Supplementary materials for

A pathway independent multi-modular ordered control system based on thermosensors and CRISPRi improves bioproduction in *Bacillus subtilis*

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Table. S1 Primers used in this study

Primer	Sequence
futc-F1	AGAGGCGATTATGTTGGCATTG
futc-R1	CGGATAGCCCAGATCCAGATT
manB-F1	TGGCGGCATTGAAGTTACC
manB-R1	TCATCAACGGGAGGGAAGTC
ndk-F1	TCAGAGCCGTGGATGATGTT
ndk-R1	ACCTGTATTCGCAATGGTGTG
gmk-F1	CCAAGAGAGGGCGAAGTGA
gmk-R1	AAGCGTCTGTTCAACATAATCG
zwf-F1	AGCACACGCACAGCCAAT
zwf-R1	CTCCAAGAAAGGGCAACTTCAT
pfkA-F1	GGTCAGCAGCACTCGGAGAA
pfkA-R1	GCGGCAAGAAGCACAGTATT
lytC-F1	GACTTCTTTCTCAAGGAGTCC
lytC-R1	GAAACACTCCTGCCGTAAG
FL-hbs-F	CGTTCACGCACCTCGAAGTTACC
FL-hbs-R	CGGTTGCAGAAGCAAGCGAATTG
amye-D-F	CGGAAGAAACATTTGGCTAATTCCCCATTGAGGGCAAG
amye-D-R	TGTGAAGGAACTGTTCTTTTTCTTT
amye-F	CTGGATTTTTATTGCTGTTTCATTT
amye-R	AGTTCAGCTCAGTGATACCTG
amye-S-F	GATAGCTTCTCGTTCAGGCGAGCGGATAACAATTTCACACAG
amye-S-R	TCTTTTTTGTTGACATGTGTACATTTCACCTCCTTTG
amye-U-F	GCAAAACGATTCAAAACCTCTT
amye-U-R	GTGAAATTGTTATCCGCTCGCCTGAACGAGAAGCTATC
CI857-F	CAAAGGAGGTGAAATGTACACATGTCAACAAAAAAGAAACCGC
CI857-R	CCCTCAATGGGGAATTAGCCAAATGTTTCTTCCGG
Pveg-NF1	AACACCTTATTAACGTTGATATNAACACCGTGCGTGTTNACATACCTCT GGCGGTGATAA
Pveg-NF2	AACACCTTATTAACGTTGATATTNNNNNCGTGCNNNNNGACATACCTCT GGCGGTGATAA
Pveg-NF3	AACACCTTATTAACGTTGATATTAACACNNNNNGTGTTGACATACCTCT GGCGGTGATAA
Pveg-NR	AATATCAACGTTAATAAGGTGTTAGATATTTAT

H-Pveg5-F	CATGGATGAACTGTACAAATAACTGCAGGTCGACGTCC
H-Pveg5-R	CGCACGGTGTTAATATCAACGTTAATAATAGAGCGCAACGCAATTAATG
Pveg5-F	CATTAATTGCGTTGCGCTCTATTATTAACGTTGATATTAACACCGTGCG
Pveg5-R	GTCGACCTGCAGTTATTTGTACAGTTCATCCATGCCA
npre-F	CTTGCGGCAGCCATCTTCAC
npre-R	GCCATCGTCACCCACTTA
ganA-F	GTGATGTCAAAGCTTGAAAAAACG
ganA-R	TTTTCCAAAGCAATAACGCTGGA
bpr-F	TTAGGCGCATTAACAGTCGGC
bpr-R	GGATGAATAATCGCGTCAAACT
epr-F	TTGTGCAGACTCTGAAAAGGT
epr-R	ACTGAAGCCGACTAAAATCGGC
apre-F	AACATCAGGATGCTGACAAATAAAA
apre-R	AGAGGGTAAAGAGTGAGAAGC
ldh-R	TTGAAGGCAGGAAGGCT
ldh-F	GCAACAAAATTGCCTGATGG

Table. S2 Plasmids used in this study

Plasmid	Characteristics	Ref.
pHTa0	ColE1 Amp^r, Cm^r, *E. coli-B. subtilis* shuttle vector, *Δ*P$_{grac}$*::egfp*	Lab stock
pHTa1	pHTa0 derivate, P$_R$- *egfp*	This work
pHTa1-2	pHTa0 derivate, P$_{veg}$- *egfp*	This work
pHTa2	pHTa0 derivate, P$_{veg1}$- *egfp*	This work
pHTa3	pHTa0 derivate, P$_{veg2}$- *egfp*	This work
pHTa4	pHTa0 derivate, P$_{veg3}$- *egfp*	This work
pHTa5	pHTa0 derivate, P$_{veg4}$- *egfp*	This work
pHTa6	pHTa0 derivate, P$_{veg5}$- *egfp*	This work
pP43-mCherry	ColE1 Amp^r, RepB Km^r, *E. coli-B. subtilis* shuttle vector, mCherry expressed from P$_{43}$	This work
pLCT-dCpf1	pMB1 Spec^r, Cm^r P$_{veg5}$-dCpf1 integrant expression at *nprE* locus of *B. subtilis*	This work
pLCT2-dCpf1	pMB1 Spec^r, Cm^r P$_{veg}$-dCpf1 integrant expression at *ganA* locus of *B. subtilis*	This work

Table S3. Sequences of genetic parts used in this study

Name	Sequence
P_{veg}	TTATTAACGTTGATATAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGT
P_{veg1}	TTATTAACGTTGATATAATTTAAATTTTATTTGACATAACACCGTGCGTGTTGTACAATAAATGT
P_{veg2}	TTATTAACGTTGATATTAACACCGTGCGTGTTGACAAAAATGGGCTCGTGTTGTACAATAAATGT
P_{veg3}	TTATTAACGTTGATATAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTAGTAACACCGTGCGTGTTG
P_{veg4}	TTATTAACGTTGATATTAACACCGTGCGTGTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTAGTACCTCTGGCGGTGATAA
P_{veg5}	TTATTAACGTTGATATTAACACCGTGCGTGTTGACATACCTCTGGCGGTGATAATACAATAAATGT
P_{veg32}	TTATTAACGTTGATATTTATTGCGTGCATGTTGACATACCTCTGGCGGTGATAATACAATAAATGT
P_{veg34}	TTATTAACGTTGATATTAACACGGGTAGTGTTGACATACCTCTGGCGGTGATAATACAATAAATGT
P_{veg37}	TTATTAACGTTGATATTGGCGGCGTGCTCGTTGACATACCTCTGGCGGTGATAATACAATAAATGT
zwf-crRNA1	GCAAAACGAAAATTGTATCCGTC
zwf-crRNA2	TGCGCGGACAATATCATGCTGGT
zwf-crRNA3	GTGAAAGACGGCTTACACTGGTG
pfkA-crRNA1	CAACGGATACGCGGGATTGATCA
pfkA-crRNA2	CATGTGTAGGTGTACCGGGTACA
pfkA-crRNA3	AAGAAGAAACAAATCTTGAAACT
lytC-crRNA1	GCCGATAACTCAGTGAAAAGAGT
lytC-	AACGGCTTCTTATATGCAGACGC

crRNA2

lytC-crRNA3

TCAGTATACATGCAAATGCTAAT

ATGTCAACAAAAAAGAAACCGCTGACACAAGAACAACTGGAAGATGCAAG

AAGACTGAAAGCAATTTATGAAAAAAAGAAAAATGAACTGGGCCTGTCACA

AGAATCAGTTGCAGATAAAATGGGCATGGGCCAAAGCGGCGTTGGCGCA

CTGTTTAATGGCATTAATGCACTGAATGCATATAATGCAGCACTGCTGACA

AAAATTCTGAAAGTTTCAGTTGAAGAATTTTCACCGTCAATTGCAAGAGAA

ATTTATGAAATGTATGAAGCAGTTTCAATGCAACCGTCACTGAGATCAGAA

TATGAATATCCGGTTTTTTCACATGTTCAAGCGGGCATGTTTTCACCGAAA

CI^{857} CTGAGAACATTTACAAAAGGCGATGCAGAAAGATGGGTTTCAACAACAAA

AAAAGCATCAGATTCAGCATTTTGGCTGGAAGTTGAAGGCAATTCAATGA

CAGCACCGACGGGCTCAAAACCGTCATTTCCGGATGGCATGCTGATTCTG

GTTGATCCGGAACAAGCAGTTGAACCGGGCGATTTTTGCATTGCAAGACT

GGGCGGCGATGAATTTACATTTAAAAAACTGATTAGAGATAGCGGCCAAG

TTTTTCTGCAACCGCTGAATCCGCAATATCCGATGATTCCGTGCAATGAAT

CATGCTCAGTTGTTGGCAAAGTTATTGCATCACAATGGCCGGAAGAAACA

TTTGGCTAA

ATGCTAACTTGCTTTAAAGCTTATGATATTCGCGGGAAACTAGGCGAAGAA

CTGAATGAAGATATCGCCTGGCGCATTGGGCGTGCCTATGGCGAATTTCT

CAAACCGAAAACCATTGTGTTAGGCGGTGATGTCCGCCTCACCAGCGAAA

CCTTAAAACTGGCGCTGGCGAAAGGTTTACAGGATGCGGGCGTCGATGT

GCTGGATATCGGTATGTCCGGCACCGAAGAGATCTATTTCGCCACGTTCC

ATCTCGGCGTGGATGGCGGCATTGAAGTTACCGCCAGCCATAATCCGAT

GGATTATAACGGCATGAAACTCGTGCGCGAGGGGGCTCGCCCGATCAGC

GGGGATACCGGACTGCGCGATGTCCAGCGTCTGGCAGAAGCCAACGACT

manB TCCCTCCCGTTGATGAAACCAAACGCGGTCGTTATCAGCAAATCAATCTG

CGTGACGCTTACGTTGATCACCTGTTCGGTTATATCAACGTCAAAAACCTC

ACGCCGCTCAAGCTGGTGATTAACTCCGGGAATGGCGCGGCGGGGCCG

GTGGTGGACGCCATTGAAGCCCGCTTTAAAGCCCTCGGCGCACCGGTGG

AATTAATCAAAGTACACAACACGCCGGACGGCAATTTCCCCAACGGTATT

CCTAACCCGTTGCTGCCGGAATGCCGCGACGACACCCGTAATGCGGTCA

TCAAACACGGCGCGGATATGGGCATTGCCTTTGATGGCGATTTTGACCGC

TGTTTCCTGTTTGACGAAAAAGGGCAGTTTATCGAGGGCTACTACATTGTC

GGCCTGCTGGCAGAAGCATTCCTCGAAAAAAATCCCGGCGCGAAGATCA

TCCACGATCCGCGTCTCTCCTGGAACACCGTTGATGTGGTGACCGCCGC

GGGCGGCACTCCGGTGATGTCGAAAACCGGACACGCCTTTATTAAAGAA

CGTATGCGCAAGGAAGACGCCATCTACGGTGGCGAAATGAGCGCCCACC

ACTATTTCCGTGATTTCGCTTACTGCGACAGCGGCATGATCCCGTGGCTG

CTGGTCGCCGAACTGGTGTGTCTGAAAGGAAAAACGCTGGGCGAACTGG

TGCGCGACCGGATGGCGGCGTTTCCGGCAAGCGGTGAGATCAACAGCAA

ACTGGCGCACCCCGTTGAGGCGATTAATCGCGTCGAACAGCATTTTAGCC

GCGAGGCGCTGGCGGTGGATCGCACCGATGGCATCAGCATGACCTTTGC

CGACTGGCGCTTTAACCTGCGCTCCTCCAACACCGAACCGGTGGTGCGG

TTGAATGTGGAATCGCGCGGTGATGTACCGCTGATGGAAGAAAAGACAAA

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TCAATATGCGTTTGCGAAAAGCCTGCAAAAACACTCAAATACGCCGGTTC

TGCTGGATATTACGTCGTTTGATTGGTCAGATAGAAAAATGCAACTGGAAC

TGTTTCCGATCGATCTGCCATATGCAAGCGAAAAAGAAATTGCAATCGCA

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TTTTGATGCGATCAGTCCTCTGATTAAACAAACATTTACACTGCCGCCGCC

GCCGCCTGAAAATGGCAACAATAAAAAGAAAGAAGAGGAATATCACCGCA

futC

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GAGGCGATTATGTTGGCATTGGCTGCCAACTGGGCATTGATTATCAAAAA

AAAGCACTGGAATATATGGCAAAAAGAGTTCCGAATATGGAACTGTTTGTT

TTTTGCGAAGATCTGACATTTACACAAAATCTGGATCTGGGCTATCCGTTT

ATGGATATGACAACAAGAGATAAAGAAGAAGAAGCATATTGGGATATGCT

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ACATTGGCTGTTTGGCCATGAAAATATTCTGTGCAAAGAATGGGTTAAAAT

TGAATCACATTTTGAAGTTAAATCACAAAAATATAATGCATAA

ATGAAAGAAAGAGGGTTATTAATCGTTCTCTCAGGTCCCTCAGGAGTTGG

TAAAGGAACGGTTCGACAAGCGATCTTTTCGCAGGAAGACACAAAATTTG

AATATTCGATTTCAGTAACCACAAGAAGTCCAAGAGAGGGCGAAGTGAAC

gmk

GGAGTCGATTATTTTTTCAAAACAAGAGACGAATTCGAGCAAATGATTGCG

GACAACAAGCTGCTTGAATGGGCAGAGTATGTCGGCAATTATTACGGCAC

GCCAGTCGATTATGTTGAACAGACGCTTCAAGATGGAAAAGACGTCTTTTT

	AGAAATTGAAGTTCAAGGGGCTCTTCAAGTGAGAAATGCTTTCCCGGAAG
	GCCTGTTTATTTTCCTTGCGCCTCCAAGCCTTTCTGAACTGAAAAACAGAA
	TCGTGACACGAGGAACAGAAACAGACGCTCTGATTGAAAATCGAATGAAA
	GCCGCAAAAGCTGAGATCGAAATGATGGATGCTTATGACTATGTCGTTGA
	AAACGATAATGTCGAAACGGCTTGCGATAAAATCAAAGCAATCGTTCTTGC
	TGAACATTTGAAGCGTGAACGCGTTGCACCAAGATATAAGAAAATGCTGG
	AGGTTGAATAA
	ATGATGGAAAAGACTTTTATCATGGTGAAACCAGACGGTGTCCAACGTCA
	GCTCATTGGGGACATTTTATCTAGATTCGAACGTAAGGGCTTACAATTAGC
	TGGCGCCAAGTTAATGAGAGTGACTGAACAAATGGCTGAGAAACACTACG
	CCGAACATCAAGGTAAGCCTTTCTTCGGAGAGCTCGTTGAGTTTATTACTT
	CAGGACCTGTATTCGCAATGGTGTGGGAAGGCGAAAATGTCATTGAAGTG
	ACGAGACAGCTGATCGGGAAAACAAACCCTAAAGAAGCTTTACCTGGTAC
	GATTCGTGGGGATTATGGCATGTTTGTCGGAAAAAACATCATCCACGGCT
	CTGATTCTCTCGAAAGTGCAGAACGCGAGATTAACATTTTCTTTAAGAATG
	AAGAATTAGTATCATATCAGCAGCTTATGGCAGGCTGGATCTATTAATCAT
	TGTGAAAATAAGCAACAGAGCCGGCAACATATAACGCCGCCGGTTTTTGT
	TCTGTTTTGTAAAGGTTTACATTAAAGTGAATAAAGGGTTGAATGGCAGTG
ndk	TGCATCAGACCTCTGCCTTTCCCAACGATCTAAAATCAGCTGAAAAGCTG
	ATTTTTTTATTGAAACAATACAATTTCCTGATAAAACAAGCTATACTAGGAC
	AAAAAGACAGCGAGACTGGGAGAGTAGAGATGGATACATACAGCGTATTT
	ACAACGAAATGGAAACAATTAACCGGAGTCGATTTAACACTATATAAAGAA
	GCACAAATGAAGAGAAGGCTAACGTCACTTTATGAGAAAAAGGGGTTCCA
	AAGCTTTAAGGACTTTGCTGCGGCATTGGAAAAGGATCAAGCTCTCTTAA
	ATGAAACATTGGACAGAATGACGATCAATGTTTCAGAATTTTATCGTAATT
	ATAAAAGATGGGAAGTTCTTGAGACTGCAATTCTGCCGTTAATCAAGACCT
	CTAGGCCTTTAAAAATTTGGAGTGCCGCCTGCTCAACGGGGGAGGAGCC
	ATACACGCTTGCCATGCTTTTGGACCAGCAAAAAGGTCTTCCGGGCTATC
	AGATTTTAGCGACGGATATTGATGAAAAAGCATTGGAAAAAGCGAAAAAA
	GGCGTTTATCAGGAGCGGTCTTTACA

Table S4 Hill-equation fitting parameters of gene expression fine-tuning

Promoter	y_{min}	y_{max}	K	n	R^2
P_{veg5}	19.97805 ± 14.5704	2378.71118 ± 29.95501	38.21927 ± 0.04872	62.02255 ± 2.92182	0.99959

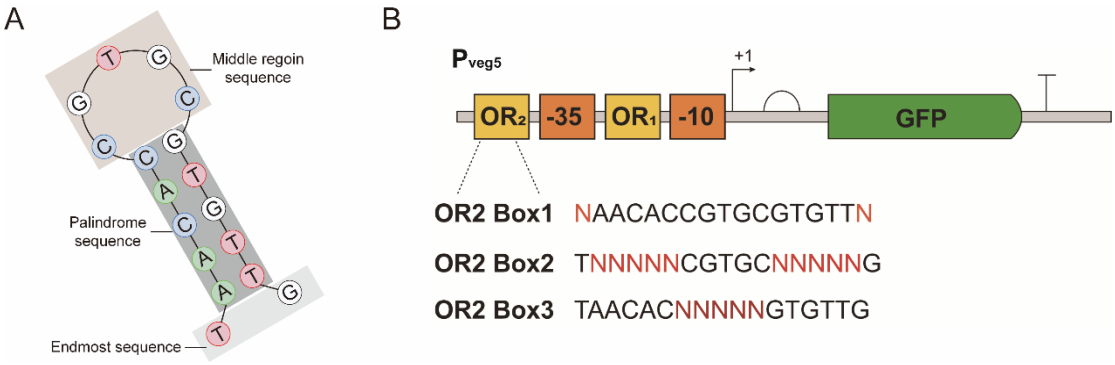

Figure S1 (A) Modular OR_2 sequence, including endmost sequence module (T and G), palindrome sequence module (AACAC and GTGTT) and middle region sequence module (CGTGC). (B) Three type of promoter mutation libraries at the OR_2 region.

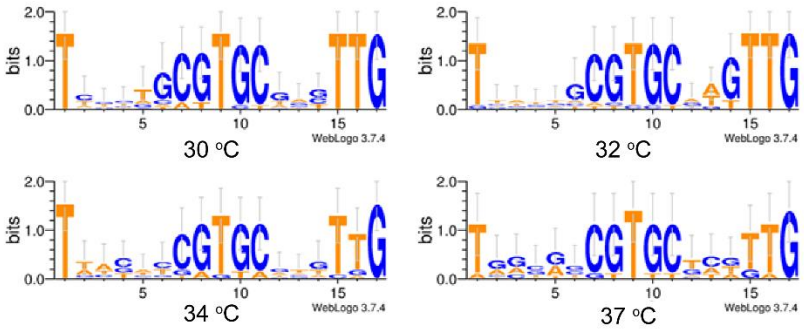

Figure S2 Sequence features at OR_2 region of promoter variants with different transition points (30, 32, 34 and 37 °C).

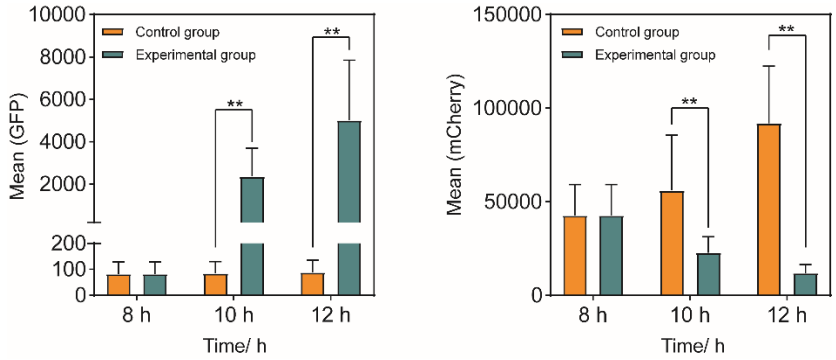

Figure S3 The switching effects of bifunctional temperature-responsive genetic circuits. The control group was cultured under constant temperature fermentation at 32 °C for 12 h and the experimental group was cultured by using single-switch control (0-8 h at 32 °C and 8-12 h at 39 °C).

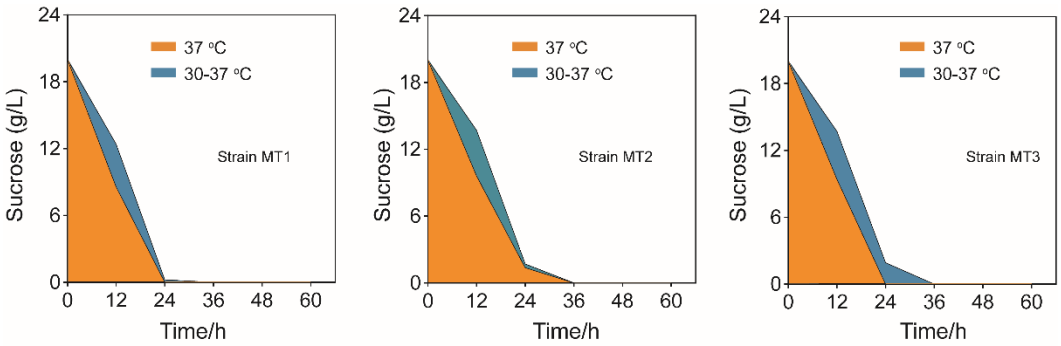

Figure S4 The sucrose concentration of strain MT1, MT2 and MT3 under different bioprocesses, including constant temperature fermentation at 37 °C for 60 h and single-switch control (0-12 h at 30 °C and 12-60 h at 37 °C).

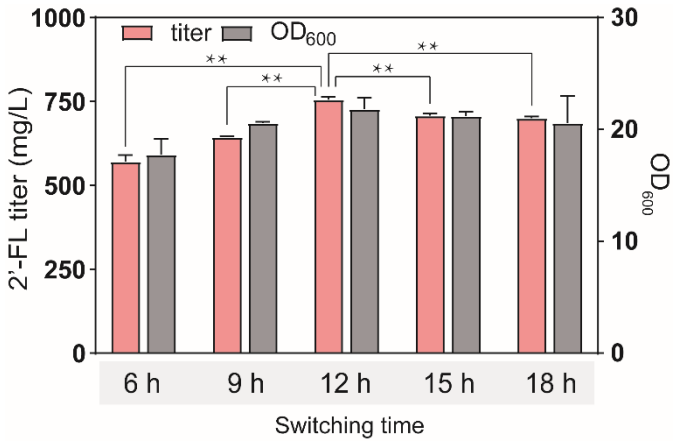

Figure S5 The cell growth and 2'-FL titer of strain MT3 (*manB* and *futC* were driven by P_{veg34}) under single-switch control from 30 to 37 °C with different switching times (6, 9, 12, 15 and 18 h). The statistical analysis is based on Student's *t*-test. All data were the average of three independent studies with standard deviations. $^{**}P < 0.01$.

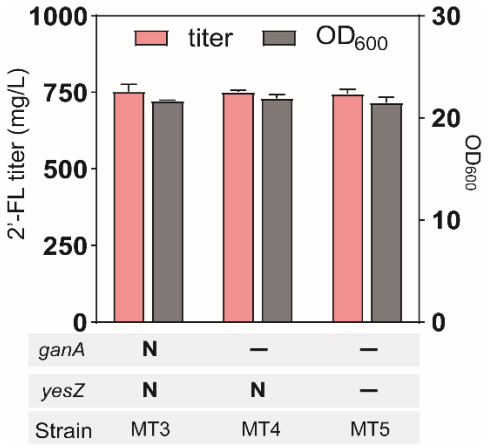

Figure S6 OD_{600} and 2'-FL titer of strain MT3, MT4 and MT5 with single-switch control (0-12 h at 30 °C and 12-60 h at 37 °C). The strain MT4 was obtained by knocking out gene *ganA* of strain MT3, and MT5 was obtained by knocking out *yesZ* of strain MT4. 'N' means no knockout and '-' means knockout. All data were the average of three independent studies with standard deviations.

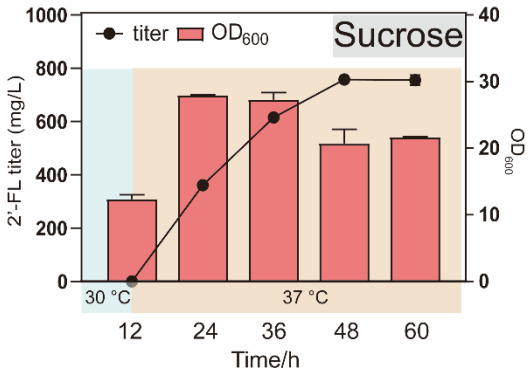

Figure S7 The fermentation process of strain MT3. Trends of OD_{600} and 2'-FL titer in shake flask with single-switch control. Data are presented as mean ± s.d. of three replicates.

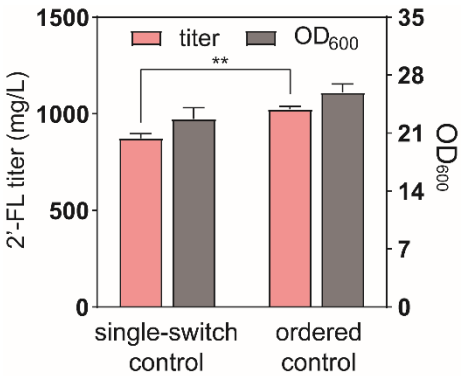

Figure S8 OD_{600} and 2'-FL titer of strain MT14 under different control strategies, including

single-switch control and ordered control (0-12 h at 30 °C ,12-18 h at 34 °C and 12-60 h at 37 °C). The statistical analysis is based on Student's *t*-test. All data were the average of three independent studies with standard deviations. $^{**}P < 0.01$.

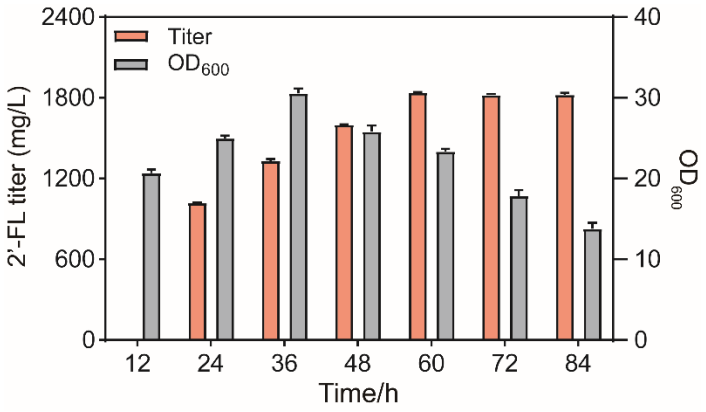

Figure S9 The OD_{600} and 2'-FL titer of strain MT17 for 84 h. All data were the average of three independent studies with standard deviations.

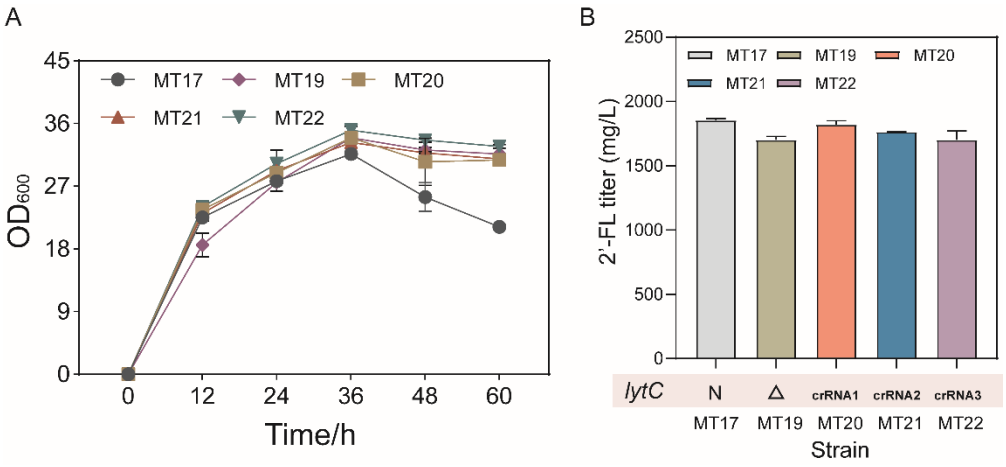

Figure S10 (A) The OD_{600} of recombinant strains with knocking out or knocking down *lytC* in shake flask. (B) The 2'-FL production of recombinant strains in shake flask. "N" means the native gene *lytC* was not engineered. "△" means the gene *lytC* was knocked out. The strain MT19 was obtained by knocking out *lytC* directly from the control strain MT17. Three crRNAs that targeted to different positions on *lytC*, driven by the promoter P_{veg5}, were integrated into the genome of MT17, resulting in the strains MT20 (crRNA1), MT21 (crRNA2) and MT22 (crRNA3), respectively. All data were the average of three independent studies with standard

deviations.

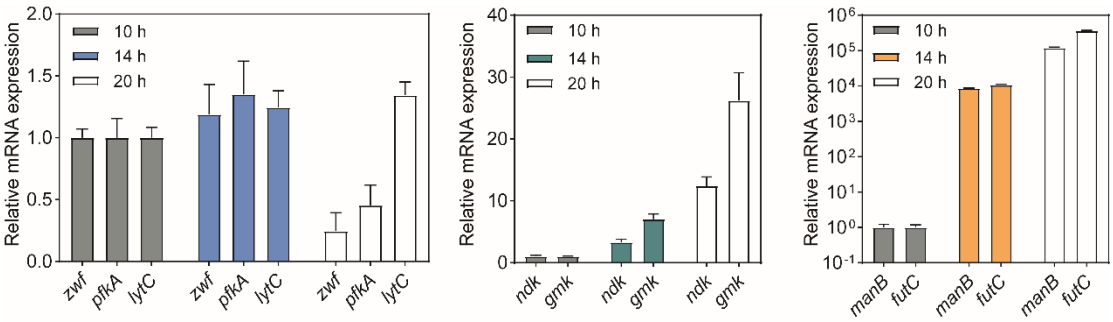

Figure S11 Relative normalized expression of genes *manB*, *futC*, *ndk*, *gmk*, *lytC*, *zwf* and *pfkA* in strain MT17 by using multi-modular ordered control (0-12 h at 30 °C, 12-18 h at 34 °C and 18-80 h at 37 °C). Data are presented as mean ± s.d. of three replicates.

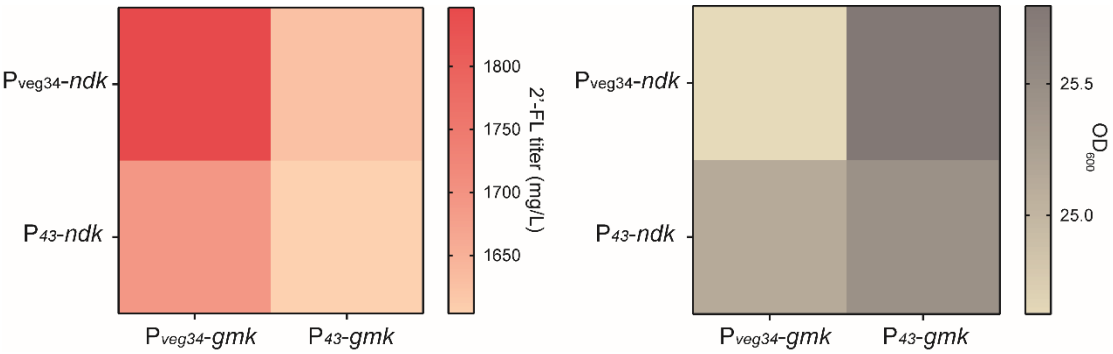

Figure S12 The cell growth and 2'-FL titer of GTP recycling-modified strains, including MT17, and the recombinant strains with constitutive overexpression of GTP supply by promoter P_{43}. The statistical analysis is based on Student's *t*-test. All data were the average of three independent studies with standard deviations.