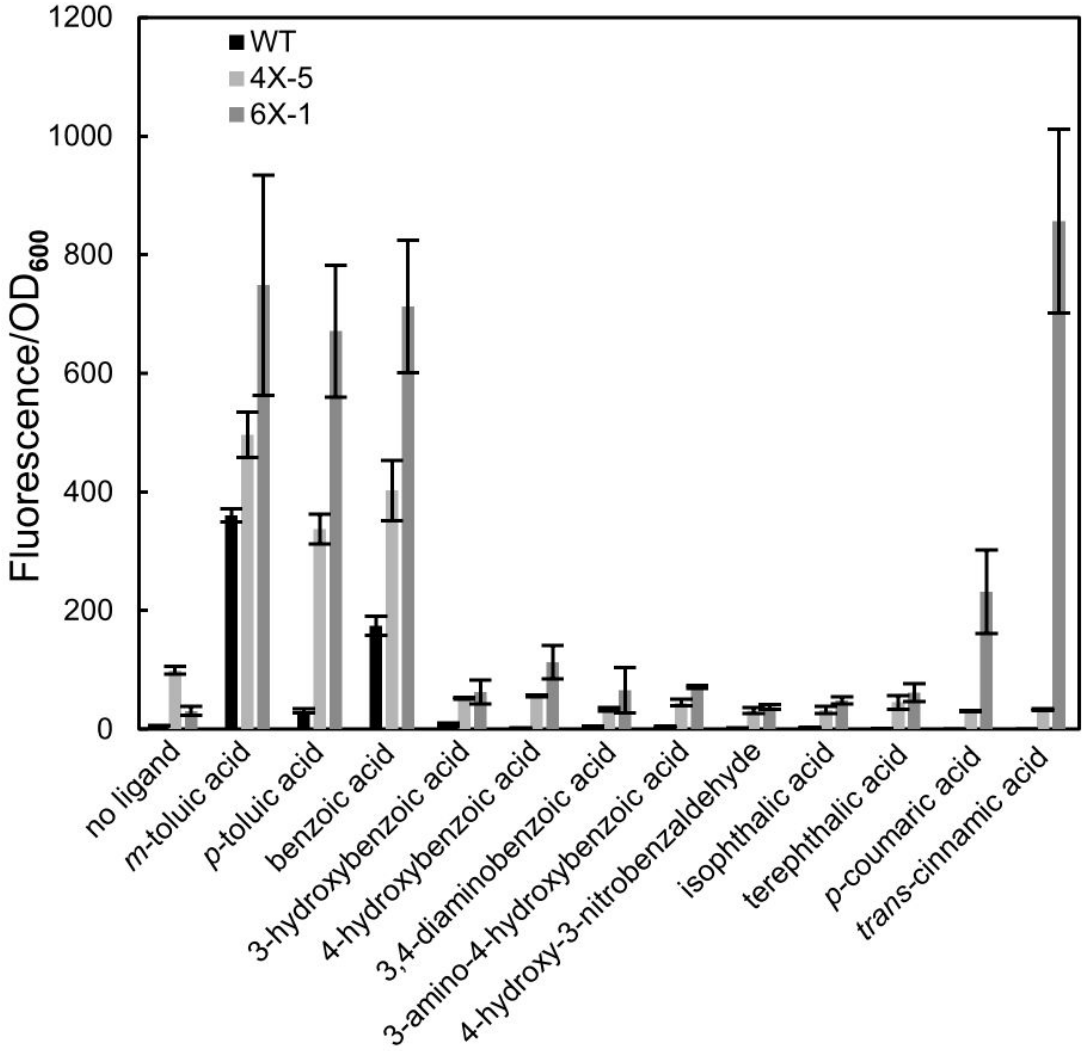
Supplementary Information

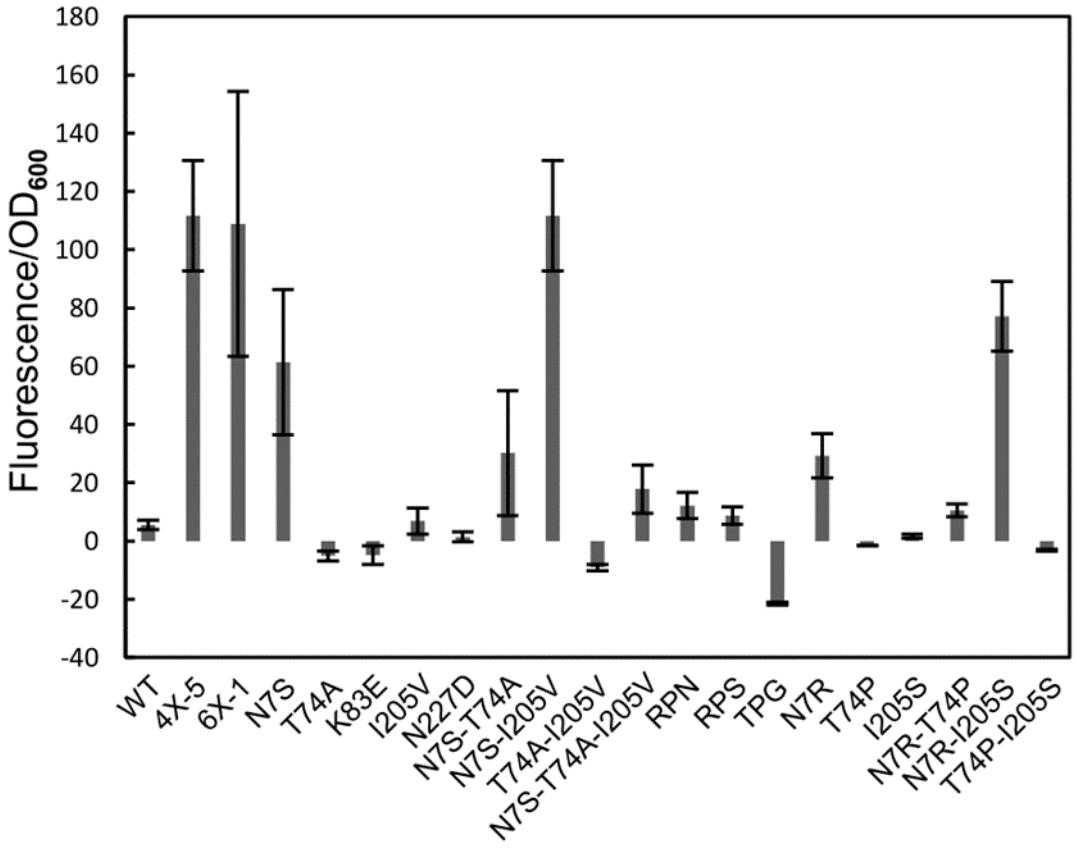
Switching the ligand specificity of the biosensor XylS from *meta* to *para*-toluic acid through directed evolution exploiting a dual selection system

Yuki Ogawa[1], Kento Ueno[1], Yohei Katsuyama[1,2] and Yasuo Ohnishi[1,2]

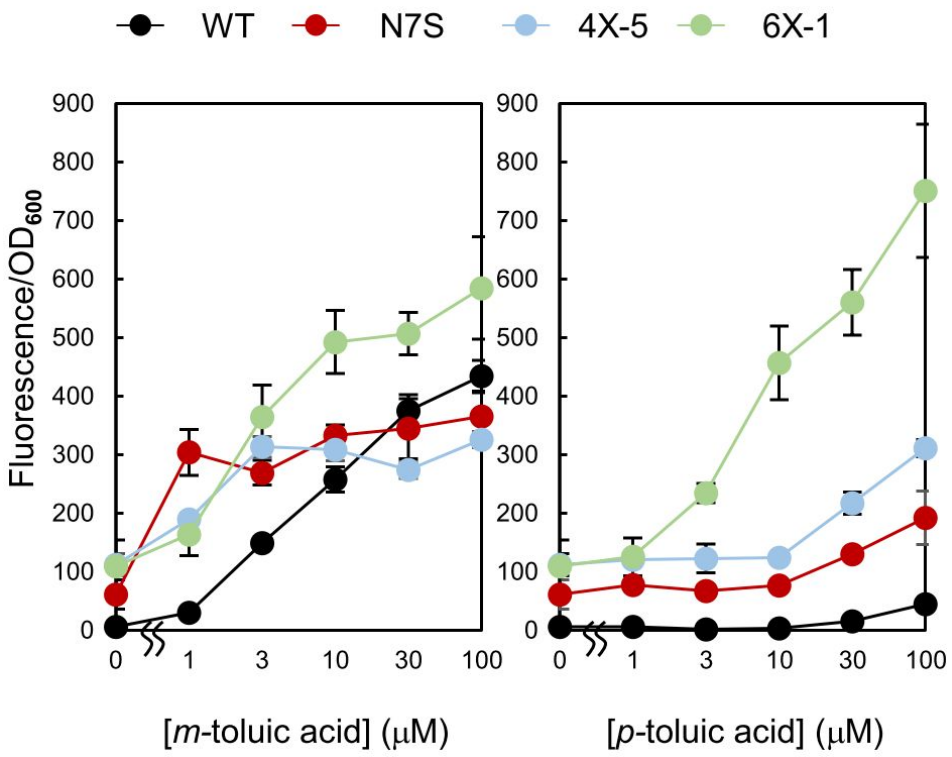
[1]Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[2]Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan



Supplementary Figure 1. Sensitivity of WT XylS and two XylS mutants to various aromatic compounds. *E. coli* strains harboring pCDFlacXylS (WT, 4X-5, or 6X-1) and pCOLAPmmCherry were cultivated for 10 h in the presence of each aromatic compound (100 μM). The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture.



Supplementary Figure 2. The induction levels of WT XylS and all XylS mutants in the absence of any external ligand. *E. coli* strains harboring pCDFlacXylS variants and pCOLA*Pm*mCherry were cultivated for 10 h. The fluorescence intensity of each culture was measured in triplicate and normalized by the OD_{600} value of each culture. The induction levels of the following XylSs, which showed large experimental errors in the first measurement in triplicate, were measured in more than nine biological replicates; WT, 4X-5, 6X-1, N7S, I205V, N7S-I205V, N7S-T74A-I205V, RPN, RPS, N7R, N7R-T74P, and N7R-I205S.



Supplementary Figure 3. *m*-Toluic acid and *p*-toluic acid sensitivity of WT XylS and XylS mutants with N7S replacement (XylS-N7S, 4X-5, and 6X-1). *E. coli* strains harboring pCDFlacXylS (WT, XylS-N7S, 4X-5, or 6X-1) and pCOLAPmmCherry were cultivated for 10 h in the presence of each ligand (0, 1, 3, 10, 30, and 100 μM). The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture. The following is supplementary discussion mainly on the N7S mutation.

The induction level of XylS-N7S in the absence of any ligand was higher than that of WT XylS, indicting that N7S mutation is responsible for increasing the basal activity. Interestingly, even a low concentration of *m*-toluic acid provoked a distinct increase of induction level, showing that N7S mutation conferred hyper-sensitivity to a low concentration of *m*-toluic acid. XylS-N7S also showed a higer sensitivity to *p*-toluic acid than WT XylS, although a high concentration of *p*-toluic acid was requierd for the distinct induction unlike the hyper-sensitivity to *m*-toluic acid. Because neither *m*-toluic acid nor *p*-toluic acid decreased the increased basal activity of XylS-N7S, neither compound acted as an antagonist toward XylS-N7S. Note that *ortho*-substituted toluene derivatives, such as *o*-xylene and *o*-chlorotoluene, act as antagonists toward TodS, the sensor kinase of a two-component regulatory system in *Pseudomonas putida*, which autophospholylates and

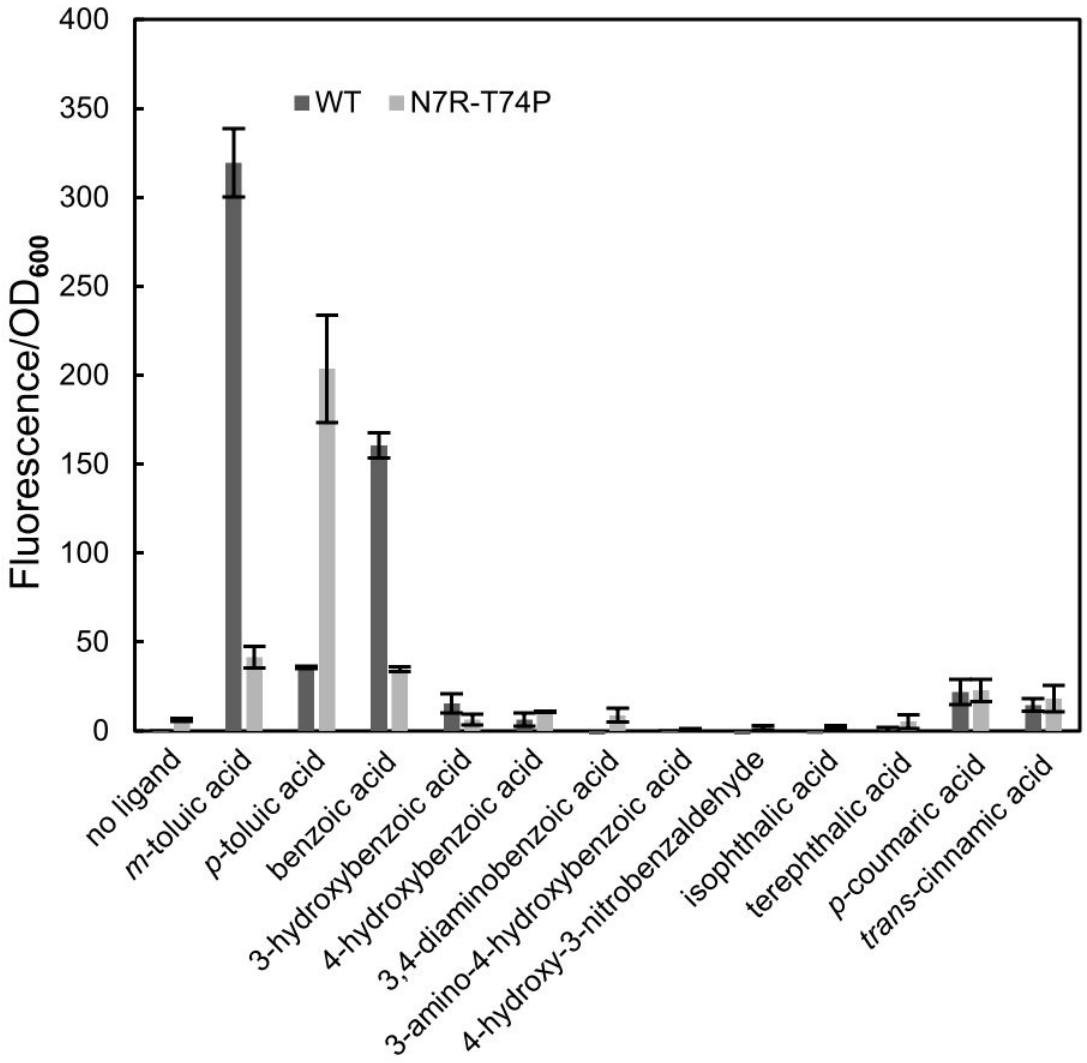
induces the signal transduction system to activate transcription of the target genes of its cognate response regulator TodT when it binds to agonistic compounds represented by toluen.[1]

Valla's group also studied the induction level of XylS-N7S by measuring the level of ampicillin resistance of a XylS-N7S-producing strain that carries *bla* under the control of *Pm* promoter.[2] In their study, XylS-N7S showed almost the same basal activity, i.e., the same induction level (ampicillin resistance, up to approximately 35 μg/ml) as WT XylS in the absence of *m*-toluic acid, while it showed about 1.5-times higher induction level (up to approximately 900 μg/ml) than WT XylS (up to approximately 600 μg/ml) in the presence of *m*-toluic acid (1 mM). This result is apparently inconsistent with our result, especially in regard to the basal activity of XylS-N7S; in our experiment, XylS-N7S showed a higher basal activity and higher induction levels only in the presence of low concentrations of *m*-toluic acid (1, 3, and 10 μM) compared with WT-XylS. However, the inconsistency may be explained by the differences of *E. coli* strains and assay systems between the two experiments.

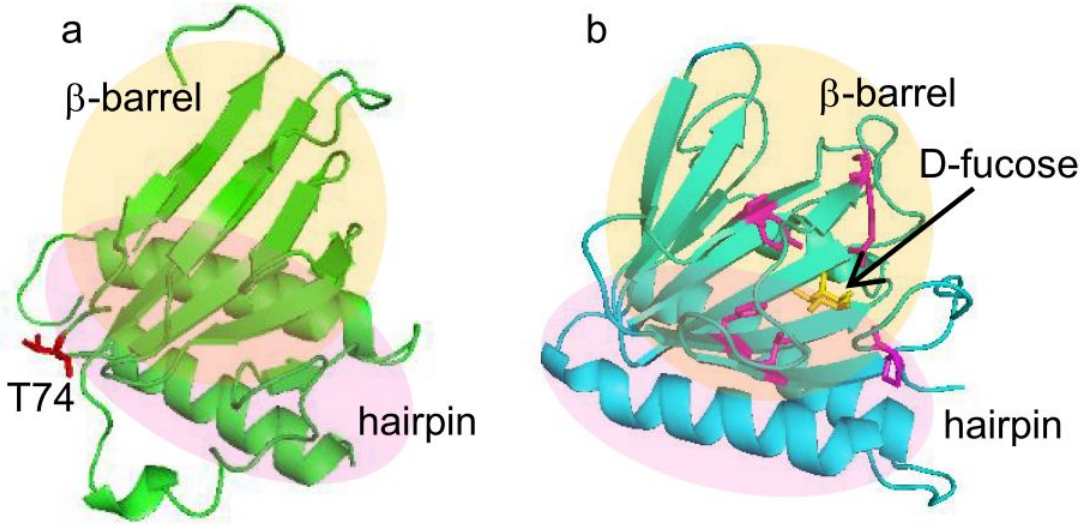
4X-5 having the N7S and I205V mutations showed a higher basal activity than XylS-N7S. In addition, 4X-5 showed increased *p*-toluic acid sensitivity compared with XylS-N7S. In contrast, *m*-toluic acid sensitivity was not so different between 4X-5 and XylS-N7S. From these results, we assume that I205V accounts for the higher sensitivity of 4X-5 to *p*-toluic acid. 6X-1 having the N7S, T74A, K83E, I205V, and N227D mutations showed a similar basal activty to 4X-5, but showed greatly increased *p*-toluic acid sensitivity in the presense of more than 3 μM *p*-toluic acid compared with XylS-N7S. In the presense of more than 10 μM *m*-toluic acid, 6X-1 also showed considerably increased *m*-toluic acid sensitivity compared with XylS-N7S and 4X-5. From these results, we assume that any of the three newly introduced mutations (T74A, K83E, and N227D) plays an important role for further increasing *p*-toluic acid sensitivity of 6X-1. Our assumptions described above were examined by the following experiment shown in Figure 5.

(1) Busch, A., Lacal, J., Martos, A., Ramos, J. L., and Krell, T. (2007) Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals. *Proc. Natl. Acad. Sci. U. S. A. 104*, 13774–13779.

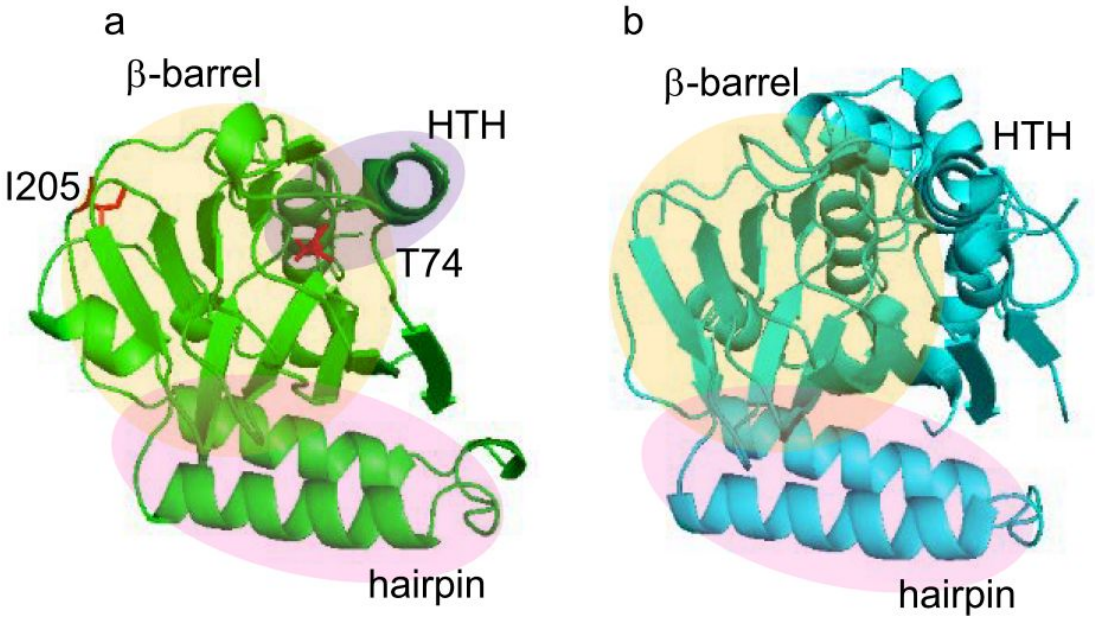
(2) Vee Aune, T. E., Bakke, I., Drabløs, F., Lale, R., Brautaset, T., and Valla, S. (2010) Directed evolution of the transcription factor XylS for development of improved expression systems. *Microb. Biotechnol. 3*, 38–47.



Supplementary Figure 4. Sensitivity of WT XylS and XylS-N7R-T74P to various aromatic compounds. *E. coli* strains harboring pCDFlacXylS (WT or N7R-T74P) and pCOLAPmmCherry were cultivated for 10 h in the presence of each aromatic compound (100 μM). The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture.



Supplementary Figure 5. Model of XylS (a) based on its homology with the structure of the N-terminal domain of AraC (b) (PDB ID: 2AAC).



Supplementary Figure 6. Model of XylS (a) based on its homology with the structure of the N-terminal domain of CuxR (b) (PDB ID: 5NLA).

Table S1. Plasmids used in this study.

Plasmid	Relevant characteristic
pCDFlac-XylS	*Sm*^{r}; pCDFlac-1 derivative encoding *xylS*
pCOLAPmmCherry	*Km*^{r}; pCOLADuet-1 derivative encoding *Pm* and *mCherry*
pUKN009	*cat, bla, Km*^{r}; pUKN008 derivative encoding *Pm*
pCDFlac-XylS-T74A	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-T74A
pCDFlac-XylS-K83E	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-K83E
pCDFlac-XylS-I205V	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-I205V
pCDFlac-XylS-N227D	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-N227D
pCDFlac-XylS-T74A-I205V	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-T74A-I205V
pCDFlac-XylS-N7S-T74A	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-N7S-T74A
pCDFlac-XylS-N7S-T74A-I205V	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-N7S-T74A-I205V
pCDFlac-XylS-N7R	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-N7R
pCDFlac-XylS-T74P	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-T74P
pCDFlac-XylS-I205S	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-I205S
pCDFlac-XylS-N7R-T72P	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-N7R-T74P
pCDFlac-XylS-N7R-I205S	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-N7R-I205S
pCDFlac-XylS-T74P-I205S	*Sm*^{r}; pCDFlac-XylS derivative encoding *xylS*-T74P-I205S

Table S2. Primers used in this study.

No	Primer	Sequence (5' to 3') (Bold letters indicate mutation sites.)	Use
1	pCDFlac-F	CATATGGCAGATCTCAATTGGATATCGG	Amplification of CDF *ori* and streptomycin resistance gene.
2	pCDFlac-R	TCTAGAGCGGTTCAGTAGAAAAGATC	Amplification of CDF *ori* and streptomycin resistance gene.
3	Plac-F	TTCTACTGAACCGCTCTAGAGCGCAACGCAATTAATGTGAGTT	Amplification of *lac* promoter.
4	Plac-R	CAATTGAGATCTGCCATATGTGTTTCCTGTGTGAAATTGT	Amplification of *lac* promoter.
5	xylS-F	AACATATGGATTTTTGCCTGCTGAA	Cloning of *xylS*. Underline, NdeI site.
6	xylS-R	TTCTCGAGTCATGCAACTTCTTTTTTACACTG	Cloning of *xylS*. Underline, XhoI site.
7	CAT-F	AAGCTAGCATGGAGAAAAAAATCACTGGATATACC	Cloning of *cat* gene. Underline, NheI site.
8	CAT-R	TTAAGCTTACGCCCCGCCCTGCCACT	Cloning of *cat* gene. Underline, HindIII site.
9	pASKCAT-F(COLA)	CCGCGCCCCGACACCATCGAATGGCCAGA	Amplification of *cat*, *bla* and *tetR*.
10	pASKCAT-R(COLA)	ACCATCACGGAAAAAGGTTATGCTGC	Amplification of *cat*, *bla* and *tetR*.
11	pCOLA-F	TTTTTCCGTGATGGTACGACCCTGCCCTGAACCGA	Amplification of COLA *ori* and kanamycin resistance gene

12	pCOLA-R	GGTGTCGGGGCGCGGGGCATGACTAACA	Amplification of COLA *ori* and kanamycin resistance gene
13	P_{Pm}-1	GGGATAAGTCCAGCCTTGCAAGAAGCGGATACAGGAGTGCAAAAAATGGCTATCTCTAGAAAGGCCTACC	Synthesis of P_{Pm}.
14	P_{Pm}-2	TGACTCCATTATTATTGTTTCTGTTGCATAAAGCCTAAGGGGTAGGCCTTTCTAGAGATAGCCAT	Synthesis of P_{Pm}.
15	P_{Pm}-3	GTGTCCGGTTTGATAGGGATAAGTCCAGCCTTGCA	Synthesis of P_{Pm}.
16	P_{Pm}-4	CATATGTTCATGACTCCATTATTATTGTTTCTGTT	Synthesis of P_{Pm}. Underline, NdeI site.
17	P_{Pm}-F	AATTTCAGGTGGCACGTGTCCGGTTTGATAGGGAT	Amplification of P_{Pm}.
18	P_{Pm}-R	ATGTTGAATACTCATATGTTCATGACTCCATTATT	Amplification of P_{Pm}.
19	pUKN008-F	ATGAGTATTCAACATTTCCGTGTC	Amplification of pUKN008 to exchange the promoter region of ampicillin resistance gene.
20	pUKN008-R	GTGCCACCTGAAATTGTAAG	Amplification of pUKN008 to exchange the promoter region of ampicillin resistance gene.
21	P_{Pm}-F-2	GCATTAGGTGTCCGGTTTGATAGGGATA	Amplification of P_{Pm} for construction of pCOLAPmmcherry.
22	P_{Pm}-R-2	GCCCATGGGTTCATGACTCCATTATTAT	Amplification of P_{Pm} for construction of pCOLAPm
23	pCOLAPm-F	CATGAACCCATGGGCAGCAGCCATCAC	Amplification of the DNA fragment harboring kanamycin resistance gene

			and COLA *ori* for construction of pCOLAPmmcherry-1.
24	pCOLAPm-R	CCGGACACCTAATGCAGGAGTCGCATA	Amplification of the DNA fragment harboring kanamycin resistance gene and COLA *ori* for construction of pCOLAPmmherry-1.
25	pCOLAPmmCherry-1-F	CATGAACAGGGCAGCAGCCATCACCAT	Insertion of A to recover missing A in P_{Pm} of pCOLAPmmCherry
26	pCOLAPmmCherry-1-R	GCTGCCCTGTTCATGACTCCATTATTA	Insertion of A to recover missing A in P_{Pm} of pCOLAPmmCherry.
27	sqPCR-F	CCAGGCTTTACACTTTATGC	For semi-quantitative PCR to estimate the amount of pCDFlac-1 and pCDFlacXylS and sequence analysis of XylS.
28	sqPCR-R	CTTCGGCTTCCCCTGGAGAG	For semi-quantitative PCR to estimate the amount of pCDFlac-1 and pCDFlacXylS.
29	epp-xylS-F-1	ATTTCACACAGGAAACACAT	Introduction of random mutations into *xylS* by error-prone PCR.
30	epp-xyls-R-1	GTTTCTTTACCAGACTCGAG	Introduction of random mutations into *xylS* by error-prone PCR.
31	pCDFlac-F-1	CGCTGCTGCGAAATTTGAAC	Amplification of linear pCDFlac-1.

32	pCDFlac-R-1	ATGTGTTTCCTGTGTGAAAT	Amplification of linear pCDFlac-1.
33	epp-xyls-F-2	TGTGAGCGGATAACAATTTCACACAGG	Introduction of random mutations into *xylS* by error-prone PCR.
34	epp-xyls-R-2	GCGGTTTCTTTACCAGACTC	Introduction of random mutations into *xylS* by error-prone PCR.
35	pCDFlac-F-2	GAACGCCAGCACATGGACTC	Amplification of linear pCDFlac-1.
36	pCDFlac-R-2	ATGTGTTTCCTGTGTGAAATTGTTATCCGC	Amplification of linear pCDFlac-1.
37	seq-xyls-R	TGCTCAGCGGTGGCAGCAGC	Sequence analysis of *xylS*.
38	xyls-T74A-F	CTGGAA**GCC**TGTTATCATCTGCAAAT	Site-directed mutagenesis of *xylS* to introduce T74A.
39	xyls-T74A-R	ATAACA**GGC**TTCCAGACCCGGACTAAT	Site-directed mutagenesis of *xylS* to introduce T74A.
40	xyls-K83E-F	ATTCTG**GAA**GGTCATTGTCTGTGGCGT	Site-directed mutagenesis of *xylS* to introduce K83E.
41	xyls-K83E-R	ATGACC**TTC**CAGAATAATTTGCAGATG	Site-directed mutagenesis of *xylS* to introduce K83E.
42	xyls-I205V-F	CGTGAA**GTC**TTTAGCAAAGGTAACCCG	Site-directed mutagenesis of *xylS* to introduce I205V.
43	xyls-I205V-R	GCTAAA**GAC**TTCACGGCTAACATTGCT	Site-directed mutagenesis of *xylS* to introduce I205V.
44	xyls-N227D-F	AAACGC**GAC**ATTAGCCTGGAACGTCTG	Site-directed mutagenesis of *xylS* to introduce N227D.
45	xyls-N227D-R	GCTAAT**GTC**GCGTTTCAGGTTCTCTTC	Site-directed mutagenesis of *xylS* to introduce N227D.
46	xyls-N7-satmut-F	GATTTTTGCCTGCTG**NNT**GAAAAAAGCCAGATTTTTGT	Codon randomization mutagenesis of *xylS* at N7.

47	xyls-N7-satmut-R	TTCCAGACCCGGACTAATAA	Codon randomization mutagenesis of *xylS* at N7.
48	xyls-T74-satmut-F	AGTCCGGGTCTGGAA**NNT**TGTTATCATCTGCAAATTAT	Codon randomization mutagenesis of *xylS* at T74.
49	xyls-T74-satmut-R	TTCACGGCTAACATTGCTAC	Codon randomization mutagenesis of *xylS* at T74.
50	xyls-I205-satmut-F	AATGTTAGCCGTGAA**NNT**TTTAGCAAAGGTAACCCGAG	Codon randomization mutagenesis of *xylS* at I205.
51	xyls-I205-satmut-R	CAGCAGGCAAAAATCCATAT	Codon randomization mutagenesis of *xylS* at I205.
52	xyls-N7R-F	CTGCTG**CGT**GAAAAAAGCCAGATTTTT	Site-directed mutagenesis of *xylS* to introduce N7R.
53	xyls-N7R-R	TTTTTC**ACG**CAGCAGGCAAAAATCCAT	Site-directed mutagenesis of *xylS* to introduce N7R.
54	xyls-T74P-F	CTGGAA**CCT**TGTTATCATCTGCAAATT	Site-directed mutagenesis of *xylS* to introduce T74P.
55	xyls-T74P-R	ATAACA**AGG**TTCCAGACCCGGACTAAT	Site-directed mutagenesis of *xylS* to introduce T74P.
56	xyls-I205S-F	CGTGAA**AGT**TTTAGCAAAGGTAACCCG	Site-directed mutagenesis of *xylS* to introduce I205S.
57	xyls-I205S-R	GCTAAA**ACT**TTCACGGCTAACATTGC	Site-directed mutagenesis of *xylS* to introduce I205S.