, the fluorescence (E_x 584 nm, E_m 607 nm; bottom read) of the cell culture (90 μL) and of the spent medium (90 μL) was measured using SpectraMax M2e microplate/cuvette reader [Molecular Devices (Wokingham, UK)].

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00136.

> Experimental procedures; plasmid map of pBBR1c-RFP; alignment of P$_{j5}$ and P$_{g25}$ promoters used in this study and in Gruber et al. (2014); graphical representation of rational promoter engineering strategies; L-arabinose-dose dependent induction of P$_{BAD}$ promoter; curve fitting of cell culture fluorescence vs time data; fluorescence of cell culture normalized by OD$_{600}$ value; promoter sequences; subcategories of each promoter engineering strategy; promoter activity table; specific growth rates of *C. necator* H16 carrying plasmids containing various promoters (PDF)

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T.S.W. and K.L.T. conceived and supervised the project. A.O.J. and M.G. performed the experiments and analyzed the data. T.S.W., K.L.T., and A.O.J. wrote the manuscript. All authors discussed the content of the manuscript and provided critical revisions on the manuscript.

Notes

The authors declare no competing financial interest.

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