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Switching the Ligand Specificity of the Biosensor XylS from *meta* to *para*-Toluic Acid through Directed Evolution Exploiting a Dual Selection System

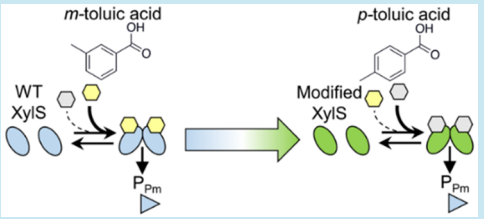
Yuki Ogawa,[†] Yohei Katsuyama,[*,†,‡] Kento Ueno,[†] and Yasuo Ohnishi[*,†,‡]

[†]Department of Biotechnology, The Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[‡]Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Supporting Information

ABSTRACT: The *Pseudomonas putida* transcriptional activator XylS induces transcription from the *Pm* promoter in the presence of several benzoic acid effectors, with *m*-toluic acid being the most effective and *p*-toluic acid being much less effective. To alter the effector specificity of XylS, we developed a dual selection system in *Escherichia coli*, which consists of (i) an artificial operon of an ampicillin resistance gene and *tetR* under *Pm* promoter control and (ii) a chloramphenicol resistance gene under *tetR* promoter control. This system enabled both positive selection to concentrate XylS mutants recognizing a desired ligand and negative selection to exclude undesired XylS mutants such as those recognizing undesired ligands and those that are active without effectors. Application of a random mutagenesis library of *xylS* to directed evolution that exploited this selection system yielded two XylS mutants that recognize *p*-toluic acid more effectively. Analysis of each missense mutation indicated three amino acid residues (N7, T74, and I205) important for *p*-toluic acid recognition. Then, a codon-randomized *xylS* library at these three residues was similarly screened, resulting in three XylS mutants with increased *p*-toluic acid-recognition specificity. Analysis of each amino acid substitution revealed that T74P attributes to both *m*-toluic acid sensitivity loss and subtle *p*-toluic acid sensitivity acquisition, and that N7R increases the overall ligand-sensitivity. Finally, the combination of these two mutations generated a desirable XylS mutant, which has a high *p*-toluic acid sensitivity and scarcely responds to *m*-toluic acid. These results demonstrate the effectiveness of the dual selection system in the directed evolution of biosensors.



KEYWORDS: *XylS, directed evolution, biosensor, ligand specificity*

Transcriptional regulators, which are capable of detecting small molecules, are useful biosensors for measuring the concentration of certain substances in the environment or *in vivo*.[1] Recently, such transcriptional regulators are also receiving research focus as tools for synthetic biology; they are used to facilitate directed evolution of enzymes and pathway engineering by enabling high-throughput selection from large libraries.[2−6] For example, several transcriptional regulators have been applied to monitor the production of useful chemicals such as naringenin,[7] butanol,[8] and mevalonate[9] to screen a strain with better productivity. In this regard, it is meaningful to extend the availability of various transcriptional regulators with different ligand specificities. However, natural transcriptional regulators do not necessarily recognize desired low molecular-weight compounds as functional ligands. Therefore, it has been of great interest to modify their ligand specificities for gaining the availability of biosensors of this type.

Directed evolution is an important strategy for engineering the ligand selectivity of transcriptional regulators.[4,10,11] In directed evolution, a target protein is engineered by a procedure that mimics the natural evolution process: proteins have evolved through the selection of mutant proteins generated by random mutations of their genes. To introduce random mutations in directed evolution experiments, various mutagenesis methods, such as error-prone PCR and genome-shuffling, are used. Various indicators, such as fluorescence intensity, colorimetry, and the growth of the host, are also used to select mutant proteins acquiring desired characteristics.[3]

In the directed evolution of biosensors, mutants with relaxed ligand specificities were often obtained; the mutants that acquired sensitivity to new ligands did not lose sensitivity to their original ligands.[12] To generate mutants that induce transcription in response to new ligands but not to their original ligands, several studies used a dual selection system combining negative selection against original ligands and positive selection toward new ligands.[13−18] Arnold and colleagues reported a biosensor (LuxR) that acquired an ability to induce transcription responses to new ligands, straight-chain acyl-homoserine lactones, but not to its original ligand, 3-oxo-hexanoyl-homoserine lactone.[13,14] In this study, a

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LuxR variant with desired ligand specificity was obtained through directed evolution by exploiting a dual selection system using an antibiotic resistance gene and a fluorescent protein gene as reporter genes.

XylS, a transcriptional activator in *Pseudomonas putida*, is a member of the XylS/AraC family.[19−22] XylS consists of 321 amino acids; the *N*- and *C*-terminal domains are responsible for ligand recognition/dimerization and DNA binding, respectively.[23−27] XylS recognizes several aromatic compounds as ligands.[1] When XylS forms a complex with its ligand and binds to the operator sequence of *Pm* promoter (P_{Pm}) as a dimer, XylS induces transcription from the promoter.[28,29] In this way, the strong transcription from P_{Pm} is tightly controlled. Because the ligands are not metabolized and do not cause growth deterioration in usual microorganisms,[30,31] a set of XylS, its aromatic ligands, and P_{Pm} can be used as a tool for inducing gene expression in a broad range of heterologous hosts. Thus, the combination of XylS and P_{Pm} is a useful genetic tool for metabolic engineering. However, no 3D structure of XylS exists because this protein is poorly soluble and the purification of its active form has been unsuccessful.[19] Therefore, structure-based rational engineering has not been applicable to XylS. Vee Aune et al. reported engineering of XylS to induce an increased level of transcription from P_{Pm} through directed evolution; XylS mutants were selected by using the *bla* reporter gene conferring ampicillin resistance under the control of P_{Pm}.[22] The researchers obtained XylS variants with several amino acid substitutions that showed up to 10-fold increased ampicillin resistance under induction conditions. Meanwhile, Ramos's group has studied XylS comprehensively for more than 30 years and obtained several XylS mutants with altered effector specificities and classified them into the following three groups:[23,26,32,33] (i) those that exhibit broader effector specificity than wild-type (WT) XylS and show high levels of transcription when induced with both original and new effectors (e.g., R45T mutant), (ii) those that exhibit constitutive transcription from P_{Pm} in the absence of an effector (high basal activity), in some cases with subtle changes in effector specificity (W88L, S229I, D274E, and D274 V), and (iii) those that exhibit a relaxed effector specificity, including new effectors, but their induction levels with new effectors are generally low (R152G, R41H, D288V, L155F, and P256R). Some XylS mutants belonging to group (iii) have reduced or absent sensitivity to some compounds that serve as weak effectors of WT XylS. However, Ramos's group and others have not obtained XylS mutants that can respond to a new (or originally unpreferable) effector, but not to the most preferable effector, *m*-toluic acid.

The purpose of this study was to develop a directed evolution-driven engineering system of biosensors. In this study, we developed a new dual selection system by using a gene circuit composed of two transcriptional regulator genes, as well as two antibiotic resistance genes against two antibiotics as reporter genes. The feasibility of the positive and negative selections of this system was confirmed by model experiments. Then, we demonstrated its applicability by completely altering the ligand specificity of XylS so that it could effectively recognize an unpreferable ligand, *p*-toluic acid, and hardly respond to the most preferable ligand, *m*-toluic acid. Thus, our dual selection system provided a useful strategy to alter the ligand specificity of a transcriptional regulator.

RESULTS

Designing a Dual Selection System. Because structure-based rational engineering is not applicable to XylS, we used directed evolution to modify its ligand specificity. We aimed to modify the XylS ligand specificity so that it efficiently recognizes *p*-toluic acid, to which WT XylS shows weak recognition.[1,32] In addition, we sought to modify the ligand specificity so that it does not respond to *m*-toluic acid, the most preferable ligand of WT XylS. We attempted to use antibiotic resistance to select XylS mutants with desired ligand specificity through directed evolution by placing an ampicillin resistance gene (*bla*) under the control of P_{Pm}. The recombinant *Escherichia coli* strain harboring P_{Pm}-*bla* and *xylS* should become resistant and sensitive to ampicillin in the presence and absence, respectively, of its ligand. Therefore, if we cultivate *E. coli* cells harboring a XylS mutant library in a liquid medium containing *p*-toluic acid, *E. coli* cells producing mutated XylS that recognize and respond to *p*-toluic acid should grow predominantly.

However, pseudopositive strains, which produce XylS mutants capable of stimulating transcription of *bla* without any ligands or by binding to some endogenous substance, should also predominantly grow in this selection system. Therefore, a system to exclude pseudopositive XylS mutants (so-called negative selection) was required.

For this purpose, we constructed a selection system composed of two plasmids, pUKN009 and pCDFlac-XylS (Figure 1a). pCDFlac-XylS is a CDF *ori*-based plasmid harboring *xylS* under the control of *lac* promoter (P_{lac}). pUKN009 is a COLA *ori*-based plasmid for selection markers. An artificial operon containing *bla* and *tetR* (a gene encoding the transcriptional repressor TetR[34]) is located under the control of P_{Pm} and *cat* (chloramphenicol resistance gene) is

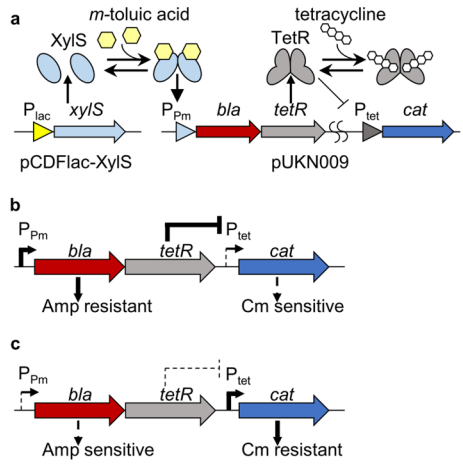

Figure 1. Construction of the dual selection system. (a) XylS induces transcription from *Pm* promoter (P_{Pm}) when it binds to the ligand. TetR represses transcription from *tetR* promoter (P_{tet}). The repression can be suppressed by the addition of tetracycline. (b) When P_{Pm} is activated by XylS, the strain should become ampicillin (Amp)-resistant and chloramphenicol (*Cm*)-sensitive. (c) When P_{Pm} is not activated by XylS, the strain should become Amp-sensitive and *Cm*-resistant. During the negative selection, the host cells producing pseudopositive XylS mutants should be *Cm*-sensitive and those producing desired XylS mutants should be *Cm*-resistant. During the positive selection, the host cells producing desired mutants should be Amp-resistant.

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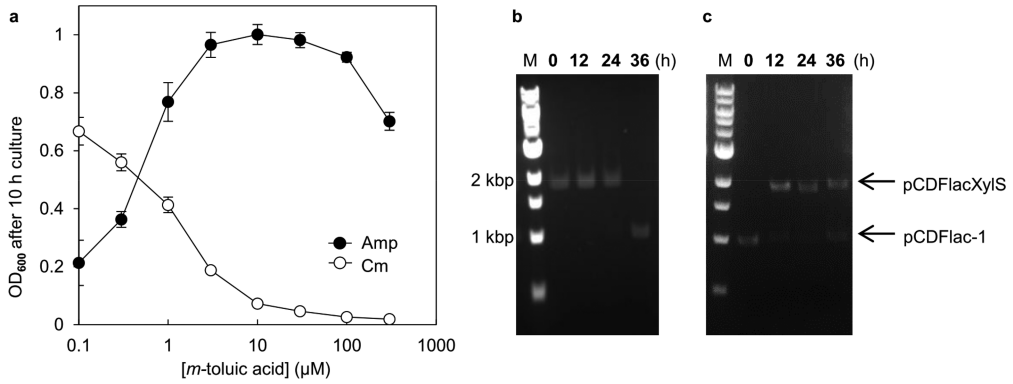

Figure 2. Model experiment for negative and positive selection. (a) The growth of *E. coli* JM109 harboring pCDFlacXylS and pUKN009 (JM109-XylS-009) in the presence of ampicillin (Amp, 350 mg/L) [black circle] and chloramphenicol (*Cm*, 34 mg/L) with a trace amount of tetracycline (Tet, 3 μg/L) [white circle] in response to various concentrations of *m*-toluic acid. The OD_{600} was measured after a 10 h cultivation. (b) Experiment mimicking the negative selection. The strain harboring pCDFlac-1 became dominant against the strain harboring pCDFlacXylS during cultivation in LB medium containing *m*-toluic acid, *Cm*, and Tet, as determined by semiquantitative PCR using the same primer pair to amplify different lengths of DNA fragments from each plasmid. (c) Experiment mimicking the positive selection. The strain harboring pCDFlacXylS became dominant against the strain harboring pCDFlac-1 during cultivation in LB medium containing *m*-toluic acid and Amp, as determined by semiquantitative PCR as described in panel b.

Table 1. Culture Conditions for the Selection of a Random-Mutagenesis *xylS* Library[a]

		negative selection			positive selection		
cycle	library size	time (h)	[Tet] (μg/L)	[*m*-toluic acid] (μM)	time (h)	[Amp] (μg/mL)	[*p*-toluic acid] (μM)
1	several hundreds	4.5	0, **3**, 10, 30	0	24	25, **50**, 100, 250, 500	100
2	~1000	4	0, **3**, 10, 30	0	24	25, 50, 100, **250**, 500	100
3	~300	5	0, **3**, 10, 30	0	13	25, 50, 100, 250, **500**	100
4	~1000	4	0, **3**, 10, 30	0	24	500	**1**, 3, 10, 30, 100
5	~300	5.5	0, **3**, 10, 30	0	31	500	1, **3**, 10, 30, 100
6	~500	8	3	0.1, 0.3, 1, 3, **10**	36	500	1, 3, 10, **30**, 100

[a]In cycles 1−5 and cycle 6 of the negative selection, the concentrations of tetracycline (Tet) and m-toluic acid, respectively, were varied. In cycles 1−3 and 4−6 of the positive selection, the concentrations of ampicillin (Amp) and p-toluic acid, respectively, were varied. Underlines indicate the concentration of additive in which the strain could not grow. Bold letters indicate the selected concentration of additive for screening.

located under the control of *tet* promoter (P_{tet}) on pUKN009. TetR binds to the operator sequence of P_{tet} with a very high affinity and represses the transcription of *cat* (Figure 1a). The repression by TetR is suppressed by the addition of tetracycline. In the presence of *m*-toluic acid, *E. coli* JM109 harboring pCDFlac-XylS and pUKN009 (JM109-XylS-009) was expected to be ampicillin-resistant and chloramphenicol-sensitive because XylS should bind to *m*-toluic acid and stimulate the expression of *bla* and *tetR* (Figure 1b). At the same time, the produced TetR should repress the expression of *cat* (Figure 1b). In contrast, in the absence of *m*-toluic acid, the recombinant strain should be ampicillin-sensitive and chloramphenicol-resistant because P_{Pm} should not be induced and P_{tet} should be active in the absence of TetR (Figure 1c). By using this system, the above-mentioned pseudopositive XylS mutants could be excluded by chloramphenicol sensitivity in the absence of external ligands (negative selection) because P_{Pm} should be activated by such pseudopositive XylS mutants even in the absence of external ligands to produce TetR, which makes the strain sensitive to chloramphenicol (Figure 1b). The mutant producing XylS that recognizes a desired ligand could be selected by ampicillin-resistance in the presence of the ligand (positive selection). It was also expected that the selection pressure could be controlled by altering the concentration of ampicillin and chloramphenicol. Furthermore, the amount of active TetR in the cells could be reduced by the addition of tetracycline.

To test the feasibility of our selection system and to optimize the experimental conditions, we carried out a model experiment using JM109-XylS-009. To find the proper growth conditions for the *E. coli* strain to show the desired phenotype, we varied concentrations of ampicillin, chloramphenicol, IPTG, and tetracycline (see Materials and Methods for details). Under the optimized condition, the growth of JM109-XylS-009 was improved as the concentration of *m*-toluic acid increased in the presence of 350 mg/L of ampicillin (Figure 2a). In addition, the growth was repressed as the *m*-toluic acid concentration increased in the presence of 34 mg/L chloramphenicol and 3 μg/L tetracycline (Figure 2a). Importantly, we revealed that TetR was produced by the basal transcription from P_{Pm} and strongly repressed the transcription of *cat* from P_{tet} even in the absence of *m*-toluic acid. Therefore, the addition of a trace amount of tetracycline was necessary for the growth of the strain in the presence of chloramphenicol.

We next prepared another recombinant *E. coli* JM109 strain harboring pCDFlac-1 and pUKN009 (JM109-empty-009). JM109-XylS-009 and JM109-empty-009 were mixed at a ratio of 10 000:1 and cultured in LB medium (100 mL) containing *m*-toluic acid, chloramphenicol, and tetracycline. In this experiment, JM109-XylS-009 mimicked a pseudopositive mutant strain, in which a mutant XylS activated P_{Pm} in the absence of a ligand. A portion (100 μL) of the culture was transferred into fresh LB medium (100 mL) every 12 h, and

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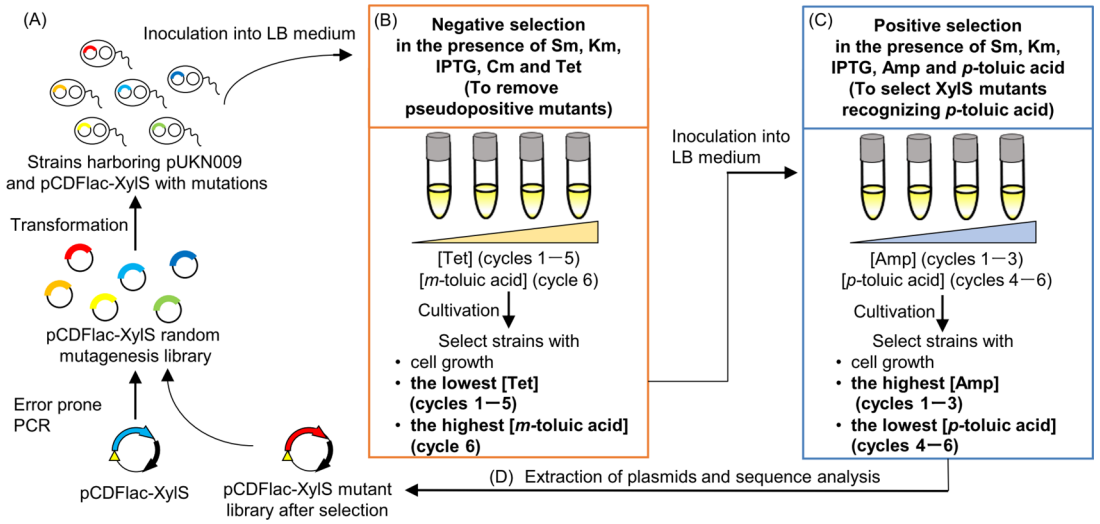

Figure 3. Screening procedure for the selection of desired XylS mutants and the removal of pseudopositive mutants. (A) Construction of a random *xylS* library and transformation of *E. coli* JM109 harboring pUKN009 with the library. (B) Cultivation of the obtained *E. coli* cells in LB medium containing chloramphenicol and tetracycline for negative selection. (C) Cultivation of the *E. coli* cells selected by step B in LB medium containing ampicillin and *p*-toluic acid for positive selection. (D) Extraction of the plasmids from the *E. coli* cells selected by step C. These plasmids are used as templates by step A in the next cycle, as well as for sequence analysis.

the plasmids were extracted from the remaining culture. Then, 1- and 2-kb regions of pCDFlac-1 and pCDFlac-XylS, respectively, were amplified by semiquantitative PCR using the same primer set to analyze the ratio of the two *E. coli* strains. The result showed that the pCDFlac-XylS-harboring strain, which imitated a pseudopositive mutant strain, was removed after a 36 h cultivation (Figure 2b). Furthermore, we similarly conducted another model experiment for positive selection. JM109-XylS-009 and JM109-empty-009 mimic strains that produce desired and undesired mutant strains, respectively. These two *E. coli* strains were mixed at a ratio of 1:10 000 and cultured in LB medium containing *m*-toluic acid and ampicillin. The result of semiquantitative PCR revealed that the pCDFlac-XylS-harboring strain, which imitated a strain producing a desired XylS mutant, grew predominantly even after a 12-h cultivation (Figure 2c). These results demonstrated that the constructed system is suitable not only for the removal of pseudopositive XylS mutants but also for the selection of XylS mutants that recognize a desired ligand.

Directed Evolution of XylS. We constructed a random mutagenesis *xylS* library by error-prone PCR as described in Materials and Methods. Then, *E. coli* harboring pUKN009 was transformed with this library. It should be noted that the numbers of transformants, which represent the size of each actual *xylS* library, were rather modest (Table 1). Therefore, we attempted to compensate for the relatively small-size library by repeating a mutagenesis−selection cycle. During directed evolution, we repeated the following screening procedure six times (Figure 3 and Table 1): (A) construction of a *xylS* mutant library on pCDFlac-XylS by error prone PCR and transformation of *E. coli* JM109 harboring pUKN009 with the library, (B) cultivation of obtained *E. coli* cells in liquid culture containing chloramphenicol and tetracycline (negative selection; the concentration of the tetracycline was changed to control the strength of the selection pressure), (C) cultivation of the *E. coli* cells obtained by step B in liquid culture containing ampicillin and *p*-toluic acid (positive selection; the concentration of the ampicillin was changed to control the strength of the selection pressure), and (D) extraction of the plasmids from the culture obtained by step C, and a mixture of the plasmids was used for a template of error prone PCR in the next cycle. At the same time, eight clones of the *xylS* mutants were applied to DNA sequencing to analyze the mutations introduced. In the negative selection (Figure 3B), we cultured the strains in the presence of various concentrations of tetracycline and selected the cells that could grow in the lowest concentration of tetracycline during cycles 1−5. In the positive selection (Figure 3C), we cultivated the strains in the presence of various concentrations of ampicillin and selected the cells that were resistant to the highest concentration of ampicillin during cycles 1−3 (Table 1). As the experiment proceeded, the strains acquired stronger resistance to ampicillin. Therefore, we fixed the concentration of ampicillin to a high level (500 mg/L) and cultivated the strains in the presence of various concentrations of *p*-toluic acid and selected the cells that grew in the lowest concentration of *p*-toluic acid during cycles 4−6 (Figure 3C). In the negative selection, to remove XylS mutants that recognize *m*-toluic acid, we cultured the strains in the presence of various concentrations of *m*-toluic acid during cycle 6 and selected the cells that grew in the highest concentration of *m*-toluic acid (10 μM) (Figure 3B). Sequence analysis of the obtained XylS mutants showed that N7S was commonly introduced into every XylS mutant after cycle 2, and that I205V was additionally introduced after cycle 4. Finally, T74A, K83E, and N227D were introduced into all XylS mutants after cycle 6 (Table 2).

Ligand Specificity of Two XylS Mutants. We selected two XylS mutants for further analysis of ligand specificity. The target mutants were 4X-5 (harboring N7S and I205V; obtained after cycle 4) and 6X-1 (harboring five missense mutations mentioned above; obtained after cycle 6). The ligand specificity of XylS mutants was analyzed using a newly constructed *E. coli* strain that harbors mutated *xylS* on pCDFlac-XylS and pCOLA*Pm*mCherry. pCOLA*Pm*mCherry is a reporter plasmid on which the mCherry gene is located under the control of P_{Pm}, and the fluorescence intensity of this reporter strain's cells should reflect the availability of a tested compound as an effector of XylS. As a result, 4X-5 was shown

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Table 2. Amino Acid Substitutions of XylS by the Mutations during the Selection of a Random-Mutagenesis *xylS* Library[a]

cycle	N7S	L36Q	T74A	K83E	I205V	N227D	E266G	others
2	7	2						3
3	8						3	1
4	6				2		3	4
5	8				8			
6	8		8	8	8	8		

[a]Mutations introduced into *xylS* during cycles 2−6 were analyzed in eight clones of each step. Of the eight clones, the number of the clones with the indicated amino acid substitution is shown.

to acquire the ability to induce transcription from P_{Pm} in response to *p*-toluic acid (Figure 4). However, it also induced

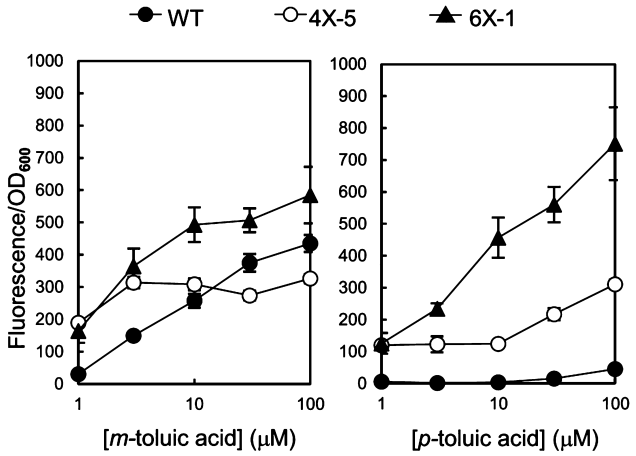

Figure 4. *m*-Toluic acid and *p*-toluic acid sensitivity of WT XylS and XylS mutants acquired from a random mutagenesis library. *E. coli* strains harboring both pCOLAPmmCherry and pCDFlacXylS (WT, 4X-5, and 6X-1) were cultivated for 10 h in the presence of various concentrations of *m*-toluic acid and *p*-toluic acid. The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture.

transcription in response to *m*-toluic acid, indicating that it acquired relaxed ligand specificity. Furthermore, 4X-5 induced transcription from P_{Pm} to some extent even in the absence of any ligand (Figure S1), indicating that it also acquired a relatively high basal activity. This result indicates that our negative selection during cycles 1−5 was not necessarily perfect. We speculate that the *E. coli* strain producing 4X-5 should survive during the negative selection owing to the leaky expression of chloramphenicol resistance gene in the presence of a small amount of tetracycline. Meanwhile, mutant 6X-1 showed higher sensitivity to *p*-toluic acid than the sensitivity of WT XylS to *m*-toluic acid (Figure 4). However, it also showed higher sensitivity to *m*-toluic acid than WT XylS. 6X-1 showed a lower expression level at a low concentration of *m*-toluic acid (1 μM) than 4X-5, indicating that the negative selection during cycle 6 was effective to remove XylS mutants that responded to a low concentration of *m*-toluic acid.

Finally, to examine the ability of the two XylS mutants to respond to other ligands, we conducted the above-mentioned fluorescence assay using other aromatic compounds. As a result, 4X-5 effectively responded only to *m*-toluic acid, *p*-toluic acid, and benzoic acid. In contrast, 6X-1 responded not only to *p*-toluic acid but also to *trans*-cinnamic acid and *p*-coumaric acid (Figure S1). These compounds have not been reported to be recognized by XylS or its mutant to date.[19]

Analysis of the Effect of Each Amino Acid Substitution on Ligand Specificity. Because all XylS mutants acquired have several amino acid substitutions, we attempted to reveal which substitution is responsible for the increased sensitivity to *p*-toluic acid. For this purpose, single amino acid substituted mutants, N7S, I205V, T74A, K83E, and N227D, were constructed by site-directed mutagenesis. Fluorescence assay using pCOLAP*m*mCherry showed that amino acid substitutions at N7S, T74A, and I205V conferred increased sensitivity to *p*-toluic acid, while those at K83E and N227D did not (Figure 5a). The induction activity of the N7S mutant in the absence of ligand was higher (60 ± 25 units) than that of WT XylS (5 ± 2 units), indicating that the N7S mutation is responsible for increasing the basal activity (Figure S2). Furthermore, in the presence of a low concentration of ligands, the N7S mutant showed higher induction activities than the basal activity; only 1 μM of *m*-toluic acid provoked a distinct increase of induction level (to 300 ± 40 units) while 1 μM of *p*-toluic acid did a relatively slight increase (to 80 ± 4 units) and 100 μM of *p*-toluic acid was required for a high induction level (200 ± 45 units) comparable to that of 1 μM of *m*-toluic acid (Figure S3; a discussion on the N7S mutation is also described in its legend). Thus, XylS-N7S gains not only increased basal activity but also (i) hypersensitivity to low concentrations of *m*-toluic acid and (ii) increased sensitivity to a high concentration of *p*-toluic acid. It is noteworthy that, in a previous study, the N7S mutation was also found from XylS mutants with increased ability to stimulate transcription from the *Pm* promoter in the presence of *m*-toluic acid (1 mM).[22]

To analyze the synergistic effects of N7S, T74A, and I205V, we constructed all possible double or triple-amino acid substituted mutants at these three amino acid residues (Figure 5b). Fluorescence assay showed that XylS-T74A-I205V had lower sensitivity to *m*-toluic acid and higher sensitivity to *p*-toluic acid than WT XylS (Figure 5b). Other mutants showed higher sensitivity to *p*-toluic acid but they also showed comparable or higher sensitivity to *m*-toluic acid compared with WT XylS. All XylS mutants harboring an N7S mutation tended to show high activity in the presence of a low concentration of ligands and even in the absence of ligand (Figure S2). This result supports the notion that N7S increases the transcription from P_{Pm} regardless of the presence of ligand.

Selection from a Codon-Randomized *xylS* Library. To investigate whether the substitutions at positions 7, 74, and 205 could be combined to acquire further improvements in *p*-toluic acid specificity, we constructed a codon-randomized *xylS* library at those positions using the NNT codon; each of the three positions was changed to any of the 15 natural amino acid residues other than Met, Gln, Lys, Glu, and Trp. *E. coli* JM109 harboring pUKN009 was transformed with the constructed codon-randomized pCDFlacXylS library. Three cycles of negative/positive selection were conducted to select XylS mutants with *p*-toluic acid specificity as described in Materials and Methods (Table 3). In the negative selection, we cultivated cells in the presence of various concentrations of *m*-toluic acid and selected the cells that grew in LB medium with the highest concentration of *m*-toluic acid. In the positive selection, we cultivated the cells in the presence of various concentrations of *p*-toluic acid and selected the cells that grew in LB medium with the lowest concentration of *p*-toluic acid. We analyzed the *xylS* sequences of eight clones after each

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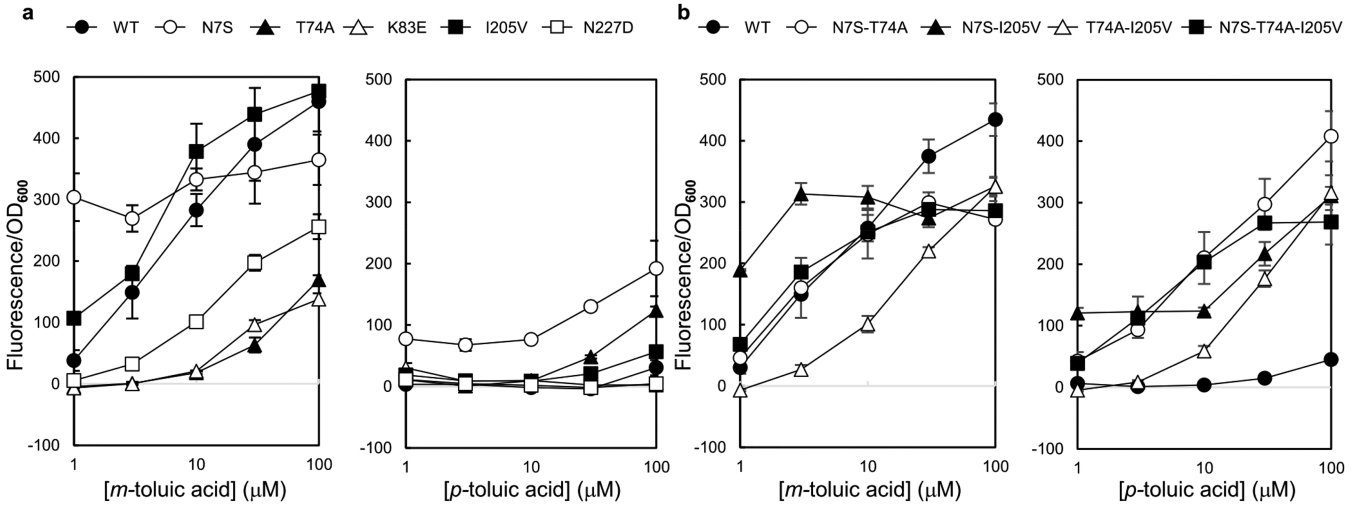

Figure 5. *m*-Toluic acid and *p*-toluic acid sensitivity of XylS mutants with single amino acid substitution (a) and double and triple mutations (b). *E. coli* strains harboring both pCOLAPmmCherry and pCDFlacXylS with a single mutation (N7S, T74A, K83E, I205V, and N227D) (a) and double and triple mutations (N7S-T74A, T74A-I205V, N7S−I205V, and N7S-T74A-I205V) (b) were cultivated for 10 h in the presence of various concentrations of *m*-toluic acid and *p*-toluic acid. The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture.

Table 3. Culture Conditions for the Selection of a Codon-Randomized *xylS* Library[a]

	negative selection			positive selection		
cycle	time (h)	[Tet] (μg/L)	[*m*-toluic acid] (μM)	time (h)	[Amp] (μg/mL)	[*p*-toluic acid] (μM)
1	24	3	1, 3, 10, 30, **100**	20	500	1, 3, 10, 30, **100**
2	24	3	1, 3, 10, 30, **100**	16	500	1, 3, 10, **30**, 100
3	24	3	1, 3, 10, 30, **100**	14	500	1, 3, 10, **30**, 100

[a]In the negative selection, the concentration of m-toluic acid was varied. In the positive selection, the concentration of p-toluic acid was varied. Underlines indicate the concentration of additive in which the strain could not grow. Bold letters indicate the selected concentration of additive for screening.

cycle. Sequence analysis showed that three XylS variants (XylS-N7R-T74P-I205N, N7R-T74P-I205S, and XylS-N7T-T75P-I205G) were enriched (Table 4). Fluorescence assay showed

Table 4. Amino Acid Substitutions of XylS by the Mutations during the Selection of a Codon-Randomized *xylS* Library[a]

cycle	N7R-T74P-I205N	N7R-T74P-I205S	N7T-T74P-I205G	others
1	3	1	1	3
2	4	1	3	
3	5	1	2	

[a]Mutations introduced into *xylS* during cycles 1−3 were analyzed in eight clones of each step. Of the eight clones, the number of the clones with the indicated triple amino acid substitutions are shown.

that two variants, XylS-N7R-T74P-I205N and XylS-N7R-T74P-I205S, responded to *p*-toluic acid much more efficiently than to *m*-toluic acid (Figure 6). Remarkably, XylS-N7R-T74P-I205S was six-times more sensitive to *p*-toluic acid than to *m*-toluic acid.

Analysis of N7R, T74P, and I205S Substitution on Ligand Specificity. The effect of the single substitution, N7R,

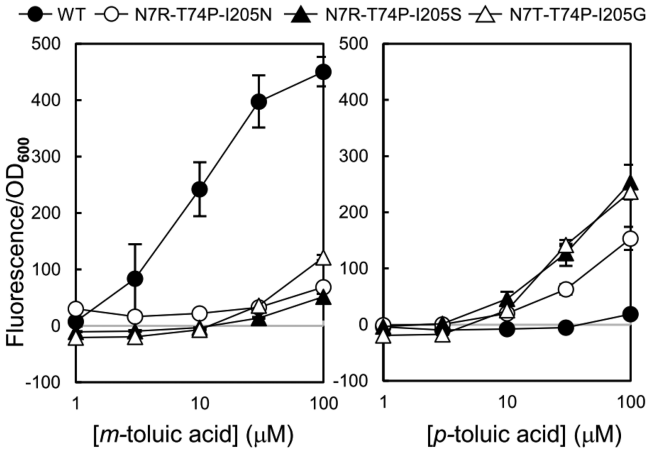

Figure 6. *m*-Toluic acid and *p*-toluic acid sensitivity of XylS mutants acquired from a codon-randomized *xylS* library. *E. coli* strains harboring both pCOLAPmmCherry and pCDFlacXylS with a triple mutation (N7R-T74P-I205N, N7R-T74P-I205S, and N7T-T74P-I205G) were cultivated for 10 h in the presence of various concentrations of *m*-toluic acid and *p*-toluic acid. The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture.

T74P, and I205S, on ligand specificity was analyzed by fluorescence assay. As a result, XylS-I205S showed slightly lower sensitivity to *m*-toluic acid and slightly higher sensitivity to *p*-toluic acid than WT XylS (Figure 7a). XylS-N7R showed increased induction activity regardless of the concentration of ligands. Interestingly, XylS-T74P completely lost the sensitivity to *m*-toluic acid, while it acquired a weak sensitivity to *p*-toluic acid.

Double mutants harboring two of these three amino acid substitutions were also constructed. Fluorescence assay showed that XylS-N7R-I205S showed higher sensitivity to both *m*-toluic acid and *p*-toluic acid than WT XylS (Figure 7b). XylS-T74P-I205S showed no sensitivity to *m*-toluic acid and subtle sensitivity to *p*-toluic acid (Figure 7b). Finally, XylS-N7R-T74P was 13-times more sensitive to *p*-toluic acid than to *m*-

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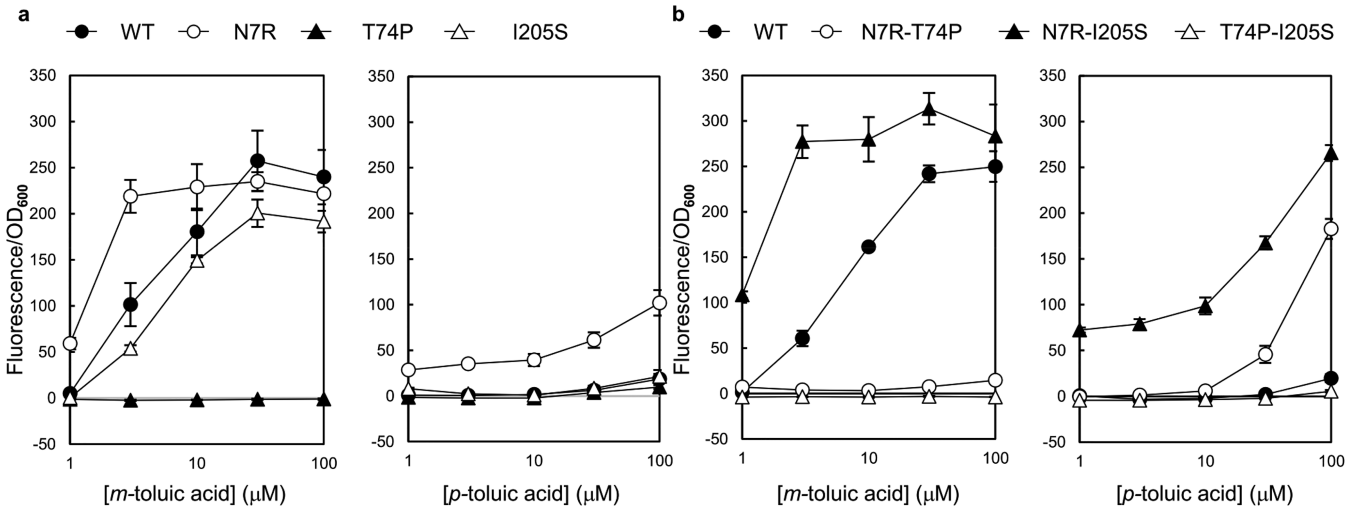

Figure 7. *m*-Toluic acid and *p*-toluic acid sensitivity of XylS mutants with a single amino acid substitution (a) and double mutation (b). *E. coli* strains harboring both pCOLAPmmCherry and pCDFlacXylS with a single substitution (N7R, T74P, and I205S) (a) and a double mutation (N7R-T74P, T74P-I205S, and N7R-I205S) (b) were cultivated for 10 h in the presence of various concentrations of *m*-toluic acid and *p*-toluic acid. The fluorescence intensity of each culture was measured and normalized by the OD_{600} value of the culture.

toluic acid. Thus, XylS-N7R-T74P was specific enough to *p*-toluic acid because WT XylS was about 13-times more specific to *m*-toluic than to *p*-toluic acid. Then, we examined whether XylS-N7R-T74P recognizes other aromatic compounds (Figure S4). As a result, in contrast to 6X-1, XylS-N7R-T74P efficiently responded only to *p*-toluic acid, indicating that this mutant is highly specific to *p*-toluic acid. It is interesting that this mutant showed reduced sensitivity to benzoic acid, which is a relatively good effector of WT XylS (Figure S4).

DISCUSSION

Our study showed that two mutations N7R and T74P completely altered the ligand specificity of XylS. Although the structural difference between *m*-toluic acid and *p*-toluic acid is slight, it is interesting that substituting only two amino acids could switch the ligand selectivity of XylS from *m*-toluic acid to *p*-toluic acid. N7R and T74P seem to have different roles in this switching of ligand selectivity. T74P causes the loss of the sensitivity to the most preferable ligand *m*-toluic acid and the acquirement of the sensitivity to *p*-toluic acid at the same time, although the *p*-toluic acid sensitivity is very low. All XylS mutants acquired from the codon-randomized *xylS* library possess T74P, indicating that our selection method was effective enough to enrich a desired mutation as well as the importance of this substitution. In contrast, N7R increases the basal transcription from P_{Pm}. This mutation probably covers the demerit of T74P, that is, the decrease in the expression level. Interestingly, a previous study on directed evolution of XylS revealed that the amino acid substitution at position 7 (N7S or N7T) is related to the increased expression level of the target gene under the control of P_{Pm} in the presence of *m*-toluic acid (1 mM), although no increase of basal activity was observed by these mutations.[22] Our results showed that mutations in this residue, N7S and N7R, increased the expression level from P_{Pm} only in the presence of low concentrations of *m*-toluic acid (1, 3, and 10 μM). It should be noted that the inconsistency may be explained by the different assay systems with different *E. coli* strains between the two studies. Anyway, these mutations at position 7 may increase the affinity of XylS to P_{Pm}, resulting in a higher level of transcription.

Because no experimental 3D structure of XylS is available, we attempted to construct a homology-based 3D model of XylS by Phyre2.[35] According to the solved structures of the N-terminal domain of AraC (PDB ID: 2AAC, 14% identity) and the full length of CuxR (PDB ID: 5NLA, 11% identity),[21,36,37] the structures of XylS were modeled. However, because the amino acid identities between XylS and AraC/CuxR are very low, these models are not expected to be so reliable that we can use them for detailed discussion of the function of each amino acid residue. Nevertheless, we expect that these models may be used for roughly estimating a possible overall structure of XylS. Both of the models consist of a β-barrel domain connected via a loop to a hairpin domain composed of two helices (Figures S5 and S6). The β-barrel forms a possible ligand binding pocket, and the hairpin forms the surface of a dimerization domain.[21,36] Both of the models show that T74 is located on a linker connecting two β-strands in the β-barrel domain (Figure S5 and S6). These positions seem to be far from the possible ligand binding site. If this is true, amino acid substitution at such positions appears to affect ligand binding indirectly. Considering that proline is much less flexible than threonine, the T74P substitution seems to cause a drastic structural change of the surrounding region, which may influence the size or the shape of the ligand binding pocket to change the ligand selectivity of XylS. Furthermore, the XylS model based on CuxR shows that I205 is located on the linker connecting the hairpin domain and the HTH motif related to DNA binding (Figure S6). In both models, N7 was absent because the amino acid residues corresponding to N7 of XylS were disordered in the structures of AraC and CuxR (Figure S5 and S6). Thus, the effects of mutations at N7, T74, and I205 were difficult to tell from the structure modeling.

CONCLUSION

We successfully generated a XylS mutant (N7R-T74P double mutant) that has high specificity toward *p*-toluic acid through directed evolution. The analysis of each amino acid substitution showed that N7R and T74P have different roles

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in acquiring *p*-toluic acid specificity. The importance of these two amino acids in the ligand specificity of XylS could not be predicted from the 3D modeling structures, which indicates the effectiveness of directed evolution using random mutagenesis. Furthermore, another XylS mutant, 6X-1, unexpectedly acquired sensitivity to new ligands such as *trans*-cinnamic acid and *p*-coumaric acid. These compounds have not been reported as ligands of XylS. Therefore, our study showed the high potential of XylS variants to be biosensors of various aromatic compounds. Because the combination of XylS and P_{Pm} is a useful genetic tool,[30,31] construction of a XylS mutant library with altered ligand specificity is useful for further application. Our selection system combining negative and positive selection steps was effective for the high-throughput screening of mutants with desired phenotypes. Thus, we provided a potent strategy for altering ligand specificity of transcriptional regulators using only antibiotic selection. This simple and effective selection system is applicable to engineering various biosensors.

MATERIALS AND METHODS

Strains, Media, Chemicals, and Other Materials. *E. coli* strain JM109 (Takara Biochemicals, Shiga, Japan) was used as a host for DNA manipulation. Luria−Bertani (LB) broth was purchased from Difco (Franklin Lakes, New Jersey). All the chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Sigma-Aldrich (St. Louis, Missouri), and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Enzymes used for DNA manipulation were purchased from Takara Bio Inc. (Shiga, Japan). Primers were purchased from Fasmac Co., Ltd. (Kanagawa, Japan), Sigma-Aldrich, Hokkaido System Science Co., Ltd. (Hokkaido, Japan), and Thermo Fisher Scientific (Waltham, Massachusetts). DNA sequences were analyzed by Fasmac Co., Ltd.

Construction of pCDFlacXylS. Plasmids and primers used in this study are listed in Tables S1 and S2, respectively. The pCDFlacXylS contains *xylS* under the control of *lac* promoter. A DNA fragment containing the CDF *ori* and streptomycin resistance gene was PCR-amplified with primers 1 and 2 using pCDFDuet-1 as a template. A DNA fragment containing *lac* promoter was PCR-amplified with primers 3 and 4 using pUC19 as a template. These two DNA fragments were connected by the In-Fusion HD Cloning kit (Takara Bio Inc.), resulting in pCDFlac-1.

Synthetic *xylS*, whose codon usage is optimized for expression in *E. coli* (Thermo Fisher Scientific), was PCR-amplified with primers 5 and 6 and cloned into pJET1.2 (Thermo Fisher scientific), resulting in pJET1.2-XylS. pJET1.2-XylS was digested with *Nde*I and *Xho*I, and the DNA fragment encoding XylS was inserted into the corresponding sites of pCDFlac-1, resulting in pCDFlac-XylS.

Construction of pUKN009. pUKN009 contains (i) an artificial operon carrying an ampicillin resistance gene (*bla*) and *tetR* under the control of P_{Pm} and (ii) a chloramphenicol resistance gene (*cat*) under the control of P_{tet}. A DNA fragment containing *cat* was PCR-amplified with primers 7 and 8 using pACYDuet-1 as a template. The PCR product was cloned into pJET1.2, resulting in pJET-CAT. pJET-CAT was digested with *Nhe*I and *Hin*dIII, and the DNA fragment containing *cat* was inserted into the corresponding sites of pASK-IBA7plus (IBA Lifesciences, Göttingen, Germany), resulting in pASK-CAT. A DNA fragment containing *cat*, *bla*, and *tetR* was PCR-amplified with primers 9 and 10 using pASK-CAT as a template. A DNA fragment containing a kanamycin resistance gene and COLA *ori* was also PCR-amplified with primers 11 and 12 using pCOLADuet-1 as a template. Both the amplified DNA fragments were connected by the In-Fusion HD Cloning kit (Takara Bio Inc.), resulting in pUKN008. P_{Pm} was synthesized by PCR using four primers 13, 14, 15, and 16. The synthesized DNA fragment containing P_{Pm} was further PCR-amplified with primers 17 and 18. The whole pUKN008 except for the promoter region of *bla* was also PCR-amplified with primers 19 and 20. Both the amplified DNA fragments were connected by the In-Fusion HD Cloning kit (Takara Bio Inc.), resulting in pUKN009.

Construction of pCOLAPmmCherry. pCOLAPmmCherry harbors the mCherry gene under the control of P_{Pm}. The synthesized P_{Pm} described above was PCR-amplified with primers 21 and 22. A DNA fragment containing a kanamycin resistance gene and COLA *ori* was also PCR-amplified with primers 23 and 24 using pCOLADuet-1 as a template. Both the amplified DNA fragments were connected by the In-Fusion HD Cloning kit (Takara Bio Inc.), resulting in pCOLAPm. pmCherry was digested with *Bam*HI and *Not*I and the mCherry gene was cloned into the corresponding sites of pCOLAPm, resulting in pCOLAPmmCherry-1. The unintended missing A at the last position of P_{Pm} on pCOLAPmmCherry-1 was inserted by mutagenesis using primers 25 and 26, resulting in pCOLAPmmCherry.

Site-Directed Mutagenesis of *xylS*. Single amino acid substituted XylS-encoding plasmid series (e.g., pCDFlac-XylS-T74A, -K83E) were constructed by inverse PCR using a pair of primers (primers 38−57) containing the corresponding mutation. Double and triple amino acid substituted XylS-encoding plasmid series (e.g., pCDFlac-XylS-T74A-I205V, -N7S-T74A-I205V) were similarly constructed by inverse PCR using an appropriate single (or double) amino acid substituted XylS-encoding plasmid as a template. The templates remaining in the reaction mixtures were digested with *Dpn*I. The PCR products were phosphorylated with T4 polynucleotide kinase (Takara Bio Inc.), and cyclized with T4 DNA ligase (Takara Bio Inc.).

Model Experiments for Positive and Negative Selection. To observe the growth of *E. coli* harboring both pCDFlacXylS and pUKN009 under the conditions for positive or negative selection, cells were precultivated in LB medium at 37 °C overnight. Then, the overnight culture was inoculated (1% v/v) into 200 μL of fresh LB medium. The LB medium contained different concentrations of *m*-toluic acid (0, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μM), 50 μM IPTG, 50 mg/L kanamycin, 50 mg/L streptomycin, and 350 mg/L ampicillin for positive selection. Instead of ampicillin, 34 mg/L chloramphenicol and 3 μg/L tetracycline were added for negative selection. The cells were incubated at 37 °C for 10 h. The OD_{600} was measured in triplicate after cultivation.

To observe the growth of *E. coli* during competitive cultivation, two *E. coli* strains were precultivated in LB medium at 37 °C overnight. One harbored pCDFlac-1 and pUKN009 and the other harbored pCDFlacXylS and pUKN009. In the model experiment for positive selection, the overnight cultures of the former and the latter strains were mixed at a ratio of 10 000:1 and subsequently inoculated (0.2% v/v) into 100 mL of fresh LB medium containing 50 μM IPTG, 50 mg/L kanamycin, 50 mg/L streptomycin, 350 mg/L ampicillin, and 100 μM *m*-toluic acid. In the model experiment for negative selection, these cultures were mixed at a ratio of

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1:10 000 and subsequently inoculated (0.2% v/v) into 100 mL of fresh LB medium containing 50 μM IPTG, 50 mg/L kanamycin, 50 mg/L streptomycin, 34 mg/L chloramphenicol, 100 μM *m*-toluic acid, and 3 μg/L tetracycline. Cells were cultivated for 36 h at 37 °C. Then, the culture was inoculated (0.1% v/v) into 100 mL of fresh LB medium supplemented with appropriate antibiotics described above every 12 h. To analyze the ratio of surviving *E. coli* strains, plasmids of the culture were extracted and analyzed by semiquantitative PCR using primers 27 and 28.

Construction of a Random Mutagenesis *xylS* Library. To construct the first generation random mutagenesis *xylS* library, we used *Taq* DNA polymerase for error-prone PCR (New England Biolabs Inc., Ipswich, Massachusetts). The reaction mixture containing 5 mM $MnCl_2$, *Taq* DNA polymerase, and primers 29 and 30 was used to amplify *xylS* from pCDFlacXylS. The linear DNA fragment of pCDFlac was also PCR-amplified with primers 31 and 32. Both the amplified DNA fragments were connected by overlap extension PCR with primers 30 and 31. The obtained DNA fragment was phosphorylated with T4 polynucleotide kinase and cyclized with T4 DNA ligase. To construct random mutagenesis libraries for the second and further generations, we used Diversify PCR Random Mutagenesis Kit (Takara Bio Inc.). The XylS-encoding DNA fragment was amplified with primers 33 and 34 and connected with the linear DNA fragment of pCDFlac that had been amplified with primers 35 and 36.

Selection from the Random Mutagenesis *xylS* Library. The ligation mixture of the first generation random mutagenesis *xylS* library was introduced into *E. coli* JM109 harboring pUKN009 and the transformants were inoculated onto LB-agar supplemented with appropriate antibiotics (50 mg/L kanamycin [the marker for pUKN009] and 50 mg/L streptomycin [the marker for pCDFlacXylS]). The plates were incubated at 37 °C overnight. The generated *E. coli* library of several hundreds of colonies was suspended into 3 mL of LB medium and applied to negative selection as follows. An appropriate amount of the mixture of the *E. coli* cells was inoculated into each fresh LB medium to give a population density of 3×10^4 cells/mL. The LB medium was supplemented with 50 μM IPTG, different concentrations of tetracycline (0, 3, 10, or 30 μg/L) [to reduce the repression of P_{tet} by TetR], and appropriate antibiotics (50 mg/L kanamycin, 50 mg/L streptomycin, and 34 mg/L chloramphenicol [to select clones expressing *cat* from P_{tet}]). Cells were cultivated at 37 °C for 4.5 h and the OD_{600} of each culture was measured. Then, we selected a culture that showed a distinct growth under the lowest concentration of tetracycline as the inoculant for the succeeding positive selection as described below. The selected culture was inoculated into a fresh LB medium to a population density of 3×10^4 cells/mL. The LB medium was supplemented with 50 μM IPTG, different concentrations of ampicillin (25, 50, 100, 250, or 500 mg/L) [to select clones expressing *bla* from P_{Pm}], appropriate antibiotics (50 mg/L kanamycin and 50 mg/L streptomycin), and 100 μM *p*-toluic acid. The cells were cultivated at 37 °C for 24 h, and the OD_{600} of each culture was measured. Then, we selected a culture that showed a distinct growth under the highest concentration of ampicillin as the source of template plasmids for the succeeding random mutagenesis as described above. We repeated such a combination of negative and positive cultures six times (cycles 1−6) with some modifications as described in Table 1. It is noteworthy that the LB medium for positive selection was supplemented with different concentrations of *p*-toluic acid (1, 3, 10, 30, or 100 μM) and the ampicillin concentration was fixed at 500 mg/L during cycles 4−6. In these cases, a culture that showed a distinct growth under the lowest concentration of *p*-toluic acid was selected for the next cycle. It is also noteworthy that the LB medium for negative selection was supplemented with different concentrations of *m*-toluic acid (0.1, 0.3, 1, 3, or 10 μM) and the tetracycline concentration was fixed at 3 μg/L to remove pseudopositive XylS mutants that recognized *m*-toluic acid in cycle 6. In this case, a culture that showed a distinct growth under the highest concentration of *m*-toluic acid was selected after an 8-h incubation. To track the mutations introduced into *xylS* during cycles 2−6, plasmids were extracted from each selected culture of positive selection. *E. coli* JM109 was transformed with the plasmids, and colonies were grown on LB agar containing 50 mg/L streptomycin [the marker for pCDFlacXylS] at 37 °C overnight. Then, eight colonies were inoculated into 2 mL of LB medium and cultivated at 37 °C for 8 h. Finally, each plasmid was extracted and the *xylS* sequence was determined using primers 27 and 37.

Fluorescence Assay. *E. coli* strains for fluorescence assay were constructed by transforming *E. coli* JM109 with both pCOLAPmmCherry and pCDFlacXylS (or its derivative encoding mutated XylS). The cells were precultivated in LB medium at 37 °C overnight. The cultures were inoculated (1% v/v) into 200 μL of fresh LB medium supplemented with 50 μM IPTG, appropriate antibiotics (50 mg/L kanamycin and 50 mg/L streptomycin) and different concentrations of *m*-toluic acid or *p*-toluic acid (1, 3, 10, 30, or 100 μM) in a microtube. Cells were incubated at 37 °C with vigorous shaking at 500 rpm for 10 h. Cells were harvested by centrifugation for 15 min at 1100*g* and resuspended in 200 μL of PBS buffer (pH 7.4−7.6). Then, the OD_{600} (optical density at 600 nm) and fluorescence (excitation wavelength of 587 nm and emission wavelength of 610 nm) values were measured in triplicate using the microplate reader Spectramax M2 (Molecular Devices, San Jose, California). The average ± standard error was plotted against the ligand concentration.

Construction of a Codon-Randomized *xylS* Library. A codon-randomized *xylS* library with mutations at N7, T74, and I205 was constructed as follows. *xylS* was divided into three fragments and each fragment was PCR-amplified with codon randomization mutagenesis primer pairs 46/47, 48/49, or 50/51. pCDFlac was PCR-amplified with primers 36 and 51 using pCDFlacXylS as a template. These four amplified DNA fragments were assembled and amplified by overlap extension PCR using primers 34 and 35 to yield a full-length of pCDFlacXylS with mutations on *xylS*. The obtained DNA fragment was phosphorylated with T4 polynucleotide kinase and cyclized with T4 DNA ligase.

Selection from the Codon-Randomized *xylS* Library. The ligation mixture of the codon-randomized *xylS* library was used to transform *E. coli* JM109 harboring pUKN009 and the transformants were inoculated onto LB agar supplemented with the appropriate antibiotics (50 mg/L kanamycin and 50 mg/L streptomycin). The plates were incubated at 37 °C overnight. The generated *E. coli* library composed of 3558 colonies was suspended into 3 mL of LB medium and applied to negative selection as follows (Table 3). An appropriate amount of the mixture of the *E. coli* cells was inoculated into each fresh LB medium to give a population density of 3×10^4

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cells/mL. The LB medium was supplemented with 50 μM IPTG, different concentrations of *m*-toluic acid (1, 3, 10, 30, or 100 μM), and appropriate antibiotics (50 mg/L kanamycin, 50 mg/L streptomycin, 34 mg/L chloramphenicol, and 3 μg/L tetracycline). The cells were cultivated at 37 °C for 24 h and the OD_{600} of each culture was measured. Then, we selected a culture that showed a distinct growth under the highest concentration of *m*-toluic acid (100 μM) as the inoculant for the succeeding positive selection as follows. The selected culture was inoculated into each fresh LB medium to a population density of 3×10^4 cells/mL. The LB medium was supplemented with 50 μM IPTG, different concentrations of *p*-toluic acid (1, 3, 10, 30, or 100 μM), and appropriate antibiotics (50 mg/L kanamycin, 50 mg/L streptomycin, and 500 mg/L ampicillin). The cells were cultivated at 37 °C for 20 h, and the OD_{600} of each culture was measured. Then, we selected a culture that showed a distinct growth under the lowest concentration of *p*-toluic acid (100 μM) as the source of plasmid variants for the transformation followed by the next negative selection. We repeated such a combination of negative and positive selection cultures three times (cycles 1−3) with some minor modifications as described in Table 3. In the positive selection during cycles 2 and 3, the cells were cultivated for 16 and 14 h, respectively. To track the mutations introduced into *xylS* during cycles 1−3, plasmids were extracted from each selected culture of positive selection, and *E. coli* JM109 was transformed with the plasmids. Colonies were grown on LB agar containing 50 mg/L streptomycin at 37 °C overnight. Then, eight colonies were inoculated into 2 mL of LB medium and cultivated at 37 °C overnight. Finally, each plasmid was extracted, and the *xylS* sequence was determined using primers 27 and 37.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00237.

Sensitivity of XylS and mutants to various aromatic compounds; induction levels of WT XylS and all XylS mutants in the absence of any external ligand; models of XylS based on homology; primers and plasmids used in this study (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: aykatsu@mail.ecc.u-tokyo.ac.jp (Y.K.).
*E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp (Y.O.).

ORCID

Yasuo Ohnishi: 0000-0001-7633-9236

Author Contributions

Y.K. and Y.Og. designed the study. Y.Og. and K. U. performed the experiments. Y.Og. and Y.K. analyzed the data. Y.Og., Y.K., and Y.Oh. wrote the paper. All authors read and commented on the manuscript, and approved the final manuscript. Y.K. and Y.Oh. supervised the study.

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Notes

The authors declare no competing financial interest.

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