Chromosome Engineering To Generate Plasmid-Free Phenylalanine- and Tyrosine-Overproducing *Escherichia coli* Strains That Can Be Applied in the Generation of Aromatic-Compound-Producing Bacteria

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ABSTRACT Many phenylalanine- and tyrosine-producing strains have used plasmid-based overexpression of pathway genes. The resulting strains achieved high titers and yields of phenylalanine and tyrosine. Chromosomally engineered, plasmid-free producers have shown lower titers and yields than plasmid-based strains, but the former are advantageous in terms of cultivation cost and public health/environmental risk. Therefore, we engineered here the *Escherichia coli* chromosome to create superior phenylalanine- and tyrosine-overproducing strains that did not depend on plasmid-based expression. Integration into the *E. coli* chromosome of two central metabolic pathway genes (*ppsA* and *tktA*) and eight shikimate pathway genes (*aroA*, *aroB*, *aroC*, *aroD*, *aroE*, *aroG^{fbr}*, *aroL*, and *pheA^{fbr}*), controlled by the T7lac promoter, resulted in excellent titers and yields of phenylalanine; the superscript “fbr” indicates that the enzyme encoded by the gene was feedback resistant. The generated strain could be changed to be a superior tyrosine-producing strain by replacing *pheA^{fbr}* with *tyrA^{fbr}*. A rational approach revealed that integration of seven genes (*ppsA*, *tktA*, *aroA*, *aroB*, *aroC*, *aroG^{fbr}*, and *pheA^{fbr}*) was necessary as the minimum gene set for high-yield phenylalanine production in *E. coli* MG1655 (*tyrR*, *adhE*, *ldhA*, *pykF*, *pflDC*, and *ascF* deletant). The phenylalanine- and tyrosine-producing strains were further applied to generate phenyllactic acid-, 4-hydroxyphenyllactic acid-, tyramine-, and tyrosol-producing strains; yield of these aromatic compounds increased proportionally to the increase in phenylalanine and tyrosine yields.

IMPORTANCE Plasmid-free strains for aromatic compound production are desired in the aspect of industrial application. However, the yields of phenylalanine and tyrosine have been considerably lower in plasmid-free strains than in plasmid-based strains. The significance of this research is that we succeeded in generating superior plasmid-free phenylalanine- and tyrosine-producing strains by engineering the *E. coli* chromosome, which was comparable to that in plasmid-based strains. The generated strains have a potential to generate superior strains for the production of aromatic compounds. Actually, we demonstrated that four kinds of aromatic compounds could be produced from glucose with high yields (e.g., 0.28 g tyrosol/g glucose).

KEYWORDS aromatic compound, chromosome engineering, phenylalanine, plasmid-free, tyrosine

Industrially important aromatic amino acids include L-phenylalanine (Phe) and L-tyrosine (Tyr). Phe is used as a precursor for aspartame production, infusion fluids, etc., whereas Tyr is used as a dietary supplement, as raw material for production of L-3,4-dihydroxyphenylalanine (DOPA), etc. (1). Phe and Tyr are commercially produced

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by fermentation in genetically modified *Escherichia coli* strains (2). Although many bacteria can produce Phe and Tyr, the synthesis pathways have been well studied in *E. coli* (3). When using glucose as the substrate, imported glucose is converted to phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) via the glycolytic pathway and pentose phosphate pathway, respectively. PEP and E4P are then condensed by 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase to form DAHP, which is an initial compound of the shikimate pathway. In the shikimate pathway, DAHP is step-by-step converted to chorismate (CA) via six reactions. CA is an intermediate in Phe, Tyr, tryptophan (Trp), folate, and ubiquinone syntheses (3, 4). In Phe synthesis, CA is converted to phenylpyruvate (PP) by CA mutase/prephenate dehydratase, encoded by *pheA*. In Tyr synthesis, CA is converted to 4-hydroxyphenylpyruvate (4HPP) by CA mutase/prephenate dehydrogenase, encoded by *tyrA*. Finally, aromatic amino acid aminotransferase, encoded by *tyrB*, transfers an amino group from glutamate to PP and 4HPP to form Phe and Tyr.

Many attempts have been made to enhance Phe and Tyr productivity in *E. coli*. An important development was the release of DAHP synthase and CA mutase from feedback inhibition by Phe and/or Tyr. For example, binding of Tyr to DAHP synthase (AroF) causes conformational change of the enzyme into an inactive form. Therefore, AroF was mutated to be insensitive to Tyr, and various positive mutants have been obtained (5, 6). Similarly, CA mutase/prephenate dehydratase (PheA) and CA mutase/prephenate dehydrogenase (TyrA) were mutated to be insensitive to Phe and Tyr, respectively (7–9). Overexpression of genes encoding feedback-resistant AroF (*aroF^{fbr}*) and PheA (*pheA^{fbr}*) or TyrA (*tyrA^{fbr}*) resulted in strains capable of producing Phe or Tyr in higher titer than the wild-type strain (10–12). Please note that in the present study, the superscript notation “*fbr*” indicates a gene encoding a feedback-resistant enzyme. Instead of *aroF^{fbr}*, feedback-resistant AroG (encoded by *aroG^{fbr}*) could be used for the generation of a Phe- or Tyr-producing strain; AroG is more thermally stable than AroF (13). A Phe-producing strain can be simply switched to become a Tyr-producing strain by altering the overexpression of *pheA^{fbr}* to *tyrA^{fbr}* (14).

Another important development in Phe and Tyr synthesis was enhancement of the availability of DAHP precursors, PEP and E4P, in cells (11, 15–17). In particular, low intracellular availability of E4P is known to result in low yield of shikimic acid, which is an intermediate in Phe and Tyr synthesis. Overexpression of *tktA* was effective to enhance E4P availability (15, 18). Enhancing the availability of PEP was also very important for Phe production. Since *E. coli* imports glucose via a PEP-dependent transfer system (PTS), one molecule of PEP is converted to pyruvate to take up one molecule of glucose into the cell. Therefore, half of the amount of synthesized PEP is consumed without relation to Phe synthesis. Two ways are known to tackle this problem: (i) PEP regeneration from pyruvate by overexpression of a PEP synthase gene (*ppsA*) and (ii) alteration of the glucose uptake system from PTS to a PTS-independent system such as galactose permease or glucose facilitator (19, 20). By enhancing the intracellular E4P and PEP availability, the maximum yield of DAHP from glucose improves from 43 to 86% (mol/mol) (15). One molecule of DAHP is synthesized from each molecule of E4P and PEP, while one molecule of Phe or Tyr is synthesized from one molecule of E4P and two molecules of PEP. Thus, the maximum yield of Phe and Tyr is also doubled (from 30 to 60% [mol/mol]) by enhancing the intracellular E4P and PEP availability.

Other important points include improvement of metabolic bottlenecks in the shikimate pathway. Through metabolic analysis and genetic engineering, the reactions catalyzed by shikimate kinase (encoded by *aroK*) and quinate/shikimate dehydrogenase (encoded by *ydiB*) were revealed to be rate-limiting in the shikimate pathway (21, 22). The reaction step catalyzed by 5-enolpyruvoylshikimate 3-phosphate (EPSP) synthase, encoded by *aroA*, is also rate limiting (23). Overexpression of the genes encoding these rate-limiting enzymes improved Phe and Tyr titer.

Deletion of specific genes also improved the yields of Phe, Tyr, and shikimic acid. Transcriptional regulator TyrR represses the transcription of several shikimate pathway

genes, including *aroF*, *aroG*, and *aroL*, and the transporter genes *aroP* and *tyrP* (24). Deletion of *tyrR* released these genes from transcriptional repression (25). Pyruvate kinase, encoded by *pykF* and *pykA*, converts PEP to pyruvate. Deletion of these genes slightly improved the shikimic acid yield (18, 26). Deletion of *tyrA* and/or *trpE* prevented Tyr and Trp synthesis from CA and enhanced Phe yield (27). In these cases, the strain became a Tyr and/or Trp auxotroph. As cell growth was limited by the supplied amounts of Tyr and/or Trp, carbon flux could be directed to Phe synthesis. Phe productivity was also improved by deletion of global regulatory gene *csrA* (28) or overexpression of *csrB* (29).

Various aromatic compounds can be produced from glucose by use of the shikimate pathway (30–36). For example, introduction of genes encoding the PP decarboxylase gene from *Azospirillum brasilense* enabled Phe-producing *E. coli* to become 2-phenylethanol- and phenylacetic acid-producing strains, and tyrosol- and 4-hydroxyphenylacetic acid-producing strains could be obtained by introduction of these genes into a Tyr-producing strain (36). DOPA and dopamine were produced in *E. coli* when tyrosinase and/or DOPA decarboxylase genes were introduced into a Tyr-producing strain (37). These compounds are resources for production of pharmaceuticals and starter compounds for various kinds of plant alkaloid, such as reticuline and tebaine (38, 39). Hence, strains capable of producing Phe and Tyr are useful for generating various aromatic compound-producing strains.

Unfortunately, most prominent Phe- and Tyr-producing strains use plasmids for the overexpression of pathway genes. When using plasmids to generate Phe- and Tyr-producing strains, plasmid compatibility is an important issue. That is, both replicons and antibiotic resistance markers in the two or more introduced plasmids required for the synthesis pathway must be different from each other. Alternatively, a plasmid might become too large and unstable if introducing genes in large number. Using plasmids also risks spreading antibiotic-resistance genes into the environment and results in a high cultivation cost because the addition of antibiotics to the culture medium is required. Therefore, plasmid-free Phe-, Tyr-, and aromatic compound-producing strains are advantageous. Several plasmid-free Phe- and Tyr-producing strains have been generated by engineering the chromosome, including introduction of pathway genes into the chromosome and deletion of unnecessary genes from the chromosome. However, most plasmid-free strains produced lower yields of Phe and Tyr than plasmid-based strains. Using plasmid-based expression, Juminaga et al. achieved the best Tyr yield, 0.43 mol/mol glucose, in minimum medium (titer of 2.1 g/liter and specific productivity of 304 $\mu\text{mol/g}$ dry cell weight [DCW]/h) (40). On the other hand, high yields of Phe, for example, 0.34 mol/mol glucose in nutrient medium (titer of 6.23 g/liter) (12) or 0.36 mol/mol glucose in minimum medium (titer of <0.5 g/liter and a specific productivity of 248 $\mu\text{mol/g}$ DCW/h) (8), were achieved using a plasmid-based Phe-producing strain. In contrast, the yields of the plasmid-free strains developed to date were <0.1 mol/mol glucose (described in detail in the Discussion). Hence, we aimed to generate plasmid-free strains capable of producing Phe and Tyr with high yields. We also determined that the yield of target aromatic compounds increased in proportion to Phe and Tyr yield when the generated Phe- and Tyr-producing strains were used to produce other aromatic compounds.

RESULTS

Generation of plasmid-free Phe- and Tyr-producing strains. In comparison to plasmid-based Phe- and Tyr-producing strains, the expression levels of pathway genes have been poor in plasmid-free strains because of the low copy numbers of the pathway genes. To circumvent this issue, we used the T7 expression system, which is a strong expression system consisting of T7 RNA polymerase and the T7 (T7lac) promoter, for overexpression of pathway genes (41).

Phe and Tyr are synthesized via the shikimate pathway (Fig. 1). DAHP is an initial compound in the shikimate pathway, and synthesis of this compound is most important for Phe and Tyr synthesis. First, a gene encoding a feedback-resistant form of DAHP

FIG 1 Synthesis pathways of phenylalanine (Phe) and tyrosine (Tyr). Abbreviations: Glc, glucose; PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DHQ, dehydroquininate; DHS, dehydroshikimate; SHIK, shikimate; S3P, shikimate 3-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate; CA, chorismate; PA, prephenate; PP, phenylpyruvate; HPP, 4-hydroxyphenylpyruvate; Glu, glutamate; 2OG, 2-oxoglutarate.

synthase, *aroG^{fbr}*, was constructed by altering GAT to AAT at positions 436 to 438 in the nucleotide sequence, which corresponded to a D-to-N change at position 146 in the amino acid sequence (D146N), and then integrated into the *adhE* locus of the *E. coli* chromosome to generate strain M-PAR-13. It is easy to switch from the synthesis of Phe to the synthesis of Tyr by overexpression of *pheA* or *tyrA*, respectively. Therefore, we initially tried to generate a Phe-producing strain and then generate a Tyr-producing strain; a gene encoding feedback-resistant PheA (*pheA^{fbr}*, S330P) was integrated into the *tyrR* locus of the M-PAR-13 chromosome to generate strain M-PAR-14. To enhance the levels of PEP and/or E4P, *ppsA* and/or *tktA* were integrated into the *ldhA* and/or *pflDC* loci of the M-PAR-14 chromosome to generate strains M-PAR-20 (*ppsA*), M-PAR-22 (*tktA*), and M-PAR-24 (*ppsA* + *tktA*). The generated strains are listed in Table 1, and the generation procedures for the strains are summarized in Fig. 2. The selected integration loci—*adhE* (alcohol dehydrogenase), *tyrR* (DNA-binding transcriptional dual regulator), *ldhA* (lactate dehydrogenase), and *pflDC* (putative formate acetyltransferase 2/pyruvate formate lyase activating enzyme)—have been shown to produce sufficient overexpression even if only a single copy of each gene is integrated. That is, in our previous study of *lacZ* reporter assay, high β -galactosidase activity was detected when a single copy of *P_{T7lac}-lacZ* was integrated into the *tyrR* locus (41). In comparison to the *tyrR* locus, the β -galactosidase activities were sufficiently high at *adhE*, *ldhA*, and *pflDC* loci (Fig. 3A). In addition, the T7-controlled pathway genes were apparently overexpressed from the respective loci in SDS-PAGE analysis (Fig. 3B). There are additional benefits of choosing these loci; for example, deletion of *tyrR* is known to increase Phe production since TyrR represses the transcription of some of the shikimate pathway genes (24). On the other hand, *adhE*, *ldhA*, and *pflDC* deletions were expected to reduce ethanol-, lactic acid-, and formic acid formation, respectively.

Strain M-PAR-14 produced 0.7 g/liter Phe from 10 g/liter glucose after 48 h of cultivation (Fig. 4). Overexpression of *ppsA* slightly increased the Phe titer in strain M-PAR-20. Surprisingly, overexpression of *tktA* dramatically decreased the Phe titer in strains M-PAR-22 and M-PAR-24. Moreover, unlike strains M-PAR-14 and M-PAR-20, strains M-PAR-22 and M-PAR-24 did not completely consume the available glucose within 48 h (<90% was consumed). Previous studies demonstrated that *tktA* was necessary to enhance the level of available E4P in cells (15, 18). Thus, we further examined *tktA* overexpression in *aroG^{fbr}*-, *pheA^{fbr}*-, and *ppsA*-overexpressing strains. We postulated that host strain MG1655(DE3) was not suitable for *tktA* overexpression. Thus, other host strains (*E. coli* K-12 and B derivatives)—BW25113(DE3), W3110(DE3), and BL21(DE3)—were tested for *tktA* overexpression along with *aroG^{fbr}*-, *pheA^{fbr}*-, and *ppsA*. Unfortunately, *tktA* overexpression dramatically decreased Phe titer in both BW25113(DE3) and W3110(DE3) as it had in MG1655(DE3); the Phe titer in BL21(DE3)

TABLE 1 Constructed plasmids and generated strains in this study

Plasmid or strain	Related genotype and feature(s) ^a
Constructed plasmids	
pET21a-FRT-aroG ^{fbr}	Carrying feedback-resistant DAHP synthase (D146N) gene from <i>E. coli</i>
pET21a-FRT-pheA ^{fbr}	Carrying feedback-resistant fused chorismate mutase P and prephenate dehydratase (S330P) gene from <i>E. coli</i>
pET21a-FRT-tyrA ^{fbr}	Carrying feedback-resistant fused chorismate mutase T and prephenate dehydrogenase (M53I and A354V) gene from <i>E. coli</i>
pET21a-FRT-tktA	Carrying transketolase 1 gene from <i>E. coli</i>
pET21a-FRT(-8TC)-tktA	Carrying transketolase 1 gene under weak T7 promoter variant
pET21a-FRT-ppsA	Carrying phosphoenolpyruvate synthase gene from <i>E. coli</i>
pET21d-FRT-aroA	Carrying 5-enolpyruvylshikimate-3-phosphate synthetase gene from <i>E. coli</i>
pET21a-FRT-aroB	Carrying 3-dehydroquinase synthase gene from <i>E. coli</i>
pET21a-FRT-aroC	Carrying chorismate synthase gene from <i>E. coli</i>
pET21a-FRT-aroD	Carrying 3-dehydroquinase dehydratase gene from <i>E. coli</i>
pET21a-FRT-aroE	Carrying dehydroshikimate reductase gene from <i>E. coli</i>
pET21a-FRT-aroL	Carrying shikimate kinase II gene from <i>E. coli</i>
pET21a-FRT-aroALC	Carrying <i>aroALC</i> artificial operon
pET21a-FRT-aroEDB	Carrying <i>aroEDB</i> artificial operon
pET21a-FRT-aroLC	Carrying <i>aroLC</i> artificial operon
pET21a-FRT-aroAC	Carrying <i>aroAC</i> artificial operon
pET21a-FRT-aroAL	Carrying <i>aroAL</i> artificial operon
pET21a-FRT-aroDB	Carrying <i>aroDB</i> artificial operon
pET21a-FRT-aroEB	Carrying <i>aroEB</i> artificial operon
pET21a-FRT-aroED	Carrying <i>aroED</i> artificial operon
LacZ-reporter assay strains [derived from BW25113(DE3)]	
AR-G161	<i>adhE</i> ::FRT-Km-FRT-P _{T7lac} - <i>lacZ</i>
AR-G162	<i>ldhA</i> ::FRT-Km-FRT-P _{T7lac} - <i>lacZ</i>
AR-G163	<i>pflDC</i> ::FRT-Km-FRT-P _{T7lac} - <i>lacZ</i>
AR-G164	<i>pykA</i> ::FRT-Km-FRT-P _{T7lac} - <i>lacZ</i>
AR-G165	<i>ascF</i> ::FRT-Km-FRT-P _{T7lac} - <i>lacZ</i>
Donor strain for P1 transduction [derived from BW25113(DE3)]	
AR-G60	<i>pflDC</i> ::FRT-Km-FRT
Donor strains for P1 transduction [derived from MG1655(DE3)]	
M-ARG3	<i>tyrR</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroG</i> ^{fbr}
M-ARG4	<i>ldhA</i> ::FRT-Km-FRT-P _{T7lac} - <i>pheA</i> ^{fbr}
M-ARG5	<i>ldhA</i> ::FRT-Km-FRT-P _{T7lac} - <i>tyrA</i> ^{fbr}
M-ARG7	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroA</i>
M-ARG10	<i>adhE</i> ::FRT-Km-FRT-P _{T7lac} - <i>ppsA</i>
M-ARG11	<i>pflBA</i> ::FRT-Km-FRT-P _{T7lac} - <i>tktA</i>
M-ARG14	<i>pflDC</i> ::FRT-Km-FRT-P _{T7lac} - <i>tktA</i>
M-ARG36	<i>pflDC</i> ::FRT-Km-FRT-P _{T7lac(-8TC)} - <i>tktA</i>
M-ARG43	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroALC</i>
M-ARG45	<i>ascF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroEDB</i>
M-ARG72	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroAL</i>
M-ARG73	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroAC</i>
M-ARG74	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroLC</i>
M-ARG75	<i>ascF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroED</i>
M-ARG76	<i>ascF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroEB</i>
M-ARG77	<i>ascF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroDB</i>
M-ARG79	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroC</i>
M-ARG80	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroD</i>
M-ARG81	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroE</i>
M-ARG82	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroL</i>
M-ARG83	<i>pykF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroB</i>
M-ARG84	<i>pykF</i> ::FRT-Km-FRT
M-ARG85	<i>ascF</i> ::FRT-Km-FRT
M-ARG99	<i>ascF</i> ::FRT-Km-FRT-P _{T7lac} - <i>aroB</i>
M-ARG103	<i>pykA</i> ::FRT-Km-FRT-P _{T7lac} - <i>tyrB</i>
M-ARG104	<i>pykA</i> ::FRT-Km-FRT

(Continued on next page)

TABLE 1 (Continued)

Plasmid or strain	Related genotype and feature(s) ^a
M-ARG105	<i>tyrB::FRT-Km-FRT</i>
M-ARG109	<i>ldhA-feaB::FRT-Km-FRT-P_{T7lac}-tyrA^{fb}</i>
M-ARG110	<i>mtlA::FRT-Km-FRT-P_{T7lac}-ppd(ab)</i>
M-ARG111	<i>pykA::FRT-Km-FRT-P_{T7lac}-yahK</i>
Phenylalanine-producing strains [derived from MG1655(DE3)]	
M-PAR-13	<i>tyrR::P_{T7lac}-aroG^{fb}</i>
M-PAR-14	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb}</i>
M-PAR-20	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA</i>
M-PAR-22	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} pflBA::P_{T7lac}-tktA</i>
M-PAR-24	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}-tktA</i>
M-PAR-66	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA</i>
M-PAR-88	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroA</i>
M-PAR-104	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} ascF::P_{T7lac}-aroEDB</i>
M-PAR-106	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}-tktA ascF::P_{T7lac}-aroEDB</i>
M-PAR-108	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA ascF::P_{T7lac}-aroEDB</i>
M-PAR-110	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} pykF::P_{T7lac}-aroALC</i>
M-PAR-114	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC</i>
M-PAR-116	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB</i>
M-PAR-118	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB</i>
M-PAR-120	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB</i>
M-PAR-140	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB</i>
M-PAR-141	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroAC ascF::P_{T7lac}-aroEDB</i>
M-PAR-142	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB</i>
M-PAR-143	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroED</i>
M-PAR-144	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEB</i>
M-PAR-145	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroDB</i>
M-PAR-147	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroC</i>
M-PAR-148	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroD</i>
M-PAR-149	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroE</i>
M-PAR-150	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroL</i>
M-PAR-151	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroB</i>
M-PAR-152	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA ΔpykF</i>
M-PAR-153	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA ΔascF</i>
M-PAR-154	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroB</i>
M-PAR-155	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroAC ascF::P_{T7lac}-aroEB</i>
M-PAR-156	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroAC ascF::P_{T7lac}-aroDB</i>
M-PAR-157	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroAC ascF::P_{T7lac}-aroB</i>
M-PAR-244	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB pykA::P_{T7lac}-tyrB</i>
M-PAR-245	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB ΔpykA</i>
M-PAR-246	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB ΔtyrB</i>
M-PAR-252	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-pheA^{fb} adhE::P_{T7lac}-ppsA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB ΔpflDC</i>
Tyrosine-producing strain [derived from MG1655(DE3)]	
M-PAR-121	<i>tyrR::P_{T7lac}-aroG^{fb} ldhA::P_{T7lac}-tyrA^{fb} adhE::P_{T7lac}-ppsA pflDC::P_{T7lac}(-8TC)-tktA pykF::P_{T7lac}-aroALC ascF::P_{T7lac}-aroEDB</i>

(Continued on next page)

TABLE 1 (Continued)

Plasmid or strain	Related genotype and feature(s) ^a
Aromatic compound-producing strains [derived from MG1655(DE3)]	
M-PAR-239	<i>tyrR</i> ::P _{T7lac} - <i>aroG</i> ^{fb} <i>ldhA</i> ::P _{T7lac} - <i>pheA</i> ^{fb} <i>adhE</i> ::P _{T7lac} - <i>ppsA</i> <i>pflDC</i> ::P _{T7lac} (-8TC)- <i>tktA</i> <i>pykF</i> ::P _{T7lac} - <i>aroALC</i> <i>ascF</i> ::P _{T7lac} - <i>aroEDB</i> <i>acs</i> ::P _{T7lac} - <i>ldhA</i> (re)
M-PAR-240	<i>tyrR</i> ::P _{T7lac} - <i>aroG</i> ^{fb} <i>ldhA</i> ::P _{T7lac} - <i>tyrA</i> ^{fb} <i>adhE</i> ::P _{T7lac} - <i>ppsA</i> <i>pflDC</i> ::P _{T7lac} (-8TC)- <i>tktA</i> <i>pykF</i> ::P _{T7lac} - <i>aroALC</i> <i>ascF</i> ::P _{T7lac} - <i>aroEDB</i> <i>acs</i> ::P _{T7lac} - <i>ldhA</i> (re)
M-PAR-241	<i>tyrR</i> ::P _{T7lac} - <i>aroG</i> ^{fb} <i>ldhA</i> ::P _{T7lac} - <i>tyrA</i> ^{fb} <i>adhE</i> ::P _{T7lac} - <i>ppsA</i> <i>pflDC</i> ::P _{T7lac} (-8TC)- <i>tktA</i> <i>pykF</i> ::P _{T7lac} - <i>aroALC</i> <i>ascF</i> ::P _{T7lac} - <i>aroEDB</i> <i>acs</i> ::P _{T7lac} - <i>tdc</i> (lb)
M-PAR-242	<i>tyrR</i> ::P _{T7lac} - <i>aroG</i> ^{fb} <i>ldhA</i> - <i>feaB</i> ::P _{T7lac} - <i>tyrA</i> ^{fb} <i>adhE</i> ::P _{T7lac} - <i>ppsA</i> <i>pflDC</i> ::P _{T7lac} (-8TC)- <i>tktA</i> <i>pykF</i> ::P _{T7lac} - <i>aroALC</i> <i>ascF</i> ::P _{T7lac} - <i>aroEDB</i> <i>pykA</i> ::P _{T7lac} - <i>yahK</i> <i>mtlA</i> ::P _{T7lac} - <i>ppd</i> (ab) Δ <i>pheA</i>

^a(ab) indicates an *Azospirillum brasilense* gene, (lb) indicates a *Lactobacillus brevis* gene, and (re) indicates a *Cupriavidus necator*/*Ralstonia eutropha* gene.

was decreased by *ppsA* overexpression even without expression of *tktA* (see Fig. S1 in the supplemental material). However, when the *tktA* expression level was controlled by P_{T7lac}(-8TC), which is a variant promoter with less than 10% of the activity of P_{T7lac}, the Phe titer and yield were slightly improved in strain M-PAR-66 compared to those in strain M-PAR-24 (in which *tktA* expression was controlled by P_{T7lac}) (Fig. 4). Overexpression of *tktA* also drastically affected the growth rate (μ) of the strains. For instance, the μ value of strain M-PAR-14 in M9M2 medium (37°C) was 0.26 h⁻¹, whereas those of strains M-PAR-22 and M-PAR-24 were less than 0.15 h⁻¹. The values returned to 0.23 h⁻¹ in strain M-PAR-66. Therefore, we concluded that *tktA* overexpression caused a serious metabolic burden on cells and thus decreased Phe titer and yield.

We hypothesized that decrease of Phe titer and yield was caused by metabolic overflow into the shikimate pathway. This problem could be circumvented by traditional metabolic engineering approaches, increasing the overexpression of bottleneck genes. Thus, each shikimate pathway gene (*aroA*, *aroB*, *aroC*, *aroD*, *aroE*, and *aroL*) was independently integrated under T7 control into the chromosome of *E. coli* overexpressing *aroG*^{fb}, *pheA*^{fb}, *ppsA*, and *tktA*; here, P_{T7lac}(-8TC) was used to control *tktA* overexpression. Although *aroL* has a paralogous gene, *aroK*, *aroL* was selected because of the lower *K_m* value of AroL for shikimic acid. Similarly, although shikimate dehydrogenase is encoded by both *aroE* and *ydiB*, *aroE* was selected since YdiB catalyzes an undesirable reaction (the reversible conversion of dehydroquinone to quinone) (40). Since AroB is hard to overexpress in an active form, the N-terminal sequence of AroB was modified as previously described (40). Among the shikimate pathway genes, integration of *aroB* (M-PAR-150) slightly increased the Phe titer and yield compared to the parental strain M-PAR-66; however, the values were considerably lower than in the non-*tktA*-overexpressing strains M-PAR-14 and M-PAR-20. We concluded that the Phe titer and yield could not be improved by integration of single shikimate pathway genes in a *aroG*^{fb}-, *pheA*^{fb}-, *ppsA*- and *tktA*-overexpressing strain.

Construction and integration of artificial operons encoding shikimate pathway genes. Strains M-PAR-14 and M-PAR-66 showed slow specific growth rates (0.23 to 0.26 h⁻¹) in mineral salts medium (M9M2) compared to that of the parental strain MG1655(DE3) (0.40 h⁻¹). We assumed that the low specific growth rates resulted from the metabolic burden. Although the host strain possessed the complete shikimate pathway driven by the endogenous promoters, overexpression of particular genes, such as *aroG*^{fb} and *pheA*^{fb} in M-PAR-14 and *aroG*^{fb}, *pheA*^{fb}, *ppsA*, and *tktA* in M-PAR-66, might perturb the normal metabolic flux in Phe synthesis. Therefore, we tried to integrate genes for the whole shikimate pathway driven by P_{T7lac} into the chromosome. Although the artificial operon should contain *aroA*, *aroB*, *aroC*, *aroD*, *aroE*, and *aroL* genes, such an operon flanked by the flippase recognition target site (FRT) and a kanamycin (Km)-resistant gene (FRT-Km-FRT cassette) seemed too large (about 7,000 bp) to be manipulated by the Red-mediated recombination strategy used in this study. Alternatively, two small artificial operons, *aroEDB* and *aroALC*, were constructed (Fig. 5A). In the *aroEDB* operon, the genes were placed in the reverse order relative to

FIG 2 Scheme showing the generation of Phe-producing strains (A) and Tyr- and aromatic compound-producing strains (B). The strains were generated by P1 transduction, e.g., strain M-PAR-13 was generated from strain MG1665(DE3) by using the P1 lysate obtained from strain M-ARG3; the lysate is described as P1(M-ARG3) in this figure.

the reaction steps in the shikimate pathway. Since the transcriptional levels of genes close to the promoter of the operon are higher than those further away (42), we used the reverse gene order relative to the enzymatic steps of the pathway to reduce the accumulation of intermediates. In the *aroALC* operon, we considered that PEP should be used for Phe synthesis as much as possible. Thus, *aroA* was placed first in the operon for the efficient incorporation of PEP into EPSP. Since the shikimate kinase-catalyzed reaction has been reported to be the rate-limiting step (21, 23), *aroL* was placed in the

FIG 3 Relationship between the chromosomal locus of P_{T7lac} -*lacZ* and the β -galactosidase activity (reporter assay). Strain AR-G65 is a BW25113 derived strain harboring P_{T7lac} -*lacZ* at the *tyrR* locus of the chromosome (41). (A) The β -galactosidase activities of the respective strains are shown as relative values when the value of strain AR-G65 was 100%. The data were obtained from three independent cultures, and error bars indicate standard deviations. (B) SDS-PAGE analysis of strains harboring T7-controlled shikimate pathway genes integrated into the respective loci of the chromosome. Cont., control strain MG1655(DE3). Combinations of integrated genes and loci are indicated as follows: M-ARG10, P_{T7lac} -*ppsA* at the *adhE* locus; M-ARG3, P_{T7lac} -*aroG^{fbr}* at the *tyrR* locus; M-ARG4, P_{T7lac} -*pheA^{fbr}* at the *ldhA* locus; M-ARG14, P_{T7lac} -*tktA* at the *pflDC* locus; M-ARG7, P_{T7lac} -*aroA* at the *pykF* locus; and M-ARG99, P_{T7lac} -*aroB* at the *ascF* locus.

second position in the operon. *aroEDB* and *aroALC* were respectively integrated into the *ascF* and *pykF* loci of the chromosome of strain M-PAR-14, which was an *aroG^{fbr}*- and *pheA^{fbr}*-overexpressing strain. The resultant strains M-PAR-104 (including *aroEDB*) and M-PAR-110 (including *aroALC*) produced more Phe than M-PAR-14 (20 and 43% increases in the Phe titer, respectively) (Fig. 6). Deletion of *pykF* (pyruvate kinase I) or *ascF* (β -glucoside-specific PTS enzyme IIBC component) from the M-PAR-14 chromosome did not significantly affect the Phe titer (<5% increase; data not shown). Strain M-PAR-116, expressing both *aroEDB* and *aroALC*, along with *aroG^{fbr}* and *pheA^{fbr}*, produced 1.54 g/liter Phe (0.15 ± 0.00 g/g glucose), which corresponded to a 120% increase of Phe titer compared to strain M-PAR-14. Subsequently, both *aroEDB* and *aroALC* operons were integrated into the *aroG^{fbr}*-, *pheA^{fbr}*-, *ppsA*-, and *tktA*-overexpressing strains M-PAR-24 (P_{T7lac} -*tktA*) and M-PAR-66 [P_{T7lac} -8TC-*tktA*]. The resultant strains M-PAR-118 and M-PAR-120 produced 1.89 and 2.01 g/liter Phe (0.19 ± 0.01 and 0.20 ± 0.00 g/g glucose), which correspond to 170 and 190% increases, respectively, in the Phe titer relative to M-PAR14. The specific growth rate of M-PAR-120 in mineral salts medium (0.43 h^{-1}) was recovered and was almost the same as that of the parental strain MG1655(DE3). In conclusion, integration of both *aroEDB* and *aroALC* operons was a way to prevent a metabolic burden and to generate a superior Phe-producing strain. The design of strain M-PAR-120 is illustrated in Fig. 5B.

By the integration of both P_{T7lac} -*aroEDB* and P_{T7lac} -*aroALC* operons, along with P_{T7lac} -*aroG^{fbr}* and P_{T7lac} -*pheA^{fbr}*, the pathway genes from PEP and E4P to PP were seamlessly overexpressed (Fig. 1). For the seamless overexpression of another gene for Phe synthesis, P_{T7lac} -*tyrB* was integrated into the *pykA* locus of strain M-PAR-120 to generate strain M-PAR-244. Deletion of *pykA* from M-PAR-120 did not affect the Phe titer and yield. Overexpression of *tyrB* decreased the Phe titer and yield by 20% (to 1.56 ± 0.15 g/liter and 0.16 ± 0.01 g/g glucose) (Fig. 6). Deletion of *tyrB*, demonstrated in strain M-PAR-246, hardly affected the Phe titer and yield (1.91 ± 0.10 g/liter and

FIG 4 Consumed glucose (Glc), Phe titer, and Phe yield by each generated Phe-producing strain for 48 h of cultivation in M9M2 medium. The Phe yield was calculated as the Phe titer/consumed Glc. The *aroG^{fb}*- and *pheA^{fb}*-overproducing strain (M-PAR-14) was modified by integrating respective genes under the control of P_{T7lac} . “*tktA*” indicates the overexpression of *tktA* under the control of the weak P_{T7} variant $P_{T7lac(-8TC)}$. The data were obtained from three independent cultures, and error bars indicate standard deviations.

0.19 ± 0.01 g/g glucose). These results indicate that overexpression of *tyrB* is not necessary in the generated strain M-PAR-120.

$P_{T7lac(-8TC)}$ -*tktA* was removed from M-PAR-120 chromosome to reduce the effect of *tktA* overexpression. The resultant strain M-PAR-252 decreased Phe titer and yield by 38% from M-PAR-120 (Fig. 6). Overexpression of *tktA* was important for high Phe titer and yield.

Minimum gene set of the artificial operons. Integration of artificial operons *aroALC* and *aroEDB* converted *aroG^{fb}*-, *pheA^{fb}*-, *ppsA*-, and *tktA*-overexpressing strain M-PAR-66 to the high-yield Phe-producing strain M-PAR-120. However, it was unclear which gene(s) was required for high titer and yield of Phe. To evaluate the contribution of each shikimate pathway gene, we designed six new artificial operons, each consisting of two shikimate pathway genes—*aroAL*, *aroAC*, *aroLC*, *aroED*, *aroEB*, and *aroDB* operons, driven by P_{T7lac} —and then substituted these for the chromosomal operons (*aroALC* or *aroEDB*, as appropriate) of strain M-PAR-120.

Replacement of *aroALC* by *aroAL* (M-PAR-140) and *aroLC* (M-PAR-142) critically decreased Phe yield (Fig. 7). Replacement of *aroEDB* by *aroED* (M-PAR-143) also decreased the Phe yield. These results indicated that *aroA*, *aroB*, and *aroC* are key genes in the *aroALC* and *aroEDB* operons, whereas *aroL* (M-PAR-141), *aroD* (M-PAR-144), and *aroE* (M-PAR-145) did not appear to be important.

To determine the minimum essential gene set from the *aroALC* and *aroEDB* operons, *aroL*, *aroD*, and *aroE* were deleted from the operons in strain M-PAR-120. The resultant strain, M-PAR-157, generated by integration of the *aroAC* operon and the *aroB* gene instead of *aroALC* and *aroEDB*, produced Phe as same as M-PAR-120 (0.2 g/g glucose).

FIG 5 Structures of constructed artificial operons *aroEDB* and *aroALC* (A) and design of the constructed Phe-producing strain M-PAR-120 (B).

We concluded that *aroA*, *aroB*, and *aroC* were the essential genes for high-level Phe production in the *aroALC* and *aroEDB* operons of strain M-PAR-120.

Confirmation of pathway gene overexpression by real-time PCR. To confirm the overexpression of the integrated pathway genes, amounts of the respective mRNAs produced during the cultivation of strain M-PAR-120 (*aroA*, *aroB*, *aroC*, *aroD*, *aroE*, *aroG^{fbr}*, *aroL*, *pheA^{fbr}*, *ppsA*, and *tktA* overexpressing) and M-PAR-157 (*aroA*, *aroB*, *aroC*, *aroG^{fbr}*, *pheA^{fbr}*, *ppsA*, and *tktA* overexpressing) were measured by real-time PCR (Table 2). The transcriptional levels of other pathway genes—*aroF*, *aroK*, *ydiB*, and *tyrB*—were also investigated. The data were standardized by threshold cycle (C_T) values of the respective pathway genes obtained from strain MG1655(DE3). All integrated pathway genes were overexpressed at the transcriptional level in M-PAR-120, whereas the transcription levels of *aroK*, *tyrB*, and the T7 RNA polymerase (T7RP) gene were the same as those in MG1655(DE3). Similarly, the seven integrated pathway genes were overexpressed in M-PAR-157. Even though T7-controlled *aroD* and *aroL* genes were not integrated into the M-PAR-157 chromosome, the transcriptional levels of these genes were 10- and 6-fold higher than in strain MG1655(DE3), respectively. Oddly, the transcriptional level of *ydiB*, encoding an isozyme of AroE, dramatically increased in both strains M-PAR-120 and M-PAR-157. Hence, whole pathway genes, except *tyrB*, were seamlessly overexpressed from DAHP to PP in both M-PAR-120 and M-PAR-157.

Kinetic parameters of strain M-PAR-120. Since strains M-PAR-120 and M-PAR-157 produced Phe with good yields, both strains were candidates for further investigation. Although strain M-PAR-157 was genetically more simple than M-PAR-120, we chose strain M-PAR-120, expecting that the constructed pathway in the strain would be more robust. That is, the transcriptional level of some pathway genes such as *aroL* was not high in M-PAR-157 (Table 2). If cultivation conditions (medium, temperature, pH, etc.) are altered, the constructed seamless pathway may become a rugged pathway in strain M-PAR-157. Hence, we decided to use strain M-PAR-120 as a Phe-producing strain thereafter. Time course experiments were performed to determine the specific productivity of Phe by strain M-PAR-120. We used two media: M9M2 minimum salts medium and M9M2-0.5Y medium, which consisted of M9M2 medium plus 0.5% (wt/vol) dried yeast extract. The Phe yield was expected to be higher in M9M2-0.5Y medium than in M9M2 medium because cell growth would be supported by the dried yeast extract. First, we optimized the cultivation conditions, including the IPTG (isopropyl- β -D-

FIG 6 Integration of artificial operons *aroALC* and/or *aroEDB* into the *aroG^{fbr}*- and *pheA^{fbr}*-overexpressing strain. The values for consumed glucose (Glc) and Phe titer by each generated strain were measured in M9M2 medium (48 h cultivation), and then the Phe yield was calculated as the Phe titer/consumed glucose. *tktA* was overexpressed under the control of P_{T7lac} (*tktA*) or $P_{T7lac(-8TC)}$ (*tktA**), and the other genes were overexpressed under the control of P_{T7lac} . Δ *tyrB* indicates *tyrB* deletion, and *tyrB* indicates *tyrB* overexpression. The data were obtained from three independent cultures, and error bars indicate standard deviations.

thiogalactopyranoside) concentration, the timing of IPTG addition (in terms of culture optical density), and the cultivation temperature (Fig. S2).

Time course experiments revealed that glucose was almost completely consumed by strain M-PAR-120 within 24 h in both media (Fig. 8A and B). M-PAR-120 grew quickly in M9M2-0.5Y medium ($\mu = 1.44 \text{ h}^{-1}$, before IPTG addition), in comparison to cultiva-

FIG 7 Integration of various artificial operons, including *aroA*, *aroB*, *aroC*, *aroD*, *aroE*, and/or *aroL*. The Phe yield was calculated as the Phe titer/consumed glucose. The respective artificial operons were controlled by P_{T7lac} . All strains overexpressed *aroG^{fbr}*, *pheA^{fbr}*, and *ppsA* under P_{T7lac} and *tktA* under $P_{T7lac(-8TC)}$. The data were obtained from three independent cultures, and error bars indicate standard deviations.

TABLE 2 Relative transcription levels of pathway genes^a

Gene	Avg relative transcription level $\pm$ SD	
	M-PAR-120	M-PAR-157
<i>aroA</i>	57 $\pm$ 10	49 $\pm$ 7
<i>aroB</i>	81 $\pm$ 5	506 $\pm$ 83
<i>aroC</i>	37 $\pm$ 5	31 $\pm$ 2
<i>aroD</i>	1,015 $\pm$ 22	10 $\pm$ 1
<i>aroE</i>	473 $\pm$ 14	1 $\pm$ 0
<i>ydiB</i>	310 $\pm$ 3	328 $\pm$ 24
<i>aroF</i>	46 $\pm$ 9	41 $\pm$ 5
<i>aroG</i>	181 $\pm$ 16	153 $\pm$ 3
<i>aroK</i>	1 $\pm$ 0	1 $\pm$ 0
<i>aroL</i>	256 $\pm$ 12	6 $\pm$ 0
<i>pheA</i>	574 $\pm$ 52	399 $\pm$ 47
<i>tyrA</i>	31 $\pm$ 9	29 $\pm$ 6
<i>tyrB</i>	1 $\pm$ 0	1 $\pm$ 0
<i>ppsA</i>	183 $\pm$ 0	127 $\pm$ 5
<i>tktA</i>	6 $\pm$ 1	4 $\pm$ 0
T7RP	1 $\pm$ 0	1 $\pm$ 0

^aThe transcription level of each gene was standardized by those obtained from MG1655(DE3). Boldfaced values were derived from overexpressing genes. The data are averages obtained from three independent cultures.

tion in M9M2 medium ($\mu = 0.42 \text{ h}^{-1}$). The final Phe titers in M9M2 and M9M2-0.5Y media were 2.3 g/liter (yield 0.23 g/g glucose) and 3.1 g/liter (yield 0.31 g/g glucose), respectively. Surprisingly, however, specific Phe productivity in M9M2-0.5Y medium (maximum of $72 \pm 7 \text{ mg g DCW}^{-1} \text{ h}^{-1}$ and average of $63 \pm 10 \text{ mg g DCW}^{-1} \text{ h}^{-1}$) was lower than that in M9M2 medium (maximum of $114 \pm 3 \text{ mg g DCW}^{-1} \text{ h}^{-1}$ and average of $90 \pm 23 \text{ mg g DCW}^{-1} \text{ h}^{-1}$), although the titer in M9M2-0.5Y medium was considerably higher than that in M9M2 medium (Fig. 8C). The specific glucose consumption rate

FIG 8 Time course analyses of M-PAR-120 cultivation in M9M2 medium (A) and M9M2-0.5Y medium (B). Circles, triangles, and squares indicate the OD₆₆₀, glucose concentrations, and Phe titers, respectively. The arrows indicate the timing of IPTG addition. Specific Phe productivity (C) and glucose consumption rate (D) over 8 to 24 h of cultivation. White and black columns indicate M9M2 and M9M2-0.5Y media, respectively. Cultivation was performed in baffled flasks under optimized conditions. The data were obtained from three independent cultures, and error bars indicate standard deviations.

TABLE 3 Comparison of recent Phe- and Tyr-producing *E. coli* strains

Strain	Target	Glucose ^b (g/liter)	Time ^c (h)	Titer (g/liter)	Yield (mol/mol glucose)	Specific productivity (μ mol/g DCW/h)	Culture condition, etc. ^d	Source or reference
Plasmid-based strains								
PB12-ev2	Phe	1	10	<0.5	0.36	248	M, Res	8
AJ12714/pHYGG	Phe	40	84	6.4	0.17	ND	M	49
WSH-Z06	Phe	35	48	6.72	0.25	ND	N, Tyr, Cit	10
VH33A	Phe	5	12	ND	0.25	ND	M, Res	17
PAPV	Phe	20	40	6.23	0.34	ND	N, Org	12
T2	Tyr	5	20	0.621	0.12	ND	M	11
T2-YK	Tyr	5	16	0.7	0.14	ND	M	21
VH33 Δ tyrR_TIR	Tyr	10	ND	0.15	0.36	359	M	57
F	Tyr	5	48	2.169	0.43	304	M	40
rpoA14 ^R	Tyr	5	48	0.902	0.18	ND	M, Phe	51
CTF06	Tyr	20	96	1.62	0.17	ND	M, Phe, Trp Pyr	35
Plasmid-free strains								
NST74	Phe	2	42	0.044	0.02	ND	M	14
AJ12714	Phe	40	84	3.7	0.10	ND	M	49
YHP05	Phe	20	48	0.52	0.03	ND	M, Tyr, Trp	27
WF123456	Phe	10	28	0.8	0.09	ND	M, Tyr, Trp	18
M-PAR-120 ^a	Phe	10 (10)	28 (24)	2.26 (3.13)	0.25 (0.34)	544 (400)	M (N)	This study
DPD4195	Tyr	2	42	0.147	0.07	ND	M, Phe, Trp	14
DPD4193	Tyr	2	42	0.18	0.09	ND	M, Phe	14
SCK5	Tyr	40	60	3.0	0.10	193	N, Cit	50
BAK11	Tyr	5	24	0.402	0.08	ND	N, Phe	58
M-PAR-121	Tyr	10	28	2.2	0.22	ND	M	This study

^aValues in parentheses were obtained from the strain cultivated in M9M2-0.5Y medium. ND, not determined.

^bInitial glucose concentration.

^cCultivation time after which the yields and titers were determined.

^dAbbreviations: M, minimum medium; N, nutrient medium (minimum medium + dried yeast extract); Cit, addition of citrate; Pyr, addition of pyruvate; Org, addition of several organic compounds; Phe, Phe auxotroph; Tyr, Tyr auxotroph; Trp, Trp auxotroph; Res, resting cell reaction.

in M9M2-0.5Y medium (maximum of 174 ± 10 mg g DCW⁻¹ h⁻¹ and average of 161 ± 21 mg g DCW⁻¹ h⁻¹) was also lower than that in M9M2 medium (maximum of 435 ± 30 mg g DCW⁻¹ h⁻¹ and average of 346 ± 125 mg g DCW⁻¹ h⁻¹). The lower specific glucose consumption rate seemed to contribute to the higher Phe yield in M9M2-0.5Y. In comparison to the plasmid-free strains described in previous studies, strain M-PAR-120 demonstrated the best yield and specific productivity in batch cultivation for Phe production (Table 3).

Application to Tyr- and aromatic compound-producing strains. Phe-producing strain M-PAR-120, harboring P_{T7lac} -*aroG^{fbr}*, P_{T7lac} -*pheA^{fbr}*, P_{T7lac} -*ppsA*, $P_{T7lac(-8TC)}$ -*tktA*, P_{T7lac} -*aroALC*, and P_{T7lac} -*aroEDB* on the chromosome, was switched to be a Tyr-producing strain by replacement of P_{T7lac} -*pheA^{fbr}* by P_{T7lac} -*tyrA^{fbr}*. The resultant strain M-PAR-121 grew well in M9M2 medium and produced Tyr with a good titer (2.2 g/liter) and yield (0.22 g/g glucose). The yield was >2-fold higher than those of the plasmid-free Tyr-producing strains that had been generated previously (Table 3).

Yields of aromatic compounds synthesized via Phe/Tyr synthesis pathways should depend on yields of Phe and Tyr. That is, superior Phe- and Tyr-producing strains have the potential to be valuable host strains for generation of various aromatic compound-producing strains. To confirm this hypothesis, the yield of phenyllactic acid was compared in phenyllactic acid-producing strains derived from two Phe-producing strains, AR-G91 and M-PAR-120, with different Phe yields. Strain AR-G91 is a derivative of strain BW25113, which was derived from *E. coli* MG1655 (41). Strain AR-G91 harbors T7-controlled *aroG^{fbr}* and *pheA^{fbr}* on the chromosome and lacks *tyrR*, whereas strain M-PAR-120 harbors T7-controlled *aroG^{fbr}*, *pheA^{fbr}*, *tktA*, *ppsA*, *aroALC*, and *aroEDB* on the chromosome and lacks *tyrR*, *adhE*, *ldhA*, *pflDC*, *pykF*, and *ascF*. Generation of a phenyllactic acid-producing strain derived from AR-G91 has been reported previously by integrating the T7-controlled lactate dehydrogenase gene from *Cupriavidus necator* into the *acs* locus on the chromosome of strain AR-G91 (36). Phenyllactic acid-producing strain M-PAR-239 was generated from M-PAR-120 in the same manner

FIG 9 Synthetic routes for aromatic compounds. The genes used for modification of M-PAR-120 were *IdhA(re)* from *Cupriavidus necator* (synonym, *Ralstonia eutropha*), *tdc(lb)* from *Lactobacillus brevis*, *ppd(ab)* from *Azospirillum brasilense*, and *yahK* from *Escherichia coli*.

(Table 1 and Fig. 2). Similarly, 4-hydroxyphenyllactic acid-, tyramine-, and tyrosol-producing strains were generated from two Tyr-producing strains, AR-G2 and M-PAR-121, with different Tyr yields, and the yields of the respective aromatic compounds were compared. The synthesis routes of the respective aromatic compounds and aromatic amino acids are illustrated in Fig. 9.

The Phe yield from strain M-PAR-120 (0.30 g/g glucose) was 2.1-fold that from AR-G91 in M9M2-0.5Y medium (0.14 g/g glucose) (Table 4). Similarly, yields of target aromatic compounds 4-hydroxyphenyllactic acid and tyrosol, and of Tyr, were also enhanced ≥ 2.0 -fold. Due to Phe accumulation, enhancement of phenyllactic acid yield did not reach 2.0-fold. Tyr accumulation was observed in tyramine production by the tyramine-producing strain derived from M-PAR-120. In both phenyllactic acid- and tyramine-producing strains derived from strain M-PAR-120, the yield of phenyllactic acid plus Phe or of tyramine plus Tyr was ≥ 2.0 -fold that obtained from the strains

TABLE 4 Relationship between yields of target aromatic compounds and Phe/Tyr

Target compound	Avg yield (g/g glucose) $\pm$ SD ^a		Enhancement (fold) ^b
	AR-G91 or its derivatives	M-PAR-120 or its derivatives	
Phe	0.14 $\pm$ 0.01	0.30 $\pm$ 0.00	2.1
Tyr	0.10 $\pm$ 0.01	0.26 $\pm$ 0.01	2.6
Phenyllactic acid	0.10 $\pm$ 0.01 (0.11 $\pm$ 0.02)	0.17 $\pm$ 0.00 (0.22 $\pm$ 0.00)	1.7 (2.0)
4-Hydroxyphenyllactic acid	0.11 $\pm$ 0.00	0.28 $\pm$ 0.00	2.5
Tyramine	0.07 $\pm$ 0.00 (0.07 $\pm$ 0.00)	0.11 $\pm$ 0.04 (0.19 $\pm$ 0.01)	1.6 (1.9)
Tyrosol	0.14 $\pm$ 0.00	0.28 $\pm$ 0.01	2.0

^aStrains were cultivated in M9M2-0.5Y medium. The data are averages obtained from three independent cultures. The values in the parentheses are the total yields of the target aromatic compounds and Phe/Tyr.

^bThe Phe yield obtained from M-PAR-120 or its derivative was divided by that obtained from AR-G91 or its derivative.

derived from strain AR-G91. These results indicate that yields of target aromatic compounds were enhanced almost proportionally to the increase of the Phe or Tyr yield.

DISCUSSION

Comparison with other Phe and Tyr producers. We modified the *E. coli* chromosome and achieved the generation of plasmid-free strains for Phe and Tyr production. Many Phe- and Tyr-producing strains have been generated by genetic manipulation with plasmids, but several strains have also been generated without plasmids. Information on recent Phe- and Tyr-producing strains is summarized in Table 3. Previous data showed that Phe and Tyr yield were higher in plasmid-based strains (0.12 to 0.43 mol/mol glucose) than in plasmid-free strains (0.02 to 0.10 mol/mol glucose). The yield tended to be high in resting cell reactions, shown in strains VH33A and PB12-ev2, because of the absence of carbon and energy flow for cell growth. The yield also tended to be high when dried yeast extract was added into media, for example, from strains WSH-Z06 and PAPV; in such cases, glucose might not be the main source for amino acid and nucleic acid synthesis. Indeed, in this study, the yield was increased from 0.25 to 0.34 mol/mol glucose in strain M-PAR-120 when dried yeast extract was added to minimal medium. Comparing data obtained from minimal media (lacking yeast extract and other organic compounds except amino acids), the average Phe or Tyr yield was 0.23 mol/mol glucose in the plasmid-based strains but only 0.07 mol/mol glucose in plasmid-free strains. The data suggested that it would be difficult to generate a superior Phe- and Tyr-producing strain by chromosome engineering alone. However, the plasmid-free strain M-PAR-120 generated in this study showed a higher Phe yield (0.25 to 0.34 mol/mol glucose) than did previous strains; this value was comparable to that from plasmid-based strains. Among plasmid-based strains, strains PAPV (0.34 mol/mol glucose) and PB12-ev2 (0.36 mol/mol glucose) showed good Phe yields; the values were far higher than the Phe yield from M-PAR-120 cultivated in M9M2 medium (0.25 mol/mol glucose). However, when dried yeast extract was added to the medium as with strain PAPV, M-PAR-120 produced Phe in yields similar to those achieved with strain PAPV (0.34 mol/mol glucose). Compared to strain PB12-ev2, M-PAR-120 demonstrated a higher specific productivity of Phe. The specific productivity of Phe by PB12-ev2 was 248 $\mu\text{mol/g DCW/h}$, whereas it was 544 $\mu\text{mol/g DCW/h}$ (maximum of 690 $\mu\text{mol/g DCW/h}$) in strain M-PAR-120. That is, strain M-PAR-120 demonstrated both a high yield and a high specific productivity of Phe. To the best of our knowledge, strain M-PAR-120 has the highest yield and the highest specific productivity of Phe among plasmid-free strains generated so far.

Promoters for chromosome engineering. The introduction of modulated pathway genes controlled under promoters with various strengths is a powerful tool in the generation of plasmid-based strains. Actually, the generation of a high-performance plasmid-based strain for tyrosine production (strain F1 in Table 3) was achieved by introducing two plasmids encoding the four modulated pathway genes controlled by three promoters (40). However, such a module technique conducted in a plasmid-based strain does not always work well in a plasmid-free strain. In fact, the tyrosine yield was not high when modules from strain F1 were introduced into the *E. coli* chromosome (43). Since the low yield was caused by low expression levels of the modulated pathway genes, all promoters in the modules were altered to P_{T7lac} ; the resulting strain increased the Tyr yield up to 5-fold. Based on this finding, we used P_{T7lac} as the strongest promoter that worked in *E. coli*.

Chromosomal loci of T7-controlled pathway genes. Since the expression level of integrated genes depends on the chromosomal locus, the choice of the locus is an important factor for generating superior Phe-producing strains. The chromosomal locus is thought to alter gene expression in two ways. First, gene expression decreases with the distance of a gene from the origin of replication (*oriC*) (44). Second, local sequences at some positions have a strong effect on the integrated genes, and the integrated gene is completely silenced at several locations (45). According to these studies, we

thought that the pathway genes should be integrated into well-known loci around *oriC*. However, the strategy was difficult to apply in this study due to the technical limitations relevant to Km marker recycling using flippase (FLP)/FRT recombination. That is, if all pathway genes are integrated into the loci around *oriC*, the FRT sequences become dense. Since FRT sequences are targeted by FLP, undesirable deletions of chromosomal regions might occur. Alternatively, we chose several loci as candidates for gene integration, with the following expectations: (i) enhancement of Phe productivity (*tyrR* and *pykF*), (ii) depression of by-product accumulation (*ldhA*, *adhE*, and *pflDC*), and (iii) no effect on Phe productivity (*ascF*). Next, referring to the data set of KEIO collection (46), we confirmed that deletion of the respective genes did not have an impact on *E. coli* growth. Finally, we observed the locus effect by using a *lacZ*-reporter assay and SDS-PAGE (Fig. 2). As a result, all loci were determined to be suitable for gene overexpression in this study.

Key genes for engineering a Phe-producing strain. Key genes in the Phe and Tyr synthesis pathways have been investigated previously. The enzyme encoded by *aroG* (*aroF*) catalyzes the first committed step, which addresses carbon flow to aromatic amino acid synthesis, while PheA and TyrA determine whether there will be synthesis of Phe and Tyr at the CA node (1, 10, 41). Since these gene products are inhibited by aromatic amino acids, feedback-resistant forms have to be overexpressed to enhance Phe and Tyr production. *tktA* and *ppsA* were also identified as critical genes for supplying the precursor compounds, E4P and PEP, for DAHP synthesis in the shikimate pathway (15, 18). Key genes have also been surveyed in the shikimate pathway, which includes *aroA*, *aroB*, *aroC*, *aroD*, *aroE*, *ydiB*, *aroK*, *aroL*, and *tyrB*. Enhancing the expression levels of candidate genes *aroA* and *aroL* *in vivo*, which were selected after *in vitro* analyses, increased the Phe titer (23). Combinational overexpression of both genes was more effective in increasing the Phe titer than overexpression of the genes individually. Combinational overexpression of *aroK* and *ydiB* resulted in the best Phe yield in an *aroG*-, *pheA*-, *tktA*-, and *ppsA*-overexpressing background (21). The study also demonstrated that a gene that did not contribute to an increased Phe titer by overexpression became a key gene when other shikimate genes were overexpressed in combination. This result suggests that expression levels of multiple genes should be simultaneously adjusted for optimization of the shikimate pathway. Juminaga et al. demonstrated that the Tyr titer was dramatically changed according to control of the expression levels of two central pathway genes and nine shikimate pathway genes by manipulating plasmid copy numbers and promoters (40). However, these data were obtained from plasmid-based strains, and such data could not be found in the literature for plasmid-free strains. In the other words, combinatorial overexpression of the pathway genes was investigated to achieve a high yield of Phe/Tyr production in the plasmid-based strains, whereas such investigations have not been performed enough in the plasmid-free strains. It might be caused by some technical issues for chromosomal engineering, including difficulty in strain generation, low expression levels of target genes (low copy numbers), and taking a long time for strain generation. Actually, the overexpression of *aroL* in an *aroG*^{fbr}-, *pheA*^{fbr}-, *tktA*-, and *ppsA*-overexpressing strain did not contribute to increasing a Phe yield (strain M-PAR-150 in Fig. 4). More importantly, the overexpression of *tktA* dramatically decreased the Phe yield in plasmid-free strains (see M-PAR-22 in Fig. 4), which has not been reported in the plasmid-based strains.

In the present study, we focused on the essential genes in the shikimate pathway to achieve higher a Phe yield from a plasmid-free strain. The individual overexpression of target genes—*aroA*, *aroB*, *aroC*, *aroD*, *aroE*, and *aroL*—did not include novelty, but the combinatorial overexpression of these genes include novelty in the plasmid-free strain. We integrated all of the shikimate pathway genes driven by P_{T7lac}, i.e., P_{T7lac}-*aroA*, P_{T7lac}-*aroB*, P_{T7lac}-*aroC*, P_{T7lac}-*aroD*, P_{T7lac}-*aroE*, and P_{T7lac}-*aroL*, into the chromosome of *aroG*^{fbr}-, *pheA*^{fbr}-, *tktA*-, and *ppsA*-overexpressing *E. coli* and then deleted the genes to identify key genes (to be more precise, the large operons *aroALC* and/or *aroEDB* were replaced by smaller operons such as *aroAC*, *aroEB*, and so on). Combinational deletion

of two or three genes which were determined to be nonessential genes (*aroD*, *aroE*, and *aroL*) revealed that the minimum key gene set for a chromosomally engineered and plasmid-free Phe- (or Tyr-) producing strain consisted of T7-controlled *aroG^{fbr}*, *pheA^{fbr}*, *tktA*, *ppsA*, *aroA*, *aroB*, and *aroC*, along with several gene deletions, including *tyrR*. This combination of gene overexpression has not been reported so far in plasmid-based Phe- and Tyr-producing strains. In a study by Dell and Frost, AroA-, AroB-, AroC- and AroL-catalyzed reactions were identified as rate-limiting steps in the shikimate pathway (47). Although overexpression of *aroL* was not necessary for generating the superior Phe-producing strain M-PAR-157 in the present study, the transcriptional level of *aroL* was increased by *tyrR* deletion (Table 2) (24). The step catalyzed by YdiB, which is an isoform of AroE, was also proposed as a rate-limiting step (21, 22). Transcriptional analysis of strain M-PAR-157 revealed unexpected *ydiB* overexpression in this strain. As a result, the generated strain M-PAR-157 overexpressed the previously reported rate-limiting genes *aroL* and *ydiB*. The generated strain in the present study will now be modified by other metabolic engineering approaches related to global regulators (28), amino acid transport (48), stress response (49), and glucose transport (20, 26) to generate the highest performance of Phe- and Tyr-producing strains. In addition, the expression level of each gene should be tuned to optimize the metabolic flow toward Phe and Tyr (50). This could be performed using a combinatorial metabolic engineering approach, such as global transcription machinery engineering or multiplex automated genomic engineering (51, 52).

Aromatic compound-producing strains. In the present study, the generated Phe- and Tyr-producing strains were applied to generate several strains for production of other aromatic compounds. The yields of the aromatic compounds increased in proportion to the increase in Phe or Tyr yield (Table 4). Tyrosol, which is a valuable chemical for the synthesis of pharmaceuticals, was produced at 2.8 g/liter from 10 g/liter glucose with a yield of 0.28 g/g glucose (0.36 mol/mol glucose). The yield was much higher than that observed in previous studies (53–55). Other aromatic compounds have been successfully produced using engineered *E. coli*, e.g., phenol (35), DOPA (56, 57), salvianic acid A (58), caffeoyl and coniferyl alcohol (59), 4-hydroxymandelic acid (60), and phenylacetic acid and 4-hydroxyphenylacetic acid (36). These compounds require one to three synthetic steps beyond 4-hydroxyphenylpyruvate, Tyr, or Phe. The Phe- and Tyr-producing strains generated here might be valuable as host strains to produce such aromatic compounds with higher titers and yields.

MATERIALS AND METHODS

Bacterial strains. *E. coli* DH5 α was used as the host strain for gene cloning. *E. coli* MG1655 (ME7986) was obtained from the National Institute of Genetics of Japan (National BioResource Project), and its λ DE3 lysogen, MG1655(DE3), was used as the host strain for generating Phe- and Tyr-producing strains.

Media and growth conditions. Strains were cultivated overnight in 5 ml of Luria-Bertani (LB) medium (10 g polypeptone, 5 g dried yeast extract, and 10 g NaCl per liter [pH 7.0]). Preculture (1% vol/vol) was inoculated into 5 ml (test tube) or 100 ml (baffled flask) of M9M2 medium [10 g glucose, 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 4 g NH₄Cl, 246.5 mg MgSO₄·7H₂O, 14.7 mg CaCl₂·2H₂O, 13.9 mg FeSO₄·7H₂O, 3.71 μ g (NH₄)₆Mo₇O₂₄·4H₂O, 24.73 μ g H₃BO₃, 7.14 μ g CoCl₂·6H₂O, 2.39 μ g CuSO₄·5H₂O, 15.83 μ g MnCl₂·4H₂O, 2.88 μ g ZnSO₄·7H₂O, and 10 mg thiamine hydrochloride per liter], and cultivation was started at 37°C with reciprocal shaking at 250 rpm (for test tubes) or with rotary shaking at 110 rpm (for baffled flasks) until an optical density at 660 nm (OD₆₆₀; measured using a UV-160A; Shimadzu Corp.) of 0.4 was attained. Baffled flasks were used for cultivation for time course experiments and transcriptional analysis experiments, and test tubes were used for all other experiments. Gene overexpression was induced by the addition of IPTG, and then cultivation was continued at 27°C. The timing of IPTG addition, the IPTG concentration, and the cultivation temperature after IPTG addition were varied to optimize the cultivation conditions. After optimization, the following parameters were applied: timing of IPTG addition, OD₆₆₀ = 0.4; IPTG concentration, 0.4 mM; and cultivation temperature after IPTG addition, 33°C. For instance, time course experiments, transcriptional analysis experiments, and aromatic compound production (e.g., of phenyllactic acid) were carried out under the optimized conditions.

Plasmid construction. Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA) was used to amplify genes and DNA fragments of interest. The primers used in this study are listed in Table 5. pET21a-FRT, pET21a-FRT(-8TC), and pET21d-FRT were used for gene cloning (36, 61). All plasmids constructed in this study are summarized in Table 1.

To construct pET21a-FRT-*aroG^{fbr}*, two fragments of *aroG^{fbr}* were amplified by PCR from genomic DNA of *E. coli* BW25113 using the primer pairs EcAroG-Nde/EcAroG-D146N-RM and EcAroG-D146N-FM/

TABLE 5 Primers used in this study^a

Primer	Sequence (5'–3')
Gene cloning	
EcAroG-Nde	CCAACCATATGAATTATCAGAACGACGATTTACG
EcAroG-D146N-RM	GGGGTGATCATATTGAGAACTC
EcAroG-D146N-FM	GAGTTTCTCAATATGATCACCCC
EcAroG-R	CAAAGCTTTTACCCGCGACGCGCTTTTAC
AroG-RM-Nde	GCTATTATCCATGTGCGGATCG
AroG-FM-Nde	CGATCCGCACATGGATAATAGC
EcPheA-F	CCAACCATATGACATCGGAAAACCCGTTAC
EcPheA-R	CACTCGAGTCAGGTTGGATCAACAGGCAC
EcTyrA-F	CCAACCATATGGTTGCTGAATTGACCGC
EcTyrA-R	CACTCGAGTTACTGGCGATTGTCATTGCGC
EctktA-Nde	CCAACCATATGTCCTCACGTAAAGAGCTTGCC
EctktA-Xho	CACTCGAGTTACAGCAGTTCTTTTGCTTTC
ppsA-F	CCAACCATATGTCCAACAATGGCTCGTC
ppsA-R	CACTCGAGTTATTTCTTCAGTTCAGCCAGG
EcAroA-F	CAACACCATGGAATCCCTGACGTTACAAC
EcAroA-R	CAAAGCTTTTCAAGGCTGCCTGGCTAATCC
AroB-opt-gib-F	GTTTAACTTTAAGAAGGAGATATACATATGGAGCGTATTGTGCTTACTC
AroB-gib-R	GTGGTGGTGGTGCTCGAGTTACGCTGATTGACAATCGGC
AroC-gib-F	GTTTAACTTTAAGAAGGAGATATACATATGGCTGGAAACACAATTGGAC
AroC-gib-R	GTGGTGGTGGTGCTCGAGTTACGAGCGTGAATATCAGTC
AroD-gib-F	GTTTAACTTTAAGAAGGAGATATACATATGAAAACCGTAACTGTAAAAGATC
AroD-gib-R	GTGGTGGTGGTGCTCGAGTTATGCTGGTGTAAAATAGTTAATAC
AroE-gib-F	GTTTAACTTTAAGAAGGAGATATACATATGAAAACCTATGCTGTTTTTGG
AroE-gib-R	GTGGTGGTGGTGCTCGAGTCACGCGGACAATTCCTCC
AroL-gib-F	GTTTAACTTTAAGAAGGAGATATACATATGACACAACCTCTTTTCTGATC
AroL-gib-R	GTGGTGGTGGTGCTCGAGTCAACAATTGATCGTCTGTGCC
ALCorEDB-F	GAAATTAATACGACTCACTATAGGGGAATTG
ALC-aroA-R	TCAGGCTGCCTGGCTAATC
ALC-aroL-F	GATTAGCCAGGCAGCCTGACTTTAAGAAGGAGATATACATATGACAC
ACL-aroL-R	GGATGGCCTCCTTAGATCCTCAACAATTGATCGTCTGTGC
ALC-aroC-F	GGATCTAAAGGAGGCCATCCATGGCTGGAACACAATTGG
ALCorEDB-R	AGCCAACCTCAGCTTCCTTTC
aroALC-F1	ATGGAATCCCTGACGTTACAAC
aroALC-R1	TTACCAGCGTGGAATATCAGTCTTC
pET21a-ALC-F	CTGATATTCACGCTGGTAACCTCGAGCACCACCACCACC
pET21a-ALC-R	GTAACGTCAGGGATTCCATGGTAATATCTCCTTCTTAAAGTTAAAC
EDB-aroE-R	CATATGTATATCTCCTTCTTAAAGTCACGCGGACAATTCCTCC
EDB-aroD-F	CGTGACTTTAAGAAGGAGATATACATATGAAAACCGTAACTGTAAAAGACC
EDB-aroD-R	GGATGGCCTCCTTTAGATCCTTATGCCTGGTGTAAAATAGTTAATAC
ALC-aroB-F	ATGGAGCGTATTGTCGTTACTCTGGGCGAACGTAGCTACC
EDB-aroB-F2	GGATCTAAAGGAGGCCATCCATGGAGCGTATTGTCGTTACTC
aroEDB-F1	ATGGAAACCTATGCTGTTTTTGG
aroEDB-R1	TTACGCTGATTGACAATCGGC
pET21a-EDB-F	CGATTGTCAATCAGCGTAACTCGAGCACCACCACCACC
pET21a-EDB-R	CAAAAACAGCATAGGTTTCCATATGTATATCTCCTTCTTAAAGTTAAAC
aroLC-DB-F	CTTAAAGTTAAACAAAATTATTCTAGAGG
aroLC-R	AAGGAGATATACATATGACACAACCTC
aroAC-F	CTTAAAGTCAGGCTGCCTGGC
aroAC-R	AAGGAGGCCATCCATGGCTGGAAAC
aroAL-F	TCAACAATTGATCGTCTGTGCC
aroAL-ED-R	CTCGAGCACCACCACCAC
aroDB-R	AAGGAGATATACATATGAAAACCGTAAC
aroEB-F	TCACGCGGACAATTCCTCC
aroEB-R	GGATCTAAAGGAGGCCATCCATG
aroED-F	TTATGCCTGGTGTAAAATAGTTAATACC
Amplification of integration cassette	
Delta-adhE-F	CGAGCAGATGATTTACTAAAAAGTTTAAACATTATCAGGAGAGCATTATGATTCCGGGGATCCGTCGACC
Delta-adhE-FRT-R	CCGTTTATGTTGCCAGACAGCGCTACTGATTAAGCGGATTTTTTCGCTTTATTCGCCAATCCGGATATAG
Delta-ldhA-F	TATTTTTAGTAGCTTAAATGTGATTCAACATCACTGGAGAAAGCTTATGATTCCGGGGATCCGTCGACC
Delta-ldhA-FRT-R	CTCCCTCGGAATGCAGGGGAGCGGCAAGATTAAACAGTTCGTTCCGGGCAATTCGCCAATCCGGATATAG
Delta-pflBA-F	CGAAGTACGCAGTAAATAAAAAATCCACTTAAGAAGGTAGGTGTTACATGATTCCGGGGATCCGTCGACC
Delta-pflBA-FRT-R	CTCAATAAAGTTGCCGCTTTACGGGGAATTAGAACATTACCTTATGACCATTCGCCAATCCGGATATAG
Delta-pflDC-F	CTTCTCCGCTCGCAAGGGCGGGTTTCGCTTTCCACAGGAGTTCCTCATGATTCCGGGGATCCGTCGACC
Delta-pflDC-FRT-R	AGGCGGTTACTGCCACCAGGTATGCCATTTTAACTCCACGGTAACCTGATTCCGCCAATCCGGATATAG

(Continued on next page)

TABLE 5 (Continued)

Primer	Sequence (5'–3')
Delta-pykF-F	GAAAGCAAGTTTCTCCATCCTTCTCAACTTAAAGACTAAGACTGTCATGATTCCGGGGATCCGTCGACC
Delta-pykF-FRT-R	GATATACAAATTAATTACAAAAGCAATATTACAGGACGTGAACAGATGCATTCCGCAATCCGGATATAG
Delta-pykF-R	GATATACAAATTAATTACAAAAGCAATATTACAGGACGTGAACAGATGCTGTAGGCTGGAGCTGCTTCG
Delta-ascF-F	GACTGATAACAACATACATCTACCCTACTGATAACAGGATAAAATCCGATGATTCCGGGGATCCGTCGACC
Delta-ascF-FRT-R	ACTTTCTGGAAATACTGACATTTTCATCCTCAATTAAGACTTACTTCTTTATTCGCCAATCCGGATATAG
Delta-ascF-R	ACTTTCTGGAAATACTGACATTTTCATCCTCAATTAAGACTTACTTCTTTGTAGGCTGGAGCTGCTTCG
Delta-pykA-F	TTTCATGTTCAAGCAACACCTGGTTGTTTCAGTCAACGGAGTATTACATGATTCCGGGGATCCGTCGACC
Delta-pykA-FRT-R	TGGCGTTTTTCGCCGCATCCGGCAACGTACTTACTCTACCGTTAAATACGATTCCGCCAATCCGGATATAG
Delta-pykA-R	TGGCGTTTTTCGCCGCATCCGGCAACGTACTTACTCTACCGTTAAATACGTTAGGCTGGAGCTGCTTCG
Delta-tyrB-F	GTTTATTGTGTTTTAACCACTGCCGTAACCTGGAGAACCATCGCGTGATTCCGGGGATCCGTCGACC
Delta-tyrB-R	GCTGGGTAGTCCAGCCTGCTTCTGCAATTACATCACCAGCAAAACGCTGTAGGCTGGAGCTGCTTCG
Delta-mtlA-F	TCGGGCTTCAGCCTGCGCGACAGCAAACATAAGAGGGGTGTTTTATGATTCCGGGGATCCGTCGACC
Delta-mtlA-FRT-R	CTTCTCCATGTGGAGAGGGTGGGATTGGATTACTTACGACCTGCCAGCAGATTCCGCCAATCCGGATATAG
Delta-tyrR-F	GTGTCATATCATATTAATTGTTCTTTTTCAGGTGAAGGTTCCCATGATTCCGGGGATCCGTCGACC
Delta-tyrR-FRT-R	TGGTGTGCAACCATCAGGCATATTGCGGCTTACTCTTCTGTTCTTCTTCTGATTCCGCCAATCCGGATATAG
Delta-ldhA-feaB-F	AAATATCTGTTTTAACTAATTGGCGTTGCAGTACATGCAACGCCAATTAGATTCCGGGGATCCGTCGACC
Delta-ldhA-feaB-FRT-R	TGCCGTTTTTACTTATGAGCGAACCAAGATTAATACCGTACACACACCGAATTCCGCCAATCCGGATATAG
Check for integration	
k1	CAGTCATAGCCGAATAGCCTC
k2	CGGTGCCCTGAATGAAGTGC
U-adhE	CTGAATCACGTTAGTCCGAAG
D-adhE	TGCAGGCCGTGCCAGTCATCC
U-ldhA	CTCCGTCTGCGGCAATTC
D-ldhA	GTCTGTTTTGCGGTCGC
U-pflBA	CCGCAATGGTCAATGGGGAC
D-pflBA	CGTTATTCTCCAGAGGTTTCATCAGC
U-pflDC	CAGTGGTGACGGCTGTTTGTC
D-pflDC	CGTTATTGAAAGGCTATGACCTGAAG
U-pykF	CAAAATCAGACAAATAACGC
D-pykF	GAGCTGCGTCATCTTTAG
U-ascF	GTAATTCATAAAATAAATGCCGTGG
D-ascF	CGTAGCGGCTGAAAACTCCACC
U-pykA	ACGCATGAGTTGTATGAATTGTAG
D-pykA	GTAAGGGGATATTATTTACCCG
U-tyrB	CAGTGTGGTGAACGGTCG
D-tyrB	CGCTTGCTGTTTTGCCGAG
U-mtlA	GCCAGAAGGGAGTCAGGCTG
D-mtlA	GATCAACGACATCATCACCATGC
U-tyrR	GTTTAATTAATCGCATCGCCACGC
D-tyrR	CGTAAGTTTAACCAACTGGCAACTG
U-ldhA-feaB	CAGCGTTAACTGGTTCGCGGTC
D-ldhA-feaB	GCGCCAACAGATTCTTCG

^aPrimers for amplification of integration cassette were selected by target chromosomal locus. For example, the primer pair delta-pykF-F/delta-pykF-FRT-R was selected when a gene would be integrated into the *pykF* locus of the chromosome (a gene cloned by pET21a-FRT was used as a template DNA). In such cases, gene integration was checked after Red recombination by the primer pair k1/U-pykF (about 1,000 bp) in addition to the primer pair k2/D-pykF (about 1,200 bp plus the length of a gene). For gene deletion, delta-pykF-R was used instead of delta-pykF-FRT-R, along with pKD13 as a template DNA.

EcAroG-R, respectively. These fragments were assembled by overlap extension PCR by using the primer pair EcAroG-Nde/EcAroG-R (first PCR). Next, the internal NdeI site of the amplicon was deleted. Two fragments of *aroG^{fb}* were amplified from the amplicon from the first PCR using the primer pairs EcAroG-Nde/AroG-RM-Nde and AroG-FM-Nde/EcAroG-R, respectively. These amplicons were assembled by overlap extension PCR using the primer pair EcAroG-Nde/EcAroG-R. The obtained amplicon was digested with NdeI and HindIII and then introduced into the corresponding sites of pET21a-FRT to generate pET21a-FRT-aroG^{fb}.

To construct pET21a-FRT-pheA^{fb} and pET21a-FRT-tyrA^{fb}, *pheA^{fb}* and *tyrA^{fb}* were amplified from pARO28 and pARO56 using the primer pairs EcPheA-F/EcPheA-R and EcTyrA-F/EcTyrA-R, respectively, and then each NdeI and XhoI doubly digested amplicon was introduced into the corresponding sites of pET21a-FRT (41).

For pET21a-FRT-tktA and pET21a-FRT(-8TC)-tktA, *tktA* was amplified by PCR from genomic DNA of *E. coli* BW25113 using the primer pair EctktA-Nde/EctktA-Xho. The obtained amplicon was digested with NdeI and XhoI and then introduced into the corresponding sites of pET21a-FRT and pET21a-FRT(-8TC) to generate pET21a-FRT-tktA and pET21a-FRT(-8TC)-tktA, respectively.

To construct pET21a-FRT-ppsA, *ppsA* was amplified by PCR from genomic DNA of *E. coli* BW25113 using the primer pair ppsA-F/ppsA-R. The obtained amplicon was digested with NdeI and XhoI and then introduced into the corresponding sites of pET21a-FRT to generate pET21a-FRT-ppsA.

To construct pET21d-FRT-aroA, *aroA* was amplified by PCR from genomic DNA of *E. coli* BW25113 using the primer pair EcAroA-F/EcAroA-R. The obtained amplicon was digested with NcoI and HindIII and then introduced into the corresponding sites of pET21d-FRT to generate pET21d-FRT-aroA.

To construct pET21a-FRT-aroB, pET21a-FRT-aroC, pET21a-FRT-aroD, pET21a-FRT-aroE, and pET21a-FRT-aroL, we amplified *aroB*, *aroC*, *aroD*, *aroE*, and *aroL* by PCR from the genomic DNA of *E. coli* BW25113 using the primer pairs AroB-opt-gib-F/AroB-gib-R, AroC-gib-F/AroC-gib-R, AroD-gib-F/AroD-gib-R, AroE-gib-F/AroE-gib-R, and AroL-gib-F/AroL-gib-R, respectively. Each amplicon was assembled with NdeI- and XhoI-digested pET21a-FRT using Gibson Assembly Master Mix (New England BioLabs, Inc., Ipswich, MA).

To construct pET21a-FRT-aroALC, DNA fragments containing *aroA*, *aroL*, and *aroC* were amplified from pET21a-FRT-aroA, pET21a-FRT-aroL, and pET21a-FRT-aroC, respectively, using the primer pairs ALCorEDB-F/ALC-aroA-R (for *aroA*), ALC-aroL-F/ALC-aroL-R (for *aroL*), and ALC-aroC-F/ALCorEDB-R (for *aroC*). The fragments containing *aroA*, *aroL*, and *aroC* were assembled by overlap extension PCR using the primer pair aroALC-F1/aroALC-R1 to construct a DNA fragment containing the *aroALC* sequence. The plasmid backbone was amplified from pET21a-FRT using the primer pair pET21a-ALC-F/pET21a-ALC-R. The plasmid backbone and *aroALC* fragment were assembled using Gibson Assembly Master Mix to generate pET21a-FRT-aroALC.

To construct pET21a-FRT-aroEDB, DNA fragments containing *aroE* and *aroD* were amplified from pET21a-FRT-aroE and pET21a-FRT-aroD, respectively, using the primer pair ALCorEDB-F/EDB-aroE-R (for *aroE*) or EDB-aroD-F/EDB-aroD-R (for *aroD*). A DNA fragment containing *aroB* was first amplified from pET21a-FRT-aroB using the primer pair ALC-aroB-F/ALCorEDB-R and then reamplified from the purified amplicon using the primer pair EDB-aroB-F2/ALCorEDB-R. Fragments containing *aroE*, *aroD*, and *aroB* were assembled by overlap extension PCR using the primer pair aroEDB-F1/aroEDB-R1 to construct a DNA fragment containing the *aroEDB* sequence. Plasmid backbone was amplified from pET21a-FRT using the primer pair pET21a-EDB-F/pET21a-EDB-R. The plasmid backbone and *aroEDB* fragment were assembled using Gibson assembly master mix to generate pET21a-FRT-aroEDB.

To construct pET21a-FRT-aroLC, pET21a-FRT-aroAC, pET21a-FRT-aroAL, pET21a-FRT-aroDB, pET21a-FRT-aroEB, and pET21a-FRT-aroED, we amplified DNA fragments containing *aroLC*, *aroAC*, and *aroAL* by PCR from pET21a-FRT-aroALC using the primer pairs aroLC-DB-F/aroLC-R, aroAC-F/aroAC-R, and aroAL-F/aroAL-ED-R, respectively. DNA fragments containing *aroDB*, *aroEB*, and *aroED* were amplified by PCR from pET21a-FRT-aroEDB using the primer pairs aroLC-DB-F/aroDB-R, aroEB-F/aroEB-R, and aroED-F/aroAL-ED-R, respectively. The respective amplicons were then phosphorylated by T4 polynucleotide kinase (TaKaRa Bio, Inc., Shiga, Japan), followed by self-ligation.

Generation of strains by chromosome engineering. Gene deletion was performed by Red-mediated recombination (46, 62). Briefly, a Km cassette flanked by two flippase recognition target sites (FRTs), called here the FRT-Km-FRT cassette, was amplified from pKD13 by PCR using primers, along with 50-nucleotide homologous sequences, and then electroporated into strain MG1655(DE3)/pKD46 expressing Red recombinase (genes encoding Gam, Bet, and Exo proteins). Gene integration was also performed by Red-mediated recombination (41). Genes of interest flanked by the T7lac promoter (P_{T7lac}) and T7 terminator (T_{7T}) were amplified by PCR along with FRT-Km-FRT from pET21a-FRT- or pET21d-FRT-derived plasmids carrying the gene of interest, followed by introduction into strain MG1655(DE3)/pKD46, which expressed Red recombinase, to integrate the gene of interest into the target locus of the chromosome. PrimeStar GXL polymerase (TaKaRa Bio, Inc.) was used for PCRs, along with the primers listed in Table 5, to amplify the integration cassette. These strains are listed in Table 1 as donor strains for P1 transduction (M-ARG strains).

To generate Phe- or Tyr-producing strains, genetic traits were combined by P1 transduction, along with Km marker recycling by FLP/FRT site-specific recombination (41). Generated strains are listed as phenylalanine-producing strains, tyrosine-producing strain, or aromatic compound-producing strains in Table 1 (M-PAR strains), and the detailed processes for generating strains are summarized in Fig. 2. Here, strains AR-G40 [BW25113(DE3) *acs*::FRT-Km-FRT-*tdc(lb)*] (where *lb* indicates that it is a *Lactobacillus brevis* gene), AR-G39 [BW25113(DE3) *acs*::FRT-Km-FRT-*ldhA(re)*] (where *re* indicates that it is a *Cupriavidus necator/Ralstonia eutropha* gene), and AR-G79 [BW25113(DE3) *pheA*::FRT-Km-FRT] were as described in the previous studies (36, 41).

Analytical methods. The concentrations of Phe, Tyr, and other aromatic compounds were measured using a previously reported method (36). A high-performance liquid chromatography system fitted with a photodiode array detector (SPD-M10AVP; Shimadzu Corp.) and an octadecyl silica column (COSMOSIL 5C₁₈-MS-II, 3.0 by 150 mm; Nacalai Tesque, Inc.) was used to measure the concentration of target compounds. The compounds were eluted at a flow rate of 0.4 ml/min of mobile phase containing methanol and 0.1% trifluoroacetic acid. The methanol concentration was increased from 20% to 80% over 5 min, then maintained for 10 min. Under such conditions, tyramine (retention time, 3.2 min), Tyr (3.8 min), 4-hydroxyphenyllactic acid (5.1 min), Phe (5.2 min), 4-hydroxyphenylethanol (5.4 min), and phenyllactic acid (6.8 min) were eluted with acceptable separation. The wavelength for the detection of Phe and phenyllactic acid was 206 nm, and that for tyramine, Tyr, 4-hydroxyphenyllactic acid, and 4-hydroxyphenylethanol was 222 nm. The compounds were quantified using standard curves of the respective commercial chemicals.

Glucose concentration was colorimetrically determined using Glucose CII Test Wako (Wako Pure Chemical Industries, Ltd.). The specific productivity (q_p , in g g DCW⁻¹ h⁻¹) was calculated by the following formula, where C_p is the product concentration (g/liter), t is the cultivation time (h), and x is the dry cell weight (DCW) in g/liter (61). DCW values were calculated from OD₆₆₀ values using the formula DCW (g/liter) = OD₆₆₀ × 0.347.

$$q_p = \frac{C_p(t + \Delta t) - C_p(t)}{\Delta t} \cdot \frac{1}{(x(t) + x(t + \Delta t))/2}$$

The method for transcriptional analysis by real-time PCR was previously reported (61). Total RNA was extracted and purified using a QIAquick RNA extraction kit (Qiagen, Hilden, Germany) from cells cultivated at 33°C in M9M2 medium until the OD₆₆₀ reached 2.0 with IPTG induction at an OD₆₆₀ of 0.4. cDNA was synthesized by using a PrimeScript II first-strand cDNA synthesis kit (TaKaRa Bio, Inc.) with the primer mixture of aroAb, aroBb, aroCb, aroDb, aroEb, ydiBb, aroFb2, aroGb, aroKb2, aroLb, pheAb2, tyrAb, tyrBb, tktAb, ppsAb, RT-infBb, and RT-T7POL-R (each at 1 μM). Real-time PCR was performed as described by Báez-Viveros et al. (63), using MP-3000 (Stratagene), Thunderbird SYBR qPCR mix (Toyobo Co., Ltd., Osaka, Japan), and specific primer pairs for the respective genes. The primers used for the cDNA synthesis and the real-time PCR are described in previous studies (61, 63, 64), but the primers used for *aroF*, *aroK*, and *pheA/pheA^{fbr}* were newly prepared for the present study as follows: *aroF*, aroFa2 (ATTGCTGCTTGA GCTGGTGA) and aroFb2 (TTGCGATTCCGTGTACGAG); *aroK*, aroKa2 (TCGCGAAGAAAAGGTCATCA) and aroKb2 (GATAAACGACAACGCCACGA); and *pheA/pheA^{fbr}*, pheAa2 (GTATGCTGCCCGTCACTTTG) and pheAb2 (GCACCGGAGCTGGTATTTTC). The PCR conditions were 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The 2^{−ΔΔCT} method was used for data analysis, and the *ihfB* gene was used as a housekeeping gene to normalize the data (65).

A reporter assay using the *lacZ* gene was performed based on a previously described method (61). Each strain harboring T7-controlled chromosomal *lacZ* gene was cultivated in LB medium at 37°C until reaching an OD₆₆₀ of 0.5, and then IPTG was added at 1 mM. Cultivation was continued at 27°C for 12 h, and the culture was used for the reporter assay.

For SDS-PAGE analysis, strain was cultivated in LB medium at 37°C until the OD₆₆₀ reached 0.5, and then IPTG was added at 1 mM. Cultivation was continued at 27°C for 22 h. Methods for sample preparation and SDS-PAGE analysis were as previously described (61).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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