Engineering and application of synthetic nar promoter for fine-tuning the expression of metabolic pathway genes in Escherichia coli

Hee Jin Hwang¹, Sang Yup Lee² and Pyung Cheon Lee¹*

Abstract

Background: Promoters regulate the expression of metabolic pathway genes to control the flux of metabolism. Therefore, fine-tuning of metabolic pathway gene expression requires an applicable promoter system. In this study, a dissolved oxygen-dependent nar promoter was engineered for fine-tuning the expression levels of biosynthetic pathway enzymes in Escherichia coli. To demonstrate the feasibility of using the synthetic nar promoters in production of biochemicals in E. coli, the d-lactate pathway consisting of one enzyme and the 2,3-butanediol (BDO) pathway consisting of three enzymes were investigated.

Results: The spacer sequence of 15 bp between the −35 and −10 elements of the upstream region of the wild-type nar promoter was randomized, fused to the GFP gene, transduced into E. coli, and screened by flow cytometry. The sorted synthetic nar promoters were divided into three groups according to fluorescence intensity levels: strong, intermediate, and weak. The selected three representative nar promoters of strong, intermediate, and weak intensities were used to control the expression level of the d-lactate and 2,3-BDO biosynthetic pathway enzymes in E. coli. When the ldhD gene encoding d-lactate dehydrogenase was expressed under the control of the strong synthetic nar promoter in fed-batch cultures of E. coli, the d-lactate titers were 105.6 g/L, 34% higher than those using the wild-type promoter (79.0 g/L). When the three 2,3-BDO pathway genes (ilvBN, aldB, and bdh1) were expressed under the control of combinational synthetic nar promoters (strong–weak–strong) in fed-batch cultures of E. coli, the titers of 2,3-BDO were 88.0 g/L, 72% higher than those using the wild-type promoter (51.1 g/L).

Conclusions: The synthetic nar promoters, which were engineered to have strong, intermediate, and weak intensities, were successfully applied to metabolic engineering of d-lactate and 2,3-BDO pathways in E. coli. By controlling expression levels of d-lactate and 2,3-BDO pathway enzymes using the synthetic nar promoters, the production of d-lactate and 2,3-BDO was increased over that using the wild-type promoter by 34 and 72%, respectively. Thus, this synthetic promoter module system will support the improved production of biochemicals and biofuels through fine-tuning of gene expression levels.

Keywords: nar promoter, Oxygen-dependent promoter, Lactate, 2,3-Butanediol, Promoter engineering

*Correspondence: pclee@ajou.ac.kr
¹ Department of Molecular Science and Technology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 16944, South Korea
Full list of author information is available at the end of the article

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Background

Synthetic biology aims to develop desired biological system through the rational design of synthetic parts/modules, including promoters, RNAs, and scaffolds [1–4]. Gene expression can be controlled utilizing several factors, such as promoters, transcription factors, and plasmid copy numbers [5, 6]. Among them, promoter engineering has been proposed as one of the most efficient ways of fine-tuning transcriptional control in Escherichia coli, Corynebacterium glutamicum, Bacillus subtilis, and yeasts [3–10]. For example, the E. coli strain with engineered L-phenylalanine-responsive promoter could produce a fourfold higher titer of phenylalanine than wild-type promoter [11], and the engineered tac promoter library could decrease leakage of antibody fragment expression in E. coli [12].

Recently, a dissolved oxygen (DO)-dependent nar promoter was successfully applied to express the d-lactate, 2,3-butanediol (BDO), and 1,3-propanediol (PDO) pathway enzymes in E. coli [13]. However, when a multi-enzyme biosynthetic pathway was reconstructed in heterologous host cells, individual expression of each pathway enzyme needed to be finely controlled; assembly or organization of multi-enzyme systems could significantly influence metabolic channeling, and thus suboptimal assembly or organization would cause accumulation of unwanted metabolic intermediates in multi-step enzyme reactions [14, 15]. Even single-enzyme metabolic pathways also should be considered for fine-tuning of expression, because expression level frequently affects end-product formation due to inclusion body formation [16].

Compared to other commonly used strong promoters such as lac or araBAD, the intensity of the wild-type nar promoter is relatively weak [13]; therefore, engineering of the wild-type nar promoter was required for fine control of target pathway gene expression. In this study, in order to generate synthetic nar promoters of diverse strengths, a synthetic nar promoter library was constructed by randomization of the spacer region sequence (15 bp) located between the consensus sequence and −35 and −10 elements of the wild-type nar promoter (Fig. 1a). Characterization of the selected three synthetic promoters showing weak, intermediate, and strong intensities was performed through transcriptional expression level and GFP fluorescence intensity assays. Then, the selected three synthetic promoters were applied to the expression of the d-lactate pathway consisting of one gene and the 2,3-BDO biosynthesis pathway consisting of three genes (Fig. 1b). We demonstrated that the production of d-lactate and 2,3-BDO was improved by tuning the expression of their pathway genes under three different strengths of the synthetic nar promoters in E. coli.

Results

Construction, screening, and strength analysis of a synthetic nar promoter library of diverse strengths

The 15-bp spacer region sequence between the −35 and −10 elements of the wild-type nar promoter was randomized using degenerated primers to construct a synthetic nar promoter library of diverse strengths. The randomized synthetic promoters were fused by PCR to a DNA fragment consisting of a Shine–Dalgarno sequence, spacer, and His6-tagged GFPm as a reporter protein for screening based on the fluorescence intensity of the expressed GFPm (Fig. 1a). The randomized promoter fragments were then ligated with a pSTVM plasmid (Table 1) and then transduced into E. coli TOP10 cells.

The library (4.59 × 1010 cell size), showing diverse fluorescence intensities, was sorted into three groups (low, intermediate, and high strength) by FACS in three rounds of sorting. The 1st round sorting was carried out with two collections of the upper and lower 1% of fluorescence intensity signals. The collected cells were grown on LB+cm agar plates and submitted to 2nd round sorting. After repeating this for 3rd round sorting, clones showing high (a red line in Fig. 2a), immediate (a blue line), and low (a yellow line) fluorescence intensities were collected and further analyzed.

After reconfirmation of the GFP fluorescence intensities of 300 randomly selected clones from the 3 groups (100 clones from each group), fluorescences of 68 distinguishable clones were measured and compared based on relative fluorescence units (RFU/OD600) (Fig. 2b). Based on the normalized values of RFU/OD600, synthetic nar promoters were grouped into strong (> 5000 RFU/OD600), intermediate (1000–2000 RFU/OD600), and weak (< 1000 RFU/OD600) groups. From each group, a representative synthetic nar promoter of strong (S3-2-64), intermediate (W2U-30), and weak (W2L-29) fluorescence was chosen for further analysis. The strength of the strong synthetic nar promoter (S3-2-64) and the intermediate nar promoter (W2U-30) were 19.8 and 6.2 times higher than that of the wild-type nar promoter, respectively, while the strength of the weak nar promoter (W2L-29) was 1.8 times weaker than that of the wild-type nar promoter based on values of RFU/OD600.

Characterization of the three representative synthetic nar promoters

The three representative nar synthetic promoters were then characterized in detail by analyzing levels of transcription, protein expression, and fluorescence of GFPm (Fig. 3a). In transcriptional analysis, qRT-PCR was carried out with a cysG gene encoding siroheme synthase [17] as a reference in order to evaluate ΔΔCt values of GFPm expression under the control of the wild-type,
strong, intermediate, and weak nar promoters. The ΔΔCt values of the strong, intermediate, and weak nar promoters were 29.4 ± 5.6, 8.4 ± 1.0, and 2.3 ± 0.4, respectively. Unexpectedly, the ΔΔCt value of the weak nar promoter was positive, indicating a higher transcription level than that of the wild-type nar promoter. Next, protein expression levels were examined by western blotting with endogenous GAPDH as a reference. The protein expression levels of GFPm under the control of the strong, intermediate, and weak nar promoters were approximately 19.7, 7.11, and 0.37 times higher than that of the wild-type nar promoter. Notably, the protein expression levels of the strong and intermediate nar promoters were 4.6 and 1.7 times higher, respectively, than that of the constitutive lac promoter. Finally, the fluorescence intensities of GFPm under the control of the strong, intermediate, and weak nar promoters were 10,017 ± 915, 2305 ± 360, and 218 ± 18 RFU/OD600, respectively.

The randomly mutated spacer regions of the nine nar promoters including three representatives were sequenced and compared. One distinguishable difference between the synthetic and wild-type nar promoters was a GC content in the space sequences: lower GC contents were observed in all synthetic promoters (strong: 33.3%; intermediate: 40%; weak: 33.3%) than in the wild-type nar promoter (60%) (Fig. 3b). A conserved sequence (GTN[A/G]N) located between the −24 and −20 positions was observed in seven clones representing intermediate and weak promoters, but not in that of the strong promoter. It has been shown that strong promoters tend to have AT-rich spacers, which help flexibility and bendability of DNA structures [18]. This is true for the strong

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**Fig. 1** a Schematic diagram for construction of the synthetic nar promoter library. The 15-bp spacer sequence (indicated by consecutive 15 Ns) between the −35 and −10 elements of the wild-type nar promoter was randomized. An FNR binding site is shown in italics; the −35 and −10 elements are indicated by underlines; a red arrow indicates a transcription start site (+ 1); SD, Shine–Dalgarno sequence, GFPm, modified green fluorescence protein with His6 tag. b Reconstructed d-lactate and 2,3-butanediol (2,3-BDO) biosynthesis pathways in E. coli. The d-lactate pathway (red) consists of ldhD gene encoding d-lactate dehydrogenase (LDHD); the 2,3-BDO pathway (blue) consists of ilvBN gene encoding acetohydroxybutanate synthase/acacetolactate synthase (IlvBN), aldB gene encoding acetolactate decarboxylase (AldB), and bdh1 encoding butanediol dehydrogenase (BDH1)
Table 1  Bacterial strains and plasmids used in this study

| Strains and plasmids     | Relevant properties                                                                 | Source or reference |
|--------------------------|------------------------------------------------------------------------------------|---------------------|
| **Strains**              |                                                                                     |                     |
| *E. coli* TOP10          | F-mcrAΔ(mrr-hsdRMS-mcrBC) pS80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15  | Invitrogen          |
| **W023**                 |                                                                                     | [13, 21]            |
| *Lactobacillus citreum*  | Source for *ldhD*                                                                     | KCTC3721            |
| *Lactococcus lactis subsp. lactis* | Source for *aldB*                                                              | KCTC3899            |
| *Saccharomyces cerevisiae* | Source for *bdh1*                                                                  | ATCC 204508         |
| **Plasmids**             |                                                                                     |                     |
| pUCM                     | Cloning vector modified from pUC19; constitutive lac promoter, AmpR                 | [23]                |
| pUCM-gfpm                | Constitutive expressed gfpm gene with lac promoter                                  | This study          |
| pUCN                     | Cloning/expression vector having inducible wild-type nar promoter, AmpR             | [13]                |
| pUCN-gfpm                | Inducible expressed gfpm gene with nar promoter                                    | This study          |
| pQE-gfpm                 | Inducible expressed gfpm gene with T5 promoter                                     | [22]                |
| pUCNt                    | Cloning/expression vector having rop gene and wild-type nar promoter, AmpR          | This study          |
| pUCNtS                   | Cloning/expression vector having rop gene and strong nar promoter (S3-2-64), AmpR | This study          |
| pUCNtl                   | Cloning/expression vector having rop gene and intermediate nar promoter (W2U-30), AmpR | This study          |
| pUCNtw                   | Cloning/expression vector having rop gene and weak nar promoter (W2L-29), AmpR     | This study          |
| NrSL                     | Inducible expressed *ldhD* gene from *L. citreum* on pUCNrS                        | This study          |
| NrNL                     | Inducible expressed *ldhD* gene from *L. citreum* on pUCNtl                         | This study          |
| NrNW                     | Inducible expressed *ldhD* gene from *L. citreum* on pUCNtlW                       | This study          |
| NrSi                     | Inducible expressed *ldhD* gene from *E. coli* on pUCNtS                           | This study          |
| NrIi                     | Inducible expressed *ldhD* gene from *E. coli* on pUCNtI                           | This study          |
| NrWii                    | Inducible expressed *ldhD* gene from *E. coli* on pUCNtW                          | This study          |
| NrSa                     | Inducible expressed *aldB* gene from *L. lactis with strong nar promoter            | This study          |
| NrIa                     | Inducible expressed *aldB* gene from *L. lactis with intermediate nar promoter      | This study          |
| NrWa                     | Inducible expressed *aldB* gene from *L. lactis with weak nar promoter              | This study          |
| NrSb                     | Inducible expressed *bdh1* gene from *S. cerevisiae with strong nar promoter        | This study          |
| Nrib                     | Inducible expressed *bdh1* gene from *S. cerevisiae with intermediate nar promoter | This study          |
| NrWb                     | Inducible expressed *bdh1* gene from *S. cerevisiae with weak nar promoter          | This study          |
| pSTVM2                   | Cloning/expression vector removing *lac* promoter, CmR                              | [13]                |
| pSTVM2- SNPL-gfpm        | gfpm expression vector with synthetic nar promoter library                           | This study          |
| pSTVM-gfpm               | Constitutive expressed gfpm gene with constitutive *lac* promoter on pSTVM2         | This study          |
| Nii                      | Inducible expressed *ldhD* gene with wild-type nar promoter on pSTVM2               | [13]                |
| Si                       | Inducible expressed *ldhD* gene with strong nar promoter on pSTVM2                  | This study          |
| li                       | Inducible expressed *ldhD* gene with intermediate nar promoter on pSTVM2           | This study          |
| Wi                       | Inducible expressed *ldhD* gene with weak nar promoter on pSTVM2                    | This study          |
| NIIa                     | Individually inducible expressed *ldhD* with wild-type nar promoter on pSTVM2       | [13]                |
| SiSa                     | Individually inducible expressed *ldhD* and *aldB* genes with wild-type nar promoter on pSTVM2 | This study          |
| lii                      | Individually inducible expressed *ldhD* and *aldB* genes with intermediate nar promoter on pSTVM2 | This study          |
| SiWa                     | Individually inducible expressed *ldhD* with strong promoter and *aldB* with weak nar promoter on pSTVM2 | This study          |
| NNN                      | Inducible expressed *ldhD*, *aldB*, and *bdh1* genes under each nar promoter on pSTVM2 | [13]                |
| SWS                      | Individually inducible expressed *ldhD* with strong promoter, *aldB* with weak promoter, and *bdh1* with strong promoter | This study          |
Comparison of  
comparison of different strengths

In order to investigate the effect of nar promoter strength on metabolic pathway flux in E. coli, a d-lactate pathway consisting of one d-lactate dehydrogenase was first chosen. The ldhD gene encoding d-lactate dehydrogenase from L. citreum was cloned into pUC NrS, pUC NrI, pUC Nr W, and pUCN (Table 1), to be expressed under the control of the four nar promoters: strong, intermediate, weak, and wild-type. The four ldhD gene-expression plasmids (NrSL, strong; NrIL, intermediate; NrWL, weak; NrL, wild-type) were transduced into E. coli, and then the four recombinant E. coli strains were micro-aerobically grown in flasks containing 20 g/L glucose as a carbon source [13]. After a 20-h cultivation, d-lactate titers were measured to be 18.6 ± 0.6 in E. coli having NrL, 18.7 ± 0.4 in NrSL, 18.5 ± 0.4 in NrIL, and 18.3 ± 0.1...
in NrWL (Fig. 4a). Because d-lactate titers were similar in all four E. coli strains, two E. coli strains having NrL and NrSL were chosen and fed-batch fermentation with glucose as a carbon source was carried out to investigate the effect of the strength of nar promoters (strong vs. wild-type) on d-lactate production. When the DO-controlled fed-batch fermentation of E. coli strain having NrSL was carried out as described in our previous study [13], 105.6 g/L of d-lactate was obtained after a 23-h cultivation. The d-lactate yield and productivity were 0.71 g/g-glucose and 4.59 g/L/h, respectively (Fig. 4b). In comparison, the E. coli strain with NrL produced 79.0 g/L of d-lactate yield of 0.67 g/g-glucose and productivity of 3.47 g/L/h (Fig. 4c). This result supports that higher expression of d-lactate dehydrogenase under the control of the synthetic strong nar promoter directed more metabolic flux into d-lactate biosynthesis in E. coli. Consequently, controlling d-lactate dehydrogenase with the strong nar promoter enhanced d-lactate titers by 34% compared to those of the wild-type nar promoter (105.6 g/L vs. 79.0 g/L).

### Comparison of acetoin and 2,3-BDO production with the synthetic nar promoters of different strengths

As a second demonstration of the feasibility of using the synthetic nar promoters in production of biochemicals in E. coli, acetoin, consisting of two enzymes, and the 2,3-butanediol (2,3-BDO) pathway, consisting of three enzymes, were investigated using the three representative nar promoters. First, the production of acetoin, a 2,3-BDO pathway intermediate (Fig. 1b), was investigated. In order to reconstruct the heterologous acetoin pathway in E. coli, two acetoin pathway genes, ilvBN from E. coli and aldB from L. lactis, were modified to be expressed under the control of the three synthetic nar promoters by cloning each gene into pUCNrS, pUCNrI, and pUC-NrW. Next, the resulting 6 expression modules (Si, Ii, Wi, Sa, Ia, and Wa, where S stands for strong promoter, I for intermediate promoter, W for weak promoter, i for ilvBN,
and a for _aldB_) were combined to produce 9 expression vectors (Fig. 5a).

When 10 expression modules including a positive control module Ni–Na ( _ilvBN_ and _aldB_ expressed with a wild-type _nar_ promoter) were expressed in flask cultures of _E. coli_, the expression of the _ilvBN_ gene with strong and intermediate synthetic _nar_ promoters (Si and Ii) produced an average of 6.30 g/L of acetoin regardless of promoter strength for expressing the _aldB_ gene. However, the expression of _ilvBN_ with the weak _nar_ promoter (Wi) produced the lowest acetoin production (3.4±0.3 g/L) when the _aldB_ gene was coexpressed with the weak _nar_ promoter (Wa) (Fig. 5b). Unexpectedly, when a heterologous acetoin pathway was reconstructed in _E. coli_, the end-product acetoin was to a limited degree transformed into 2,3-BDO by unknown factors [19]. Therefore, when the total summed amount of acetoin and 2,3-BDO produced by combination of the 10 modules was taken into consideration, Si–Wa and li–Ia combinations exhibited better production than the others (8.4±0.5 and 8.1±0.6 g/L, respectively).

Next, the two selected acetoin-producing combination modules, Si–Wa and li–Ia, were assembled as independent expression modules into one plasmid pSTVM2, resulting in SiWa and liIa (Table 1). In order to reconstruct a 2,3-BDO pathway on the two-plasmid system, SiWa and liIa were coexpressed with _bdh1_ from _S. cerevisiae_ under the control of the 3 synthetic _nar_ promoters (6 expression combinations: Si, li, Wi, Sa, Ia, Wa, Sb, Ib, and Wb, where S stands for strong, I for intermediate, W for weak, i for _ilvBN_, a for _aldB_, and b for _bdh1_; Fig. 6a) in flask cultures. Among seven complementations including a wild-type _nar_ promoter module (NiNa–Nb), the SiWa–Sb combination module produced the highest titer of 2,3-BDO (9.7±0.2 g/L), followed by the SiWa–Wb module (Fig. 6b). This best combination modules along with the wild-type _nar_ promoter module as a positive control were then assembled as an independent expression module into one plasmid pSTVM2, resulting in SWS and NNN. After fed-batch cultivation of _E. coli_ expressing NNN and SWS, the SWS module produced 88.0 g/L of 2,3-BDO (Fig. 6c), while the NNN module produced 51.1 g/L of 2,3-BDO (Fig. 6d). This fine-tuning of each 2,3-BDO pathway enzyme expression enhanced the 2,3-BDO titer by 72%. Even though conversion yields (g/g-glucose) were similar [0.33 (NNN) vs. 0.35 (SWS)], volumetric productivity of 2,3-BDO obtained by expressing SWS was 1.87 g/L/h, which was 75% higher than the 1.07 g/L/h obtained by expressing NNN.

Discussion
A promoter is one cellular strategy for controlling the flux of metabolism by regulating the expression of metabolic pathway genes. Therefore, fine-tuning of metabolic pathway gene expression requires an applicable promoter system. In our previous study, a DO-dependent _nar_ promoter was successfully applied to control expressions of biochemical biosynthetic pathway enzymes in _E. coli_ [13]. However, more fine-tuning of expressions of biochemical biosynthetic pathway enzymes is necessary to enhance yield and titer of a target biochemical or
biofuel by metabolic flux channeling. Therefore, in this study, 3 synthetic nar promoters showing weak (W2L-29), intermediate (W2U-30), and strong (S3-2-64) intensities were constructed by randomization of the spacer region sequence (15 bp) located between the consensus sequence $-35$ and $-10$ elements of the wild-type nar promoter (Fig. 1a). Analyses of transcription by qRT-PCR, protein expression by western blotting, and fluorescence by a GFPm reporter protein assay were in a good correlation with the apparent strengths of the 3 synthetic nar promoters (Fig. 3a). Sequence analysis showed that these synthetic nar promoters have relatively higher AT contents than the wild-type nar promoter and higher variations at the $-24$, $-20$, and $-14$ sequence sites (Fig. 3b). The observed features of the synthetic nar promoters are well supported by other research reporting that the AT-rich sequences of the spacer region affected the strength of the promoter through structural changes [18, 20].

To evaluate the general use of synthetic promoters in biochemical production, the 3 synthetic nar promoters were used to express d-lactate and 2,3-BDO pathway enzymes. Among the 3 synthetic nar promoters, the expression of the $ldhD$ gene under the control of the strong synthetic nar promoter on a low-copy plasmid produced the highest amount (105.6 g/L) of d-lactate by fed-batch fermentation. This titer is slight lower than the 113.1 g/L of d-lactate which was obtained.

**Fig. 5** Comparison of acetoin production with the expression of $ilvBN$ and $aldB$ under control of the 3 synthetic nar promoters in flask cultures. a Combination of expression modules of $ilvBN$ and $aldB$ genes. Modules are Si, li, Wi, Sa, la, and Wa; S stands for strong promoter, i for intermediate promoter, W for weak promoter, i for $ilvBN$, and a for $aldB$. b Titer of acetoin obtained by expressing 10 modules in flask cultures. The positive control Ni–Na is a module expressing the $ilvBN$ and $aldB$ genes with a wild-type nar promoter. Black and white bars indicate acetoin and 2,3-BDO, respectively.
by fed-batch fermentation [13] by expression of the \( \text{ldhD} \) gene with the wild-type \( \text{nar} \) promoter on a high-copy plasmid. This suggests that the \( \text{ldhD} \) expression level may be highly correlated with titer of \( \alpha \)-lactate. The correlation of expression level and production titer was also high in the production of 2,3-BDO. In a similar manner to \( \alpha \)-lactate pathway gene expression, each of the three 2,3-BDO biosynthesis pathway genes (\( \text{ilvBN}, \text{aldB}, \) and \( \text{bdh1} \)) were modified to be under the control of the synthetic \( \text{nar} \) promoters of different strengths and combinatorially expressed. In the case of the acetoin pathway, which is a precursor of 2,3-BDO, controlling \( \text{ilvBN} \) gene expression was critical in biosynthesis flux because the expression of \( \text{ilvBN} \) with a weak \( \text{nar} \) promoter resulted in lower titers of acetoin than expression with strong or intermediate promoters regardless of the promoter strength for expressing the \( \text{aldB} \) gene. The acetoin pathway was further extended

Fig. 6 Comparison of 2,3-BDO production with the expression of \( \text{ilvBN}, \text{aldB}, \) and \( \text{bdh1} \) under control of the 3 synthetic \( \text{nar} \) promoters. a Combination of expression modules of \( \text{ilvBN}, \text{aldB}, \) and \( \text{bdh1} \) genes. Modules are Si, Ii, Sa, Wi, Sa, Wi, Sb, Ib, and Wb; S stands for strong promoter, I for intermediate promoter, W for weak promoter, i for \( \text{ilvBN} \), a for \( \text{aldB} \), and b for \( \text{bdh1} \). b Titer of 2,3-BDO obtained by expressing 7 modules in flask cultures. The positive control NINa-Nb is a module expressing \( \text{ilvBN}, \text{aldB}, \) and \( \text{bdh1} \) genes with a wild-type \( \text{nar} \) promoter. Fed-batch cultivation of \( \text{E. coli} \) expressing SWS (c) and NNN (d) modules. The SWS module expressed \( \text{ilvBN} \) with a strong, \( \text{aldB} \) with a weak, and \( \text{bdh1} \) with a strong \( \text{nar} \) promoter. The positive control NNN module expressed \( \text{ilvBN}, \text{aldB}, \) and \( \text{bdh1} \) genes with a wild-type \( \text{nar} \) promoter. Black square, 2,3-BDO; hollow circle, cell growth; hollow triangle, glucose; solid gray circle, DO level; red arrows, the time of DO downshift (induction).
to 2,3-BDO by controlling expression of the \textit{bdh1} gene with the 3 synthetic \textit{nar} promoters. The best combinatorial expression module for 2,3-BDO was a strong promoter for \textit{ilvBN} (Si), weak promoter for \textit{aldB} (Wa), and strong promoter for \textit{bdh1} (Sb). The expression module (Si+Wa+Sb) produced 88.0 g/L of 2,3-BDO in fed-batch fermentation, which was 72% higher than the 51.1 g/L obtained by expressing the 3 enzymes with a wild-type \textit{nar} promoter.

Conclusions
In this study, the synthetic \textit{nar} promoters, which were engineered to have strong, intermediate, and weak intensities, were successfully applied to metabolic engineering of the \textit{d}-lactate and 2,3-BDO pathways in \textit{E. coli}. By controlling expression levels of one \textit{d}-lactate enzyme and three 2,3-BDO pathway enzymes using the synthetic \textit{nar} promoters, the production of \textit{d}-lactate and 2,3-BDO was increased by 34 and 72%, respectively, compared with production using a wild-type \textit{nar} promoter. This synthetic \textit{nar} promoter module system will support the improved production of biochemicals and biofuels through the fine-tuning of gene expression levels in \textit{E. coli}.

Methods
Strains
The \textit{E. coli} TOP10 (Invitrogen, USA) strain was used for cloning and maintenance of plasmids. The \textit{E. coli} W023 [13, 21] strain was used to produce \textit{d}-lactate, acetoin, and 2,3-BDO in flask and fed-batch fermentation. The bacterial strains used in this study are listed in Table 1.

Construction of the randomized \textit{nar} promoter library
A \textit{gfpm} gene encoding GFPm was amplified by PCR from pQE-\textit{gfpm} [22] with gene-specific primers including ribosome binding site (RBS) and restriction enzyme sites, and then the PCR product was inserted between \textit{Xmal} and \textit{NotI} sites downstream of a wild-type \textit{nar} promoter on pUCN [13] and pUCM plasmids [23]. In order to randomize the spacer sequence (15 bp) between the −10 and −35 elements of the \textit{nar} promoter, two primers, \textit{Xmal}-SNPL-gfpm-F and \textit{SphI}-gfpm-R (Table 2) were designed. The \textit{Xmal}-SNPL-gfpm-F primer contains a restriction enzyme site (\textit{Xmal}), the −35 element, randomized 15-bp sequences (\textit{N}_{15}), the −10 element, and the \textit{gfpm}-specific sequence in order. The \textit{SphI}-gfpm-R primer contains a restriction enzyme site (\textit{SphI}) and the \textit{gfpm}-specific sequence. The randomized \textit{nar} promoter region and \textit{gfpm} gene amplified by PCR were cloned into the \textit{Xmal} and \textit{SphI} sites of the pSTVM2 plasmid [13], generating pSTVM2-SNPL-gfpm. The pSTVM2-SNPL-gfpm plasmids were transduced into \textit{E. coli} TOP10 cells by electroporation and the transformants were incubated in 40 mL Luria–Bertani (LB) medium supplemented with 30 μg/L chloramphenicol [24] at 30 °C with shaking at 100 rpm.

Screening of the randomized \textit{nar} promoter library
\textit{Escherichia coli} cells harboring pSTVM2-SNPL-gfpm were cultivated in 40 mL LB medium supplemented with 50 μg/mL Cm in a 100-mL flask at 30 °C with shaking at 100 rpm for 12 h. Cells were harvested by centrifugation at 7000 rpm for 10 min at 4 °C and washed twice with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_{2}HPO_{4}, and 2 mM KH_{2}PO_{4}, pH 7.4). After washed cells were resuspended with PBS buffer, cells were subjected to a fluorescent activated cell sorter (FACS; MoFlo XDP, Beckman Coulter, FL). FACS-sorted cells were directly poured into fresh LB agar plates containing Cm (50 μg/mL) and incubated at 30 °C for 12 h. Cells scraped from the plates were then cultivated in 40 mL LB medium containing Cm (50 μg/mL) in a 100-mL flask and then subjected to the next round of FACS sorting following the same procedure mentioned above. After the third round of FACS sorting, colonies on LB agar plates were randomly selected to cultivate in 200 μL of LB+Cm medium in a 96-deep-well plate at 30 °C with shaking at 100 rpm overnight.

Construction of plasmids
The nucleotide sequences of the primers used in construction of plasmids are listed in Table 2. Plasmid pUCN [13] was modified by adding the \textit{rop} gene, which resulted in a low-copy number plasmid pUCNr with a wild-type \textit{nar} promoter. Next, the wild-type \textit{nar} promoter in pUCNr was replaced by one of the three representative synthetic \textit{nar} promoters [S3-2-64 (strong), W2U-30 (moderate), W2L-29 (weak)] and amplified by PCR with each synthetic promoter-specific reverse primer and phosphorylated -10Pnar-F primer. The constructed plasmids were named pUCNrS (for S3-2-64), pUCNrW (for W2U-30, and pUCNrW (for W2L-29, weak promoter). The reporter \textit{gfpm} gene was amplified by PCR from pQE-\textit{gfpm} [22] with primers (\textit{Xmal}-gfpm-F and \textit{NotI}-gfpm-R) and cloned downstream of the \textit{lac} promoter of pUCM, generating pUCM-gfpm. To obtain pSTVM-gfpm, the PCR-amplified \textit{gfpm} gene containing the \textit{lac} promoter was inserted between the \textit{BamHI} and \textit{EcoRI} sites of the pSTVM2 plasmid [12]. The \textit{ldhD} encoding \textit{d}-lactate dehydrogenase of \textit{Leuconostoc citreum}, \textit{ilvBN}-encoding acetolactate decarboxylase of \textit{Lactococcus lactis}, and \textit{bdh1}-encoding butanedioi dehydrogenase from \textit{Saccharomyces cerevisiae} were amplified by PCR from the genomic DNAs of each strain, and then...
cloned downstream of the synthetic nar promoters of pUCNrs, pUCNrI, and pUCNrW (Table 2). For complementation experiments, the ilvBN gene was amplified by PCR with the synthetic nar promoter and a terminator, and then inserted between the PstI and BamHI sites of the pSTVM2 plasmid. To assemble two genes (ilvBN and aldB) encoding acetoin pathway enzymes and three genes (ilvBN, aldB, and bdh1) encoding 2,3-BDO pathway enzymes in pSTVM2, each gene was amplified by PCR with the synthetic nar promoter and a terminator, and then subcloned into pSTVM2 using the USER™ cloning method [25, 26].

**Flask and bioreactor fermentations**

The recombinant E. coli strains harboring a plasmid or plasmids for production of d-lactate, acetoin, and

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**Table 2 Primers used in this study**

| Primer name | Sequence (5′-3′) |
|-------------|-----------------|
| For cloning |                 |
| Xmal-SNPL-gfpm-F | TCCCCTTGCCGGCTCTTGTGTTATCAAAATCGCCANNNNNNNNNNNNNNGTATAA |
| Spnl-gfpm-R | GCCATCGCATTTAGTGGTGGGTGGTGTTGTAGAGCTCATCGATGC |
| Xmal-gfpm-F | TCCCCTTGCCGGAGGAGGATTACAAATATGTAAGGAGAAGAAACTT |
| Not-f-gfpm-R | TAAAGATCGCGCGCTTTAGTGGTGGGTGGTGTTGTAGAGCTCATCGATGC |
| ldhD-citreum-F-XbaI | CTAAGTCTAGAAGGAGGATTACAAATATGTAAGGAGAAGAAACTT |
| ldhD-citreum-R-Notl | TTCCCTTGCGCGCGCTTTACTTTTACGCAATACTT |
| ilvB-EC-F-Xbal | CTAAGTCTAGAAGGAGGATTACAAATATGTAAGGAGAAGAAACTT |
| ilvN-EC-R-Notl | TTCCCTTGCGCGCGCTTTACTTTTACGCAATACTT |
| aldB-LL-F-Xbal | CTAAGTCTAGAAGGAGGATTACAAATATGTAAGGAGAAGAAACTT |
| aldB-LL-R-Notl | TTCCCTTGCGCGCGCTTTACTTTTACGCAATACTT |
| bdh1-SC-F-Xmal | CTAAGTCTAGAAGGAGGATTACAAATATGTAAGGAGAAGAAACTT |
| bdh1-SC-R-Notl | TTCCCTTGCGCGCGCTTTACTTTTACGCAATACTT |
| Spnl-pUC-F | ACATGCATGCGCGACTGGAAGCC |
| Spnl-pUC-R | ACATGCATGCGCGACTGGAAGCC |
| Pst-pUC-F | AAAATCTGGACCGCGACTGGAAGCC |
| BamHII-pUC-R | CCGGATCCGGCGGTGTAATAACCC |
| PnarS-R | TCCCTTGCGCGCGCTTTACTTTTACGCAATACTT |
| PnarR | AAATCTGGACCGCGACTGGAAGCC |
| PnarW-R | GGAGATGTACAATATGGAATTGTTGTAAGGACCATCAG |
| -10Pnar-F | GTATATGGCCCTTAAATCCTAGA |
| pUCN-ori-fr | AGGAAGCCGAAGAGCC |
| pUCN-ori-r | GAAGATCTCGTCTTCTTCTA |
| pET-ori-R | TTGAGATCTCTTTTTCG |
| pET-rop-F | GGTGCCGATGATCGT |
| pSTVM2-pUC-sub-USER-3-F | AGACAGLUCATAAGTGGC |
| pSTVM2-pUC-sub-USER-1-R | ATGCAACUCGTAGGACAG |
| pUC-sub-USER-1-F | AGTTGCAUCGCGACTGGAAGCC |
| pUC-sub-USER-2-F | ATCCATGUCGCGACTGGAAGCC |
| pUC-sub-USER-S-F | ATATGCGAUCGCGACTGGAAGCC |
| pUC-sub-USER-2-R | ACATGCGAATGCGGTTGTAATACC |
| pUC-sub-USER-S-R | ATCGCATATGCGGTTGTAATACC |
| pUC-sub-USER-3-R | ACTGTCATGCGGTTGTAATACC |
| For qRT-PCR |                 |
| q-cysG-F | TTGGTGCCGCGGTGGTGGATGTC |
| q-cysG-R | ATGCGGTTAAGCTTGGGAATACAC |
| q-gfpm-F | AGAGGTTGAAGGTAGTGGC |
| q-gfpm-R | AGATGATCCGGGATAAACCG |

Italic and underline letters represent a restriction enzyme site.
2,3-BDO were inoculated in 4 mL LB medium supplemented with 50 μg/mL Cm or/and 100 μg/mL ampicillin (Ap) at 37 °C overnight with shaking at 250 rpm. For flask cultivation, 100-mL flasks were filled with 40 mL LB medium containing 20 g/L glucose and appropriate antibiotics, and then were inoculated with 2% (v/v) seed culture. For d-lactate production, pH of the culture media was controlled by adding 10 g/L CaCO₃. A nar promoter was induced by reducing shaking speed from 250 to 100 rpm when recombinant E. coli cells grew to an OD₆₀₀ of 1.0 at 30 °C at 250 rpm. For bioreactor fermentation, fed-batch culturing was carried out with an initial culture volume of 1.0 L of modified R [27] medium containing 20 g/L glucose, 5 g/L yeast extract, and the required antibiotics in a 3.0-L jar bioreactor BIOSTAT B (Sartorius, Germany) [28]. The temperature was maintained at 30 °C and pH was automatically controlled at 7.0 by adding 5 N NH₄OH. The DO level was controlled by supplying air or a mixture of air and pure oxygen gas. In order to induce nar promoters, cells were grown at DO level >80% (aerobic phase) until an OD₆₀₀ of 10.0 and then immediately DO level was decreased to <1–2% (microaerobic). The feeding solution consisting of 800 g/L glucose, 50 g/L yeast extract, 15 g/L tryptone, 15 g/L MgSO₄·7H₂O, and 5 g/L KH₂PO₄ [29] was periodically added when the residual glucose concentration was below 5–10 g/L. Cell growth was monitored at a wavelength of 600 nm with a SPECTRAm-max PLUS384 (Molecular Devices, USA).

**Transcriptional analysis**

Cells were grown in LB medium containing 2% (w/v) glucose until mid-exponential growth phase, and total RNA was extracted using Hybrid-R RNA purification kit (GeneAll biotechnology, Korea) according to the manufacturer’s instructions. Quantitative reverse transcription PCR (qRT-PCR) was performed using a Rotor-Gene Q (Qiagen, Germany) and SensiFAST™ SYBR No-ROX One-Step Kit (Bioline, USA). Solutions of 5.0 μL of 2× SensiFAST™ SYBR No-ROX One-Step mix, 0.1 μL of reverse transcriptase, 0.2 μL of RNase inhibitor, 0.4 mM forward and reverse primers (gfpm-qPCR-F and gfpm-qPCR-R), 2.0 μL of isolated total RNA (10 ng/μL), and 1.9 μL of diethylpyrocarbonate (DEPC)-treated water were mixed for each qRT-PCR reaction and qRT-PCR was performed as follow: 45 °C for 10 min, 95 °C for 2 min, and then 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 5 s. The value of ΔΔCₜ was averaged from triplicate measurements. The cysG gene encoding siroheme synthase was used as a reference gene and the genes expressed by the wild-type nar promoter were used as calibrators.

**Western blotting analysis**

GFPM expression under the control of nar promoters was analyzed by western blotting. The harvest cells were washed and resuspended in 20 mM Tris–HCl (pH 8.0), and then disrupted by sonication. After centrifugation, supernatants were collected, quantified using the Bradford method, and then analyzed by 15% (w/v) SDS-PAGE. For immunodetection of His-tagged GFPM, a monoclonal anti-polystyrene conjugated mouse IgG (Pierce, USA) were used according to the manufacturer’s instructions. GAPDH was used as a reference gene for quantification of proteins.

**Fluorescence analysis**

After cells were grown under aerobic conditions, GFPM protein expression under the control of nar promoters was induced by lowering DO levels through changing culture rpm of 250–100. Harvested cells were washed and resuspended in 1 mL phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4) and the fluorescence intensity of the reporter GFPM protein was measured using a SPECTRAmax Gemini plate reader (Molecular Devices, USA) with excitation at 470 nm and emission at 510 nm. Cytometric analysis was performed using a BD FACS Calibur flow cytometer (BD Biosciences, USA). GFPM was excited using a 15-mW argon ion laser (488 nm) and fluorescence emission was detected using the FL1 channel (530/30 bandpass filter).

**Metabolite analysis**

The concentrations of glucose, d-lactate, 2,3-BDO, and other metabolites were determined using an Agilent Technologies 1200 high-performance liquid chromatography equipped with a refractive index detector (Agilent, USA) and an Aminex HPX-87H column (Bio-Rad, USA) at a flow rate of 0.7 mL/min and column temperature of 50 °C using 4 mM H₂SO₄ as the mobile phase.

**Abbreviations**

GF: green fluorescent protein; 2,3-BDO: 2,3-butanediol; FACS: fluorescent activated cell sorter; RFU: relative fluorescence unit.

**Authors’ contributions**

HJJ participated in the experimental design and work, data analysis, and writing of the manuscript. SYL assisted in the experimental design. PCL assisted in the experimental design, the experimental work, data analysis, and writing of the manuscript. All authors read and approved the final manuscript.

**Author details**

¹ Department of Molecular Science and Technology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 16944, South Korea. ² Department of Chemical and Biomolecular Engineering, KAIST, Daejeon 34141, South Korea.
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