ASSOCIATED CONTENT

NOT-gate genetic circuits to control gene expression in cyanobacteria

Arnaud Taton, Amy T. Ma, Mizuho Ota, Susan S. Golden, and James W. Golden

Division of Biological Sciences, University of California San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA

FIGURES

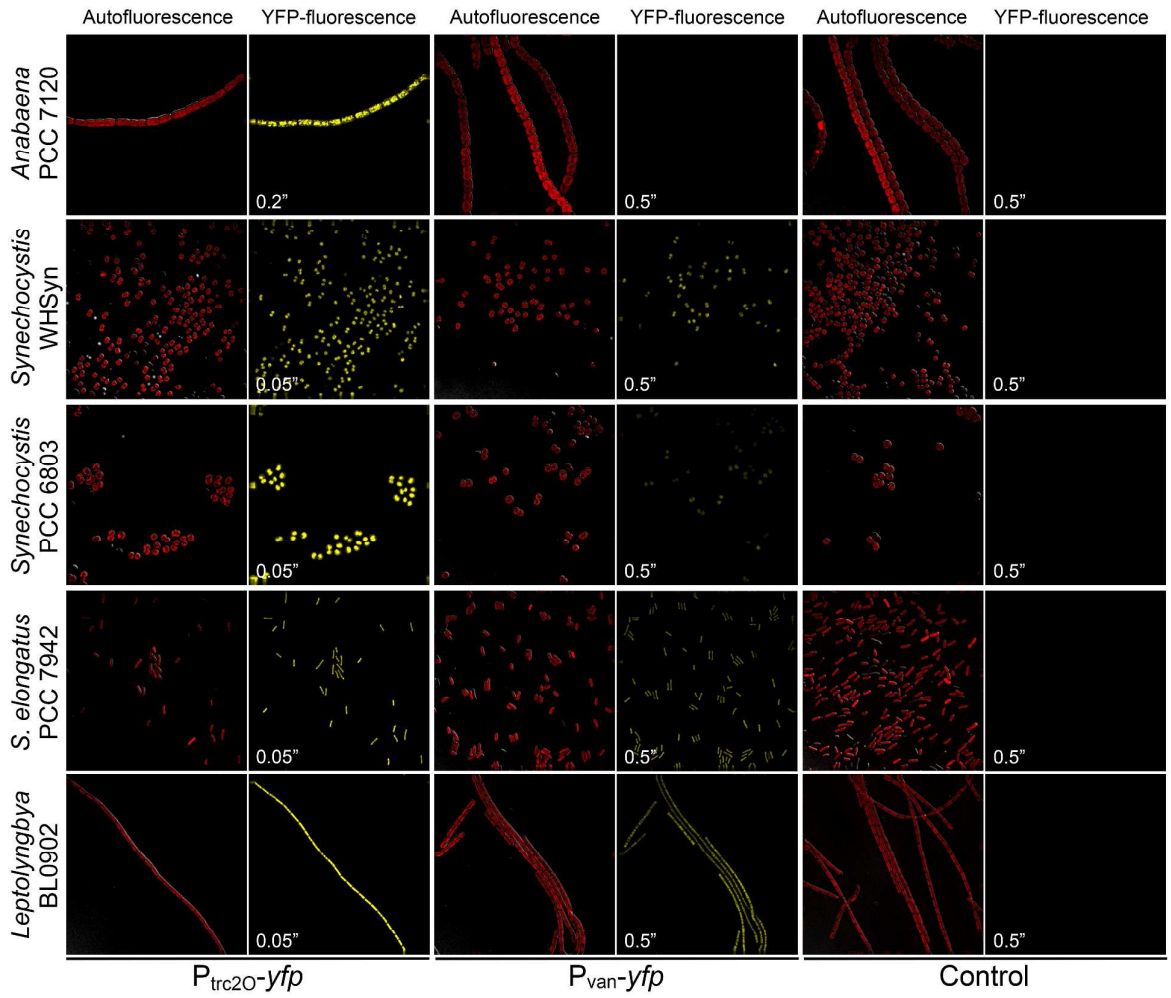

Figure S1. Photomicrographs of 5 cyanobacterial strains harboring the reporter device alone (P_{trc2O}-*yfp* or P_{van}-*yfp*) and control strains. Left images show autofluorescence of photosynthetic pigments merged with differential interference contrast (DIC); right images show YFP fluorescence. Exposure times used for each strain are indicated at the bottom left of the YFP fluorescence images.

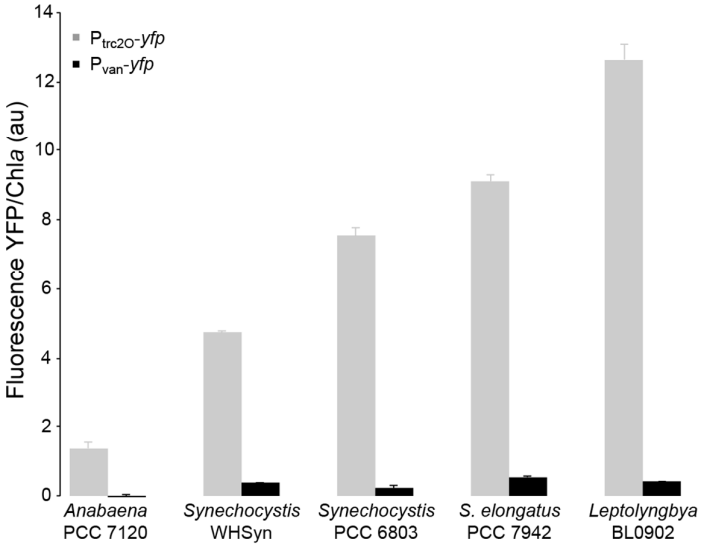

Figure S2. Comparison of P_{trc2O} and P_{van} activity in the absence of the cognate transcription repressors, $LacI^q$ and VanR, respectively. Data were obtained from triplicate cultures grown from three independent colonies adjusted to an OD_{750} of 0.1, and YFP fluorescence intensities were further normalized to chlorophyll (Chl*a*) fluorescence.

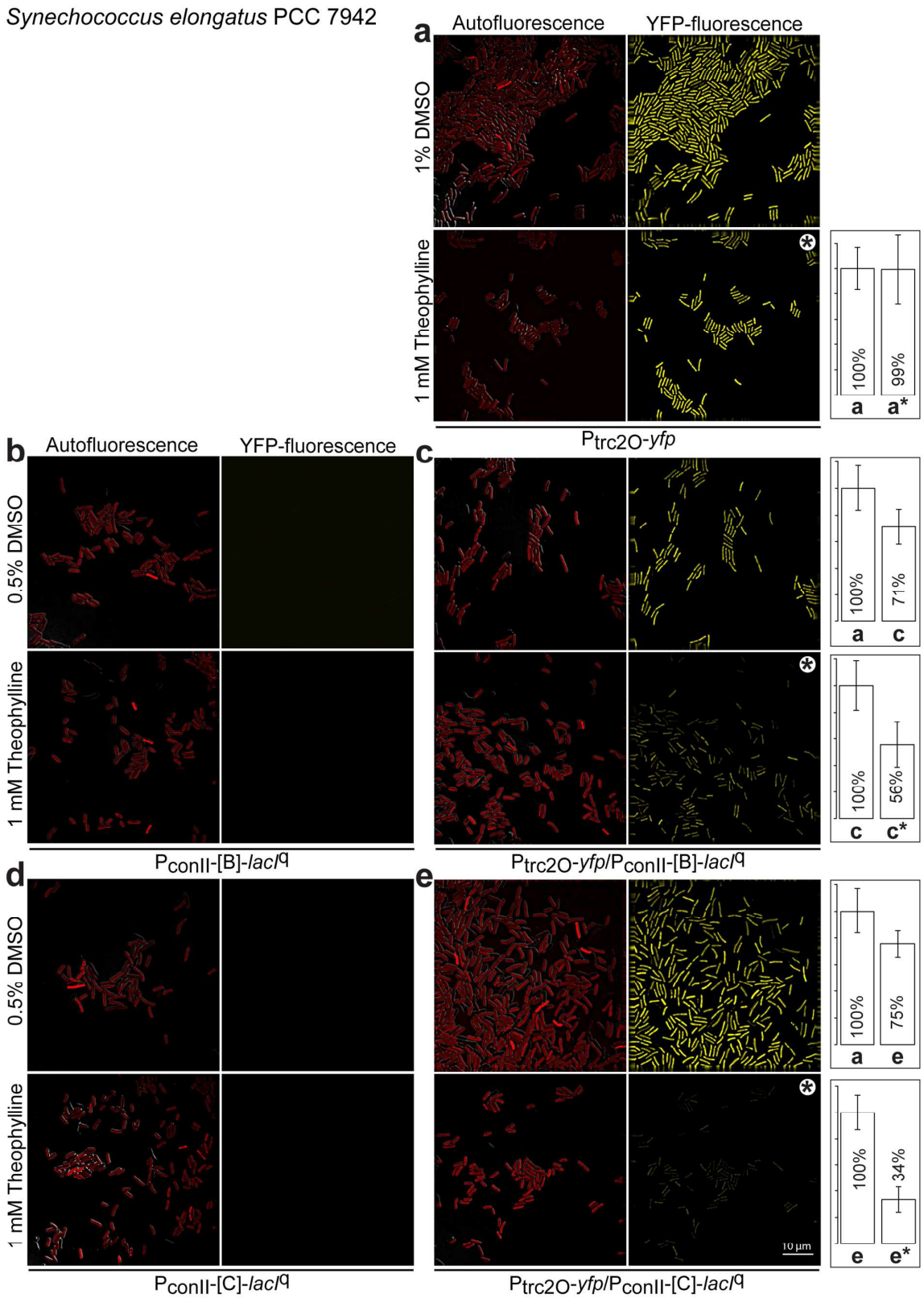

Figure S3. Photomicrographs of *S. elongatus* PCC 7942 harboring the P_{trc2O}/LacI^q NOT gates or individual devices (and antibiotic markers) in non-induced (ON state, with DMSO vehicle only) and induced (OFF state, with theophylline) conditions. (a) A strain harboring the reporter device alone; (b and d) strains harboring the repressor device alone; (c and e) strains harboring the NOT gates, where LacI^q is controlled by riboswitch B and C, respectively. Left images show autofluorescence merged with differential interference contrast (DIC); right images show YFP fluorescence. Bar graphs are paired comparisons of relative YFP fluorescence levels quantified from >30 cells picked at random from the indicated sample. The bars on the right indicate YFP fluorescence levels relative to the bars on the left, which are set at 100%. Panels and bars labeled with asterisks indicate cells in the OFF state.

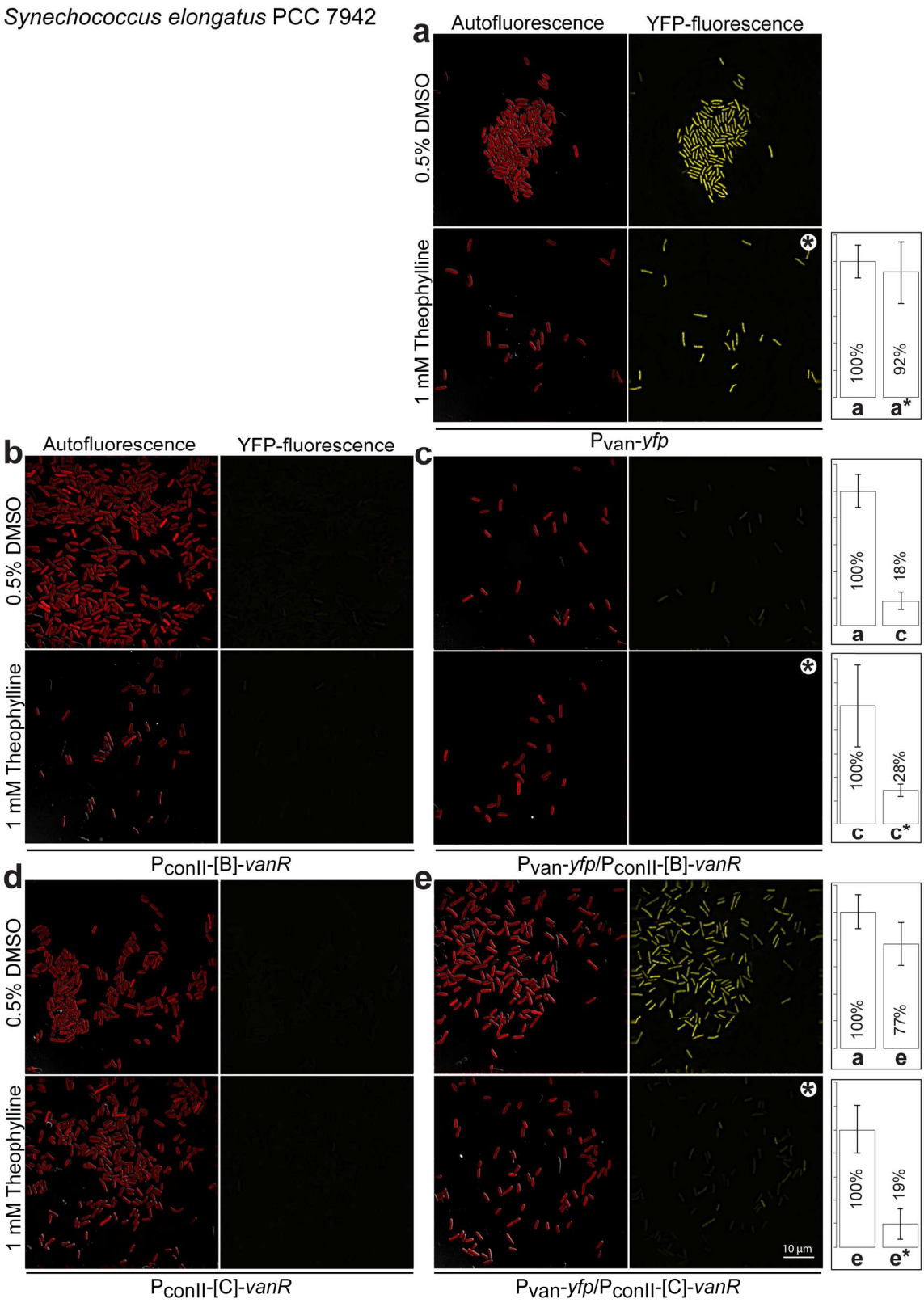

Figure S4. Photomicrographs of *S. elongatus* PCC 7942 harboring the P_{van}/VanR NOT gates or individual devices in non-induced and induced conditions. Labels and descriptions are as defined in Figure S3.

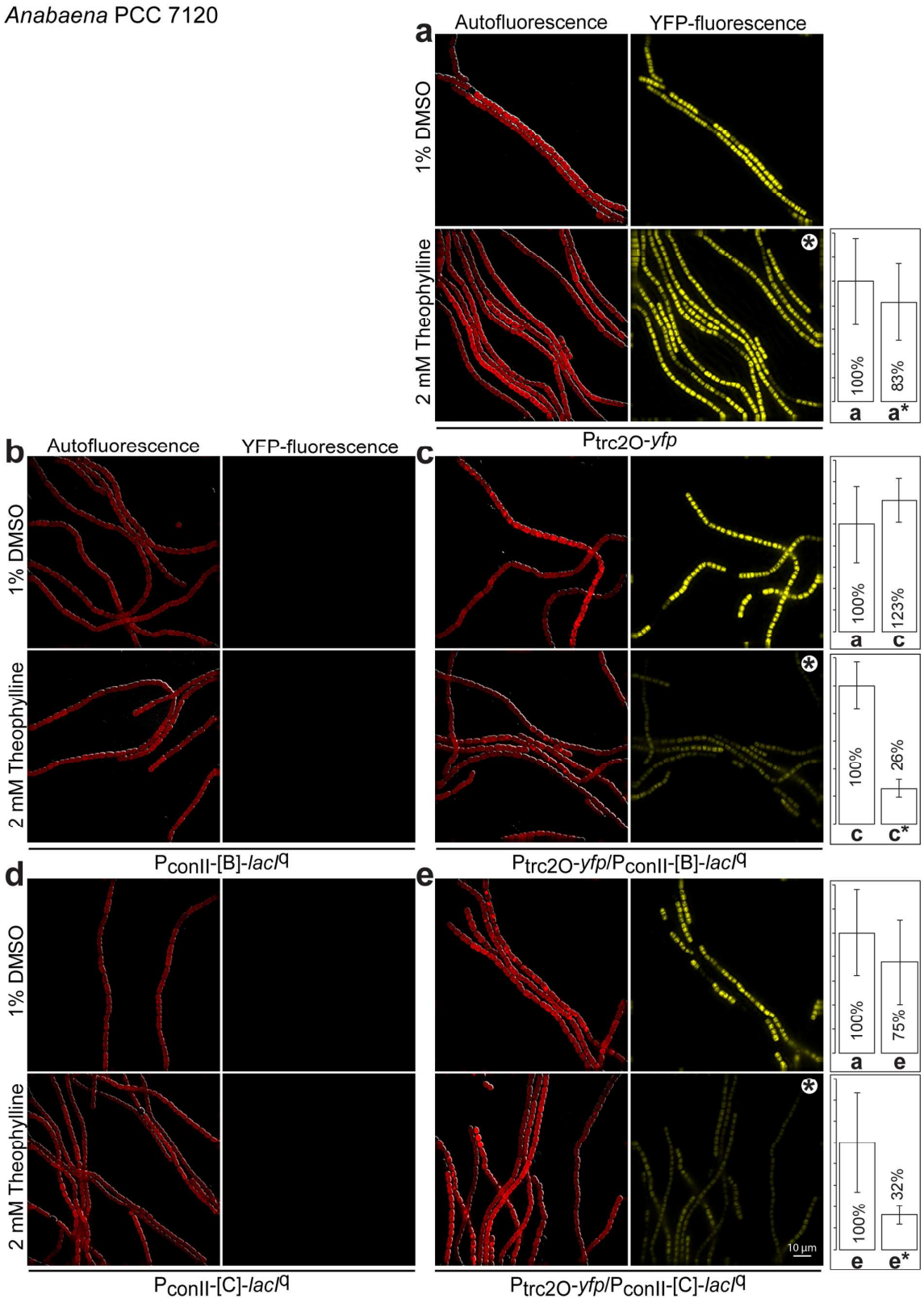

Figure S5. Photomicrographs of *Anabaena* PCC 7120 harboring the P_{trc2O}/LacI^q NOT gates or individual devices in non-induced and induced conditions. Labels and descriptions are as defined in Figure S3.

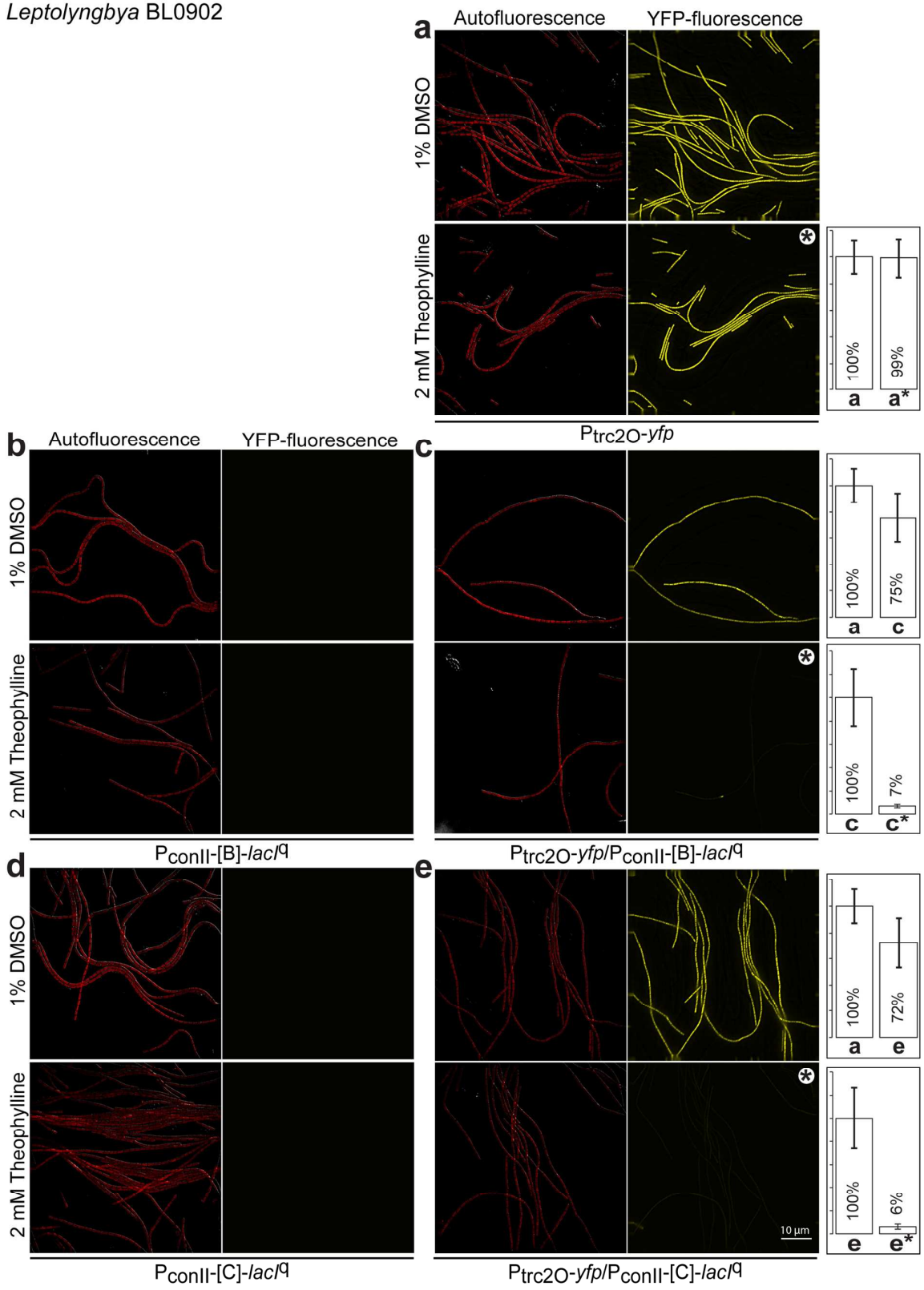

Figure S6. Photomicrographs of *Leptolyngbya* BL0902 harboring the P_{trc2O}/LacI^q NOT gates or individual devices in non-induced and induced conditions. Labels and descriptions are as defined in Figure S3.

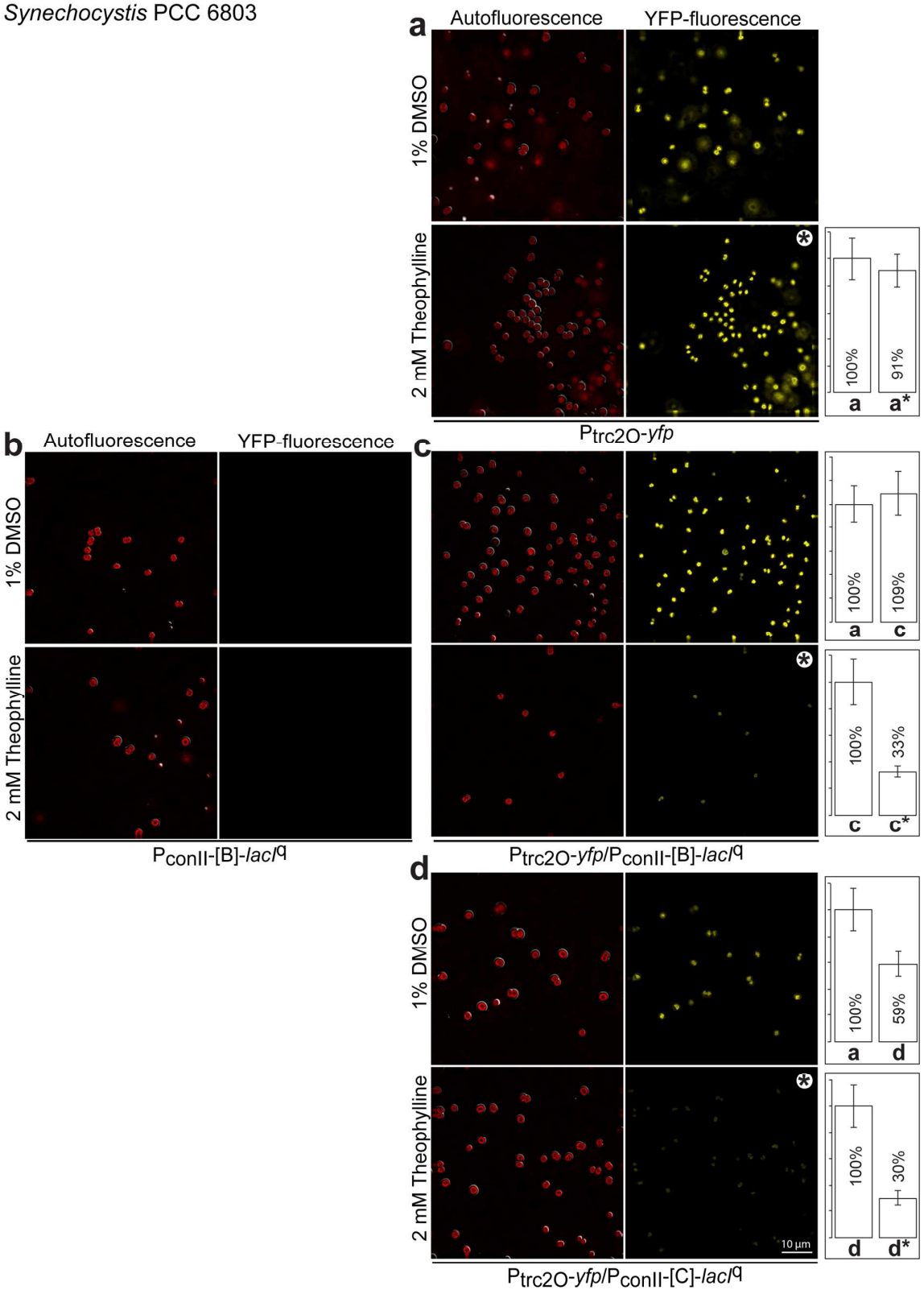

Figure S7. Photomicrographs of *Synechocystis* PCC 6803 harboring the P_{trc2O}/LacI^q NOT gates or individual devices in non-induced and induced conditions. (a) A strain harboring the reporter device alone; (b) a strain harboring the repressor device alone; (c and d) strains harboring the NOT gates, where LacI^q is controlled by riboswitch B and C, respectively. Otherwise, labels and descriptions are as in Figure S3.

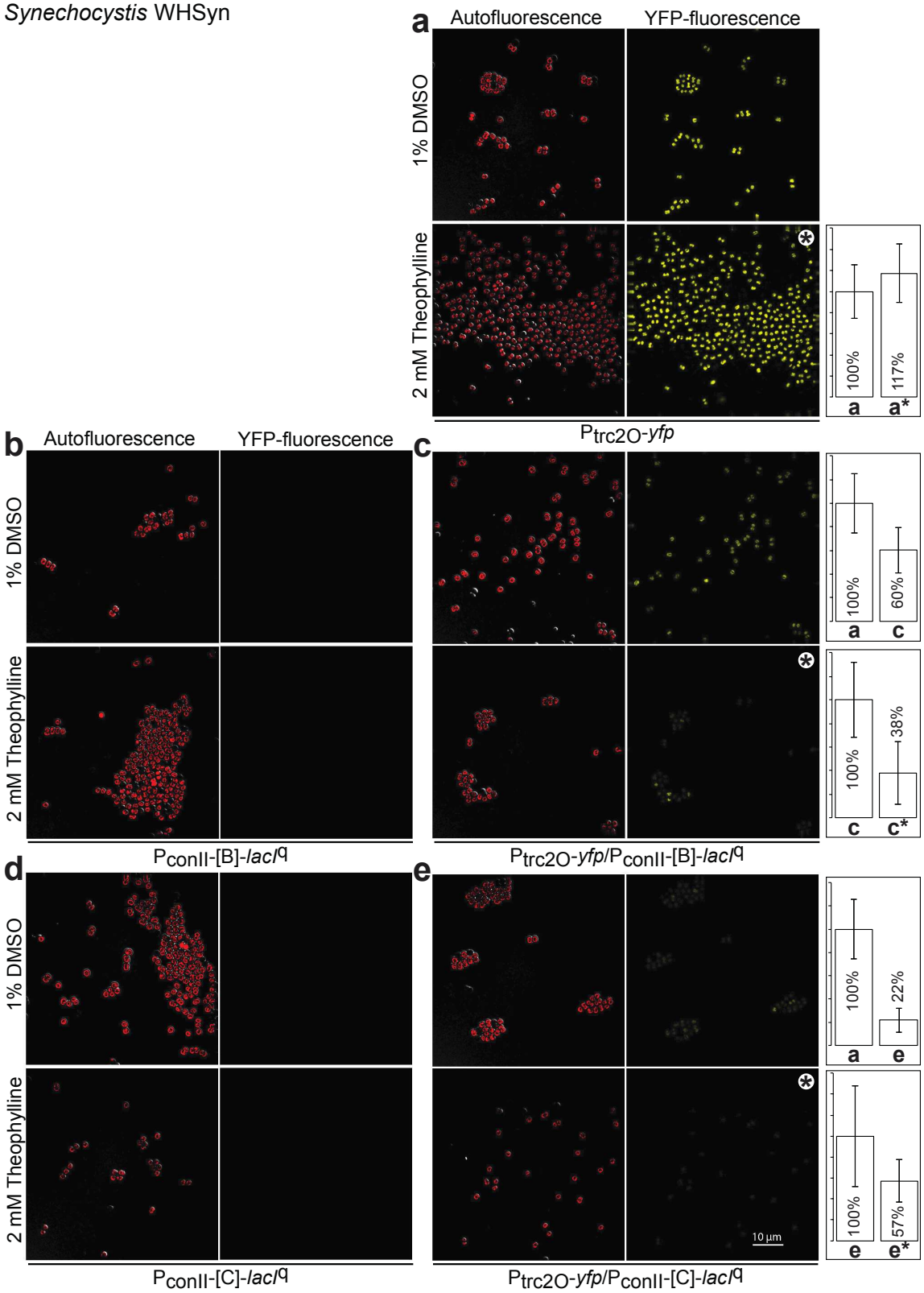

Figure S8. Photomicrographs of *Synechocystis* WHSyn harboring the P_{trc2O}/LacI^q NOT gates or individual devices in non-induced and induced conditions. Labels and descriptions are as defined in Figure S3.

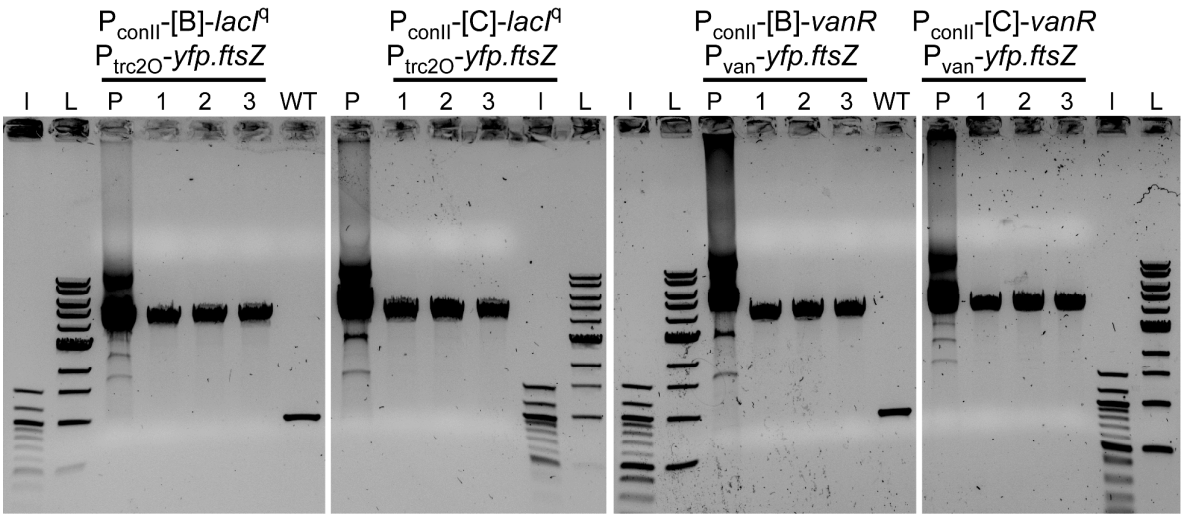

Figure S9. PCR analyses carried out on *S. elongatus* strains to confirm chromosomal integration of the recombinant DNA (Figure 1b), and complete segregation of the engineered chromosomes. PCR products of the expected size were obtained for the recombinant (4403 and 4411 bp for the *lacI*^{q} based NOT gates controlled by riboswitch B and C, respectively, and 3967 and 3975 bp for *vanR*-based NOT gates controlled by riboswitch B and C, respectively) and WT (904 bp) strains. Complete segregation is indicated by the absence of a WT band for the recombinant strains. For each recombinant strain, three colonies (labeled 1, 2 and 3) were analyzed. DNA ladder size markers are (I) 1,500, 1,200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp and (L) 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,000, 1,500, 1,000, 500 bp. PCRs carried out on plasmid DNA are indicated by P. The PCRs carried out on plasmid DNA resulted in several minor unspecific products. For unknown reasons, the size of the PCR product obtained for the plasmid harboring P_{conII}-[B]-*vanR*/P_{van}-*yfp-ftsZ* is larger than expected.

TABLES

Table S1. Plasmids used in this study

Plasmid name	Description (plasmid descriptive name)
pAM5328	Suicide plasmid for the integration of a gentamycin resistance device at NS3 of S. PCC 7942 chromosome (S7942NS3-aacC1).
pAM5006	pAM5328 backbone with the trc2O promoter driving the expression of a *yfp* reporter gene (S7942NS3-aacC1-Ptrc2O_YFP).
pAM5155	pAM5328 backbone with the trc2O promoter driving the expression of a *yfp* reporter gene fuse to a LVA degradation tag (S7942NS3-aacC1-Ptrc2O_YFP_LVA).
pAM5010	pAM5328 backbone with the van promoter driving the expression of a *yfp* reporter gene (S7942NS3-aacC1-Pvan_YFP).
pAM5154	pAM5328 backbone with the van promoter driving the expression of a *yfp* reporter gene fuse to a LVA degradation tag (S7942NS3-aacC1-Pvan_YFP_LVA).
pAM5329	Suicide plasmid for the integration of a spectinomycin/streptomycin resistance device at NS1 of S. PCC 7942 chromosome (S7942NS1-aadA).
pAM5330	pAM5329 backbone with the conII promoter driving the expression of the LacI^q transcriptional repressor by theophylline riboswitch variant B (S7942NS1-aadA-PconII_RSwB_lacI).
pAM5331	Same as pAM5330 but the translation of the LacI^q transcriptional repressor is controlled by theophylline riboswitch variant C (S7942NS1-aadA-PconII_RSwC_lacI).
pAM5332	pAM5329 backbone with the conII promoter driving the expression of the VanR transcriptional repressor controlled by theophylline riboswitch variant B (S7942NS1-aadA-PconII_RSwB_vanR).
pAM5333	Same as pAM5332 but the translation of the VanR transcriptional repressor is controlled by theophylline riboswitch variant C (S7942NS1-aadA-PconII_RSwC_vanR).
pAM5334	Suicide plasmid for the integration into S. PCC 7942 chromosome at the *ftsZ* native locus. The recombinant DNA comprises: a conII promoter driving the expression of the LacI^q transcriptional repressor by theophylline riboswitch variant B, a spectinomycin/streptomycin resistance device and the trc2O promoter driving the expression of a *yfp-ftsZ* translational fusion (UftsZ_RswB_lacI-aadA-Ptrc2O_YFP_ftsZ).
pAM5335	Same as pAM5334 but the translation of the LacI^q transcriptional repressor is controlled by theophylline riboswitch variant C (UftsZ_RswC_lacI-aadA-Ptrc2O_YFP_ftsZ).
pAM5161	Suicide plasmid for the integration into S. PCC 7942 chromosome at the *ftsZ* native locus. The recombinant DNA comprises: a conII promoter driving the expression of the VanR transcriptional repressor controlled by theophylline riboswitch variant B, a spectinomycin/streptomycin resistance device and the van promoter driving the expression of a *yfp-ftsZ* translational fusion (UftsZ_RswB_vanR-aadA-Pvan_YFP_ftsZ).
pAM5162	Same as pAM5161 but the translation of the VanR transcriptional repressor is controlled by theophylline riboswitch variant C (UftsZ_RswC_vanR-aadA-Pvan_YFP_ftsZ).
pAM5163	Broad host range autonomously replicating plasmid RSF1010Y25F carrying a kanamycin or neomycin resistance device (RSF1010Y25F-aphI)
pAM5336	pAM5163 backbone with the trc2O promoter driving the expression of the YFP reporter (RSF1010Y25F-aphI-Ptrc2O_YFP).
pAM5337	pAM5163 backbone with the van promoter driving the expression of the YFP reporter (RSF1010Y25F-aphI-Pvan_YFP).
pAM5338	pAM5163 backbone with the trc2O promoter driving the expression of the YFP reporter and the conII promoter driving the expression of the LacI^q transcriptional repressor controlled by theophylline riboswitch variant B (RSF1010Y25F-aphI-Ptrc2O_YFP-PconII_RSwB_lacI).
pAM5339	Same as pAM5338 but the translation of the LacI^q transcriptional repressor is controlled by theophylline riboswitch variant C (RSF1010Y25F-aphI-Ptrc2O_YFP-PconII_RSwC_lacI).
pAM5340	pAM5163 backbone with the van promoter driving the expression of the YFP reporter and the conII promoter driving the expression of the VanR transcriptional repressor controlled by theophylline riboswitch variant B (RSF1010Y25F-aphI-Pvan_YFP-PconII_RSwB_vanR).
pAM5341	Same as pAM5340 but the translation of the VanR transcriptional repressor is controlled by theophylline riboswitch variant C (RSF1010Y25F-aphI-Pvan_YFP-PconII_RSwC_vanR).
pAM5342	pAM5163 backbone with the conII promoter driving the expression of the LacI^q transcriptional repressor controlled by theophylline riboswitch variant B (RSF1010Y25F-aphI-PconII_RSwB_lacI).
pAM5343	Same as pAM5342 but the translation of LacI^q is controlled by theophylline riboswitch variant C (RSF1010Y25F-aphI-PconII_RSwC_lacI)
pAM5344	pAM5163 backbone with the conII promoter driving the expression of the VanR transcriptional repressor controlled by theophylline riboswitch variant B (RSF1010Y25F-aphI-PconII_RSwB_vanR).
pAM5345	Same as pAM5344 but the translation of VanR is controlled by theophylline riboswitch variant C (RSF1010Y25F-aphI-PconII_RSwC_vanR).

* backbone is defined here as homologous recombination site or replicon and antibiotic resistance device

Table S2. Strains used in this study

Synechococcus elongatus PCC 7942		
Strain	Plasmid(s)	
P_{trc2O}-*yfp*/P_{conII}-[B]-*lacI*^{q}	pAM5006	pAM5330
P_{trc2O}-*yfp-lva*/P_{conII}-[B]-*lacI*^{q}	pAM5155	pAM5330
P_{trc2O}-*yfp*/P_{conII}-[C]-*lacI*^{q}	pAM5006	pAM5331
P_{trc2O}-*yfp-lva*/P_{conII}-[C]-*lacI*^{q}	pAM5155	pAM5331
P_{van}-*yfp*/P_{conII}-[B]-*vanR*	pAM5010	pAM5332
P_{van}-*yfp-lva*/P_{conII}-[B]-*vanR*	pAM5154	pAM5332
P_{van}-*yfp*/P_{conII}-[C]-*vanR*	pAM5010	pAM5333
P_{van}-*yfp-lva*/P_{conII}-[C]-*vanR*	pAM5154	pAM5333
P_{trc2O}-*yfp*	pAM5006	pAM5329
P_{trc2O}-*yfp-lva*	pAM5155	pAM5329
P_{conII}-[B]-*lacI*^{q}	pAM5328	pAM5330
P_{conII}-[C]-*lacI*^{q}	pAM5328	pAM5331
P_{van}-*yfp*	pAM5010	pAM5329
P_{van}-*yfp-lva*	pAM5154	pAM5329
P_{conII}-[B]-*vanR*	pAM5328	pAM5332
P_{conII}-[C]-*vanR*	pAM5328	pAM5333
P_{conII}-[B]-*lacI*^{q}/P_{trc2O}-*yfp-ftsZ*	pAM5334	
P_{conII}-[C]-*lacI*^{q}/P_{trc2O}-*yfp-ftsZ*	pAM5335	
P_{conII}-[B]-*vanR*/P_{van}-*yfp-ftsZ*	pAM5161	
P_{conII}-[C]-*vanR*/P_{van}-*yfp-ftsZ*	pAM5162	

Anabaena PCC 7120, *Leptolyngbya* BL0902, *Synechocystis* PCC 6803, *Synechocystis* WHSyn	
Strain	Plasmid
P_{trc2O}-*yfp*/P_{conII}-[B]-*lacI*^{q}	pAM5336
P_{trc2O}-*yfp*/P_{conII}-[C]-*lacI*^{q}	pAM5337
P_{van}-*yfp*/P_{conII}-[B]-*vanR*	pAM5338
P_{van}-*yfp*/P_{conII}-[C]-*vanR*	pAM5339
P_{trc2O}-*yfp*	pAM5340
P_{conII}-[C]-*lacI*^{q}	pAM5341
P_{conII}-[B]-*lacI*^{q}	pAM5342
P_{van}-*yfp*	pAM5343
P_{conII}-[B]-*vanR*	pAM5344
P_{conII}-[C]-*vanR*	pAM5345

Table S3. Promoter and riboswitch sequences used in this study.

Name	Sequence
P_{conII}-[B]	accggtttcgaa**TTGACA**attaatcatcggctcg**TATAAT**ggtacc**G**GTGATACCAGCATCGTCTTGATGCCCTTGGCAGCACCcgc tgcgc**AGGGGGT**atcaacaag**ATG**
P_{conII}-[C]	accggtttcgaa**TTGACA**attaatcatcggctcg**TATAAT**ggtacc**C**ggtacctgataagataggGGTGATACCAGCATCGTCTTGA TGCCCTTGGCAGCACC**AAGGG**acaacaag**ATG**
P_{trc2O}	tttcgaattggAATTGTGAGCGCTCACAATTcgaacggttctggcaaatattctgaaatgagctg**TTGACA**attaatcatccggctc g**TATAAT**gtgtgg**A**ATTGTGAGCGGATAACAATTtcacacaaaatagaa**GGAGG**tcttaag**ATG**
P_{vanA}	ccgctcccgagaatgaatacccgcagctcagagaccccagctgacattaactcacatttacccc**TTGACA**gtgatttgaagcacaag **CAATAT**atgacct**A**cgccataAACTAACTAAGAGTTTAGGTATTTGGTTataaaatagaa**GGAGG**tcttaag**ATG**

Predicted -35, -10 and transcriptional start sites are bolded and underlined.
Operator sequences $lacO_{sym}$, $lacO_1$ and vanO are highlighted in grey.
Riboswitch aptamer sequences are underlined.
Ribosome binding sites and start codons are in bold.

MATERIALS AND METHODS

Plasmid construction

PCR amplifications were carried out with Phusion High-Fidelity DNA polymerase (Finnzymes, Thermo Scientific) or Q5 (New England BioLabs) according to the manufacturer's instructions. Plasmid preparations were carried out with the QIAprep Spin Miniprep Kit (Qiagen). Restriction digests were typically performed for 3 to 5 hours, using 5U of enzyme per µg of plasmid DNA in a final volume at least 50 times greater than the volume of enzyme added. DNA purification/concentration following PCR and restriction digests were performed with DNA Clean & Concentrator™-5 (Zymo). Nucleic acid concentrations were measured with a UV-Vis spectrophotometer NanoDrop 2000c. Assembly reactions were carried out using GeneArt Seamless Cloning and Assembly Kit (Life Technologies) using equimolar or 3:1 ratios of the insert(s) and plasmid backbone at final concentrations of DNA fragments to be assembled of about 8 ng/µl.

New reporter devices (P_{trc2O}-*yfp*, P_{trc2O}-*yfp-lva*, P_{van}-*yfp*, P_{van}-*yfp-lva*) were initially constructed as CYANO-VECTOR (http://golden.ucsd.edu/CyanoVECTOR/) compatible donor plasmids with flanking terminator (*lpp*T and *rnb*T2), GC-adaptor, and EcoRV restriction sequences in a pMA-RQ backbone (GeneArt). These devices were further released from the donor plasmids with EcoRV or ZraI and assembled in cyanobacterial shuttle plasmids as described by Taton et al.[1] with two other modular devices: S7942NS3-RK2BOM, harboring the recombination sequences of *S. elongatus* neutral site 3 (NS3), the pBR322 oriV and the RK2 bom site, and *aacC1*, a gentamycin resistance gene.

Similarly, we constructed donor plasmids carrying the conII promoter followed by riboswitch B or C, an EcoRI restriction site, a *rnb*T2 terminator, flanking GC-adaptor, and EcoRV restriction sequences in pUC19 plasmids. After being released with EcoRV, these devices were further assembled in cyanobacterial shuttle plasmids with: S7942NS1TC, harboring the recombination sequences of *S. elongatus* neutral site 1 (NS1), the pBR322 oriV and the pBR322 bom site, and *aadA*, a spectinomycin/streptomycin resistance cassette. The transcriptional repressor gene *lacI*^{q} and *vanR* were then cloned at the EcoRI site as described earlier resulting in shuttle plasmids carrying the repressor devices: P_{conII}-[B]-*lacI*^{q}, P_{conII}-[C]-*lacI*^{q}, P_{conII}-[B]-*vanR*, or P_{conII}-[C]-*vanR*.

Empty plasmids that were used to construct control strains carrying the appropriate antibiotic resistance markers were assembled as described earlier[1] using the modular devices: S7942NS3-RK2BOM, AACC1, and CCDB-SWAI harboring the *ccdB* toxic gene[2] flanked with SwaI restriction sites or the modular devices S7942NS1TC, AADA, and CCDB-SWAI. The *ccdB* gene was then removed from those 2 plasmids by performing restriction digests with SwaI and re-ligation of the linearized plasmids.

To construct self-replicating broad host range plasmids harboring the NOT gate circuits, pER003[1] harboring the RSF1010Y25F replicon, a kanamycin/neomycin resistance device (APHI), and the CCDB-SWAI module was linearized by restriction digestion with SwaI. The *ccdB* toxic gene was then replaced by a repressor device (P_{conII}-[B]-*lacI*^{q}, P_{conII}-[C]-*lacI*^{q}, P_{conII}-[B]-*vanR* or P_{conII}-[C]-*vanR*) and its cognate reporter device (P_{trc2O}-*yfp* and P_{van}-*yfp*) obtained by PCR using the plasmids described above as templates. RSF1010Y25F-based plasmids that were used to construct control strains without repressor devices and therefore constitutively expressed the reporter gene were obtained by restriction digests of the resulting plasmids with SwaI or SacI and re-ligation. In addition, we constructed a control plasmid without both repressor and reporter devices by digesting pER003 with SwaI to release the *ccdB* toxic gene and then re-ligating the linearized plasmid.

Strain selection

Cultures of recombinant strains were supplemented with appropriate antibiotics: 2 µg/ml of spectinomycin (Sp) plus 2 µg/ml of streptomycin (Sm) and/or 2 µg/ml of gentamycin (Gm) for *S. elongatus*, 25 µg/ml of Neomycin for *Anabaena* and *Leptolyngbya*; and 25 µg/ml kanamycin for both strains of *Synechocystis*.

Microscopy

Differential interference contrast (DIC) and fluorescence microscopy were performed on a DeltaVision (Applied Precision, Inc.) microscope system composed of an Olympus IX71 inverted microscope equipped with Olympus UPlanSApo 40x/0.90, 60x/1.35, and 100x/1.40 objectives, and a CoolSNAP HQ2/ICX285 camera. Tetramethylrhodamine isothiocyanate (TRITC) filters (EX555/28 and EM617/73) were used to image autofluorescence of photosynthetic pigments, and YFP (EX500/20 and EM535/30) filters were used to image YFP reporter protein fluorescence. Image acquisition, deconvolution, and analysis were performed using Resolve3D softWoRx-Acquire (Version 4.0.0).

References

(1) Taton, A., Unglaub, F., Wright, N. E., Zeng, W. Y., Paz-Yepes, J., Brahamsha, B., Palenik, B., Peterson, T. C., Haerizadeh, F., Golden, S. S., and Golden, J. W. (2014) Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria *Nucleic Acids Res*, *42*, e136.

(2) Bernard, P., Gabant, P., Bahassi, E. M., and Couturier, M. (1994) Positive-selection vectors using the F-plasmid *ccdb* killer gene *Gene*, *148*, 71-74.