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Title	New gene responsible for para-aminobenzoate biosynthesis
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Citation	Journal of Bioscience and Bioengineering, 117(2), 178-183 https://doi.org/10.1016/j.jbiosc.2013.07.013
Issue Date	2014-02
Doc URL	https://hdl.handle.net/2115/56676
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Type	journal article
File Information	SI_submit.pdf, Supplementary data

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Supporting information.

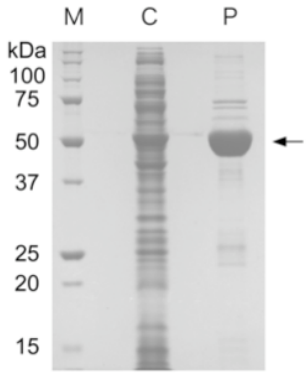
Table S1. Primes used in this study

Primers	Sequences (5' to 3')
For *NE2150* expression	
PB1	AGAGAGCTCTTAAG*AAGGAG*ATATACATATGAATCATTGCATAACT GAAAGTGAATTCAACG
PB2	ATAGGATCCTCAGGTGATCGTACTGTCCAGGCCATTC
For *NE1434* expression	
PB3 [a]	ATGGATCCATAATATTATTTCATTTCACTCTGAACAGGAGC
PB4	TTAAGCTTA**GTGATGGTGATGGTGATG**ACCACCGTGCAGGGTGG CTGCGGTT
For *CT610* expression	
PB5 [b]	GGAGAATTCTTTGCGCATAGATGAAACAGAGGAC
PB6	TATAAGCTTA**GTGATGGTGATGGTGATG**ACCACCATAAGATTGA TGACAACTACAACAAGTTCCTGG
For *pqqC* expression	
PB7	AAGAATTCTAAG*AAGGAG*ATATACATATGAGCGACGCACTGCCAAT GTCC
PB8	TATAAGCTTGGTACCTCATAGGGTGATTCCCTTGTGCCACAC
For *aroB* disruption and its confirmation	
PB9	TATATACACTCGTCTGCGGGTACAGTAATTAAGGTGGATGTCGCGTT ATGGAATTAACCCTCACTAAAGGGCGGC
BP10	CTTGATAAGCGGCCTGACCTTTCTTGTTGTTACGCTGATTGACAATC GGCAATAATACGACTCACTATAGGGCTCG
PB11	GCGGCTCTGTGAAATCCCGTGAAACG
BP12	GCTCTACGCGCTCTTCATCCACGGTTTC
For *aroC* disruption and its confirmation	
PB13	TTATAAAGATTAAGTAAACACGCAAACACAACAATAACGGAGCCGT GATGGAATTAACCCTCACTAAAGGGCGGC
BP14	AGCAGCGCAATCGCGGTTTTATTCATTTTTTACCAGCGTGGAATATC AGTAATACGACTCACTATAGGGCTCG
PB15	GAAGATATGTCCGACCTGCCAAACGAATACC
PB16	CACCGTACCCATGCCCAGATTGCTCAC
For *aroD* disruption and its confirmation	
PB17	TGGGGTTCGGTGCCTGACAGGCTGACCGCGTGCAGAAAGGGTAAAA AATGGAATTAACCCTCACTAAAGGGCGGC
PB18	GGAGGGTGTTCCCGCCGAAATATTATTGCTTATGCCTGGTGTAAAAT AGTTAATACGACTCACTATAGGGCTCG
PB19	CCGACATTTTAACCAATGGCACAAAAGTG

PB20	GCAGTAATTAACGTGGTGGCTTCCAGAATACC
For *NE1434* integration and its confirmation	
PB21	GAATTAACCCTCACTAAAGGGCGGCTCATGTTTGACAGCTTATCATC GATTAGC
PB22	TCAGCGTTACAAGTATAACACAAAGTTTTTTATGTTGTCAATATTTT TTTGATGAGATCTTCCATTCAGGTCGAGGTGGC
PB23	ATTGACAACATAAAAAACTTTGTGTTATACTTGTAACGCTGAATTCA CTCTGAACAGGAGCTTTTCATGG
PB24	TAATACGACTCACTATAGGGCTCGACTTATCAGACTTGCAGGATCA GTG
PB25	AATATTCACAGGGATCACTGTAATTAAAATAAATGAAGGATTATGT AATGGAATTAACCCTCACTAAAGGGCGGC
PB26	ATCCTTATAGCCACTCTGTAGTATTAATTAAACTTCTTTAAGTTTTGC GGTAATACGACTCACTATAGGGCTCG
PB27	CACCGCCCTTGATTTGCCCTTCTGTAG
PB28	CCAGGCACCGGCAAGATCAACAG

DNA sequences underlined indicate restriction sites of *Nde*I, *Bam*HI, *Hin*dIII, or *Eco*RI.
Bold indicates sequences encoding His-tag.
Italic includes a ribosome binding site sequences.
[a]PB3 could anneal to approximately 40 bp upstream region of the start codon of *NE1434* gene.
[b]PB5 could anneal to approximately 70 bp upstream region of the start codon of *CT610* gene

(A)



(B)

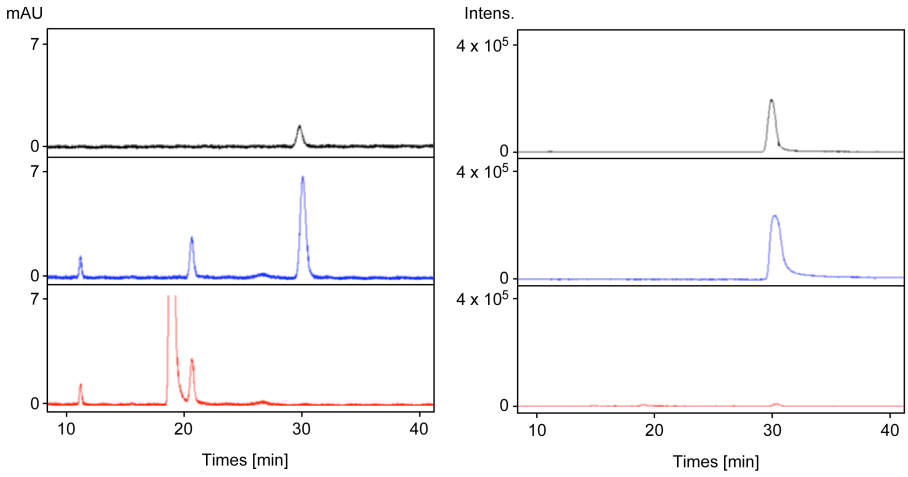


Figure S1. *In vitro* assay of the NE2150 gene product.

(A) SDS-PAGE analysis of purified *N*-terminal His-tagged NE2150 protein (56.6 kDa). M, marker; C, crude lysate (20 μg); P, purified protein (10 μg). An aliquot (20 mL) of overnight culture of *E. coli* BL21(DE3) harboring pET-NE2150 was inoculated into 1 L of fresh LB media and cultured at 30°C to OD 0.5. Then, IPTG was added at a final concentration of 0.5 mM and cultivation was continued for an additional 16 hr. After the cells were harvested and washed once with chilled buffer A (50 mM sodium phosphate, 300 mM NaCl; pH 8.0)

containing 20 mM imidazole, they were suspended in 40 mL of the same buffer. Protein purification was performed according to the manufacturer's instructions. The sample was desalted using an Amicon apparatus (Millipore, Bedford, MA, USA) with buffer B (10 mM sodium phosphate, 60 mM NaCl; pH 8.0). The purity of the recombinant enzyme was checked by SDS-PAGE.

(B) LC/MS analysis of the reaction product. Enzyme assay reported by He, Z. et al (1) was slightly modified. The assay mixture contained, in a final volume of 200 μL, 100 mM bicine (pH8.5), 10 mM chorismate, 100 mM NH_4Cl, and a suitable amount of recombinant NE2150. This mixture was incubated at 30°C for 60 min and then deproteinized using an Amicon apparatus (Millipore). The samples (2 μL) were analyzed by LC/MS (middle). Analytical conditions were the same as those used for the examination of pABA productivity. The UV chromatogram (284 nm, left column) and selected ion chromatogram for m/z = 138.056 ± 0.010 (corresponding to the $[M+H]^+$ ion for anthranilate, right column) are shown. Standard anthranilate (upper) and a control experiment without the enzyme (lower) are also shown.

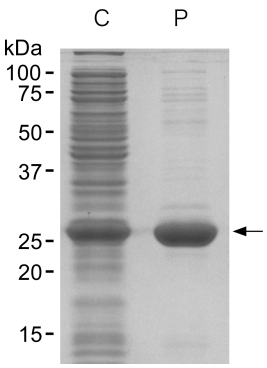
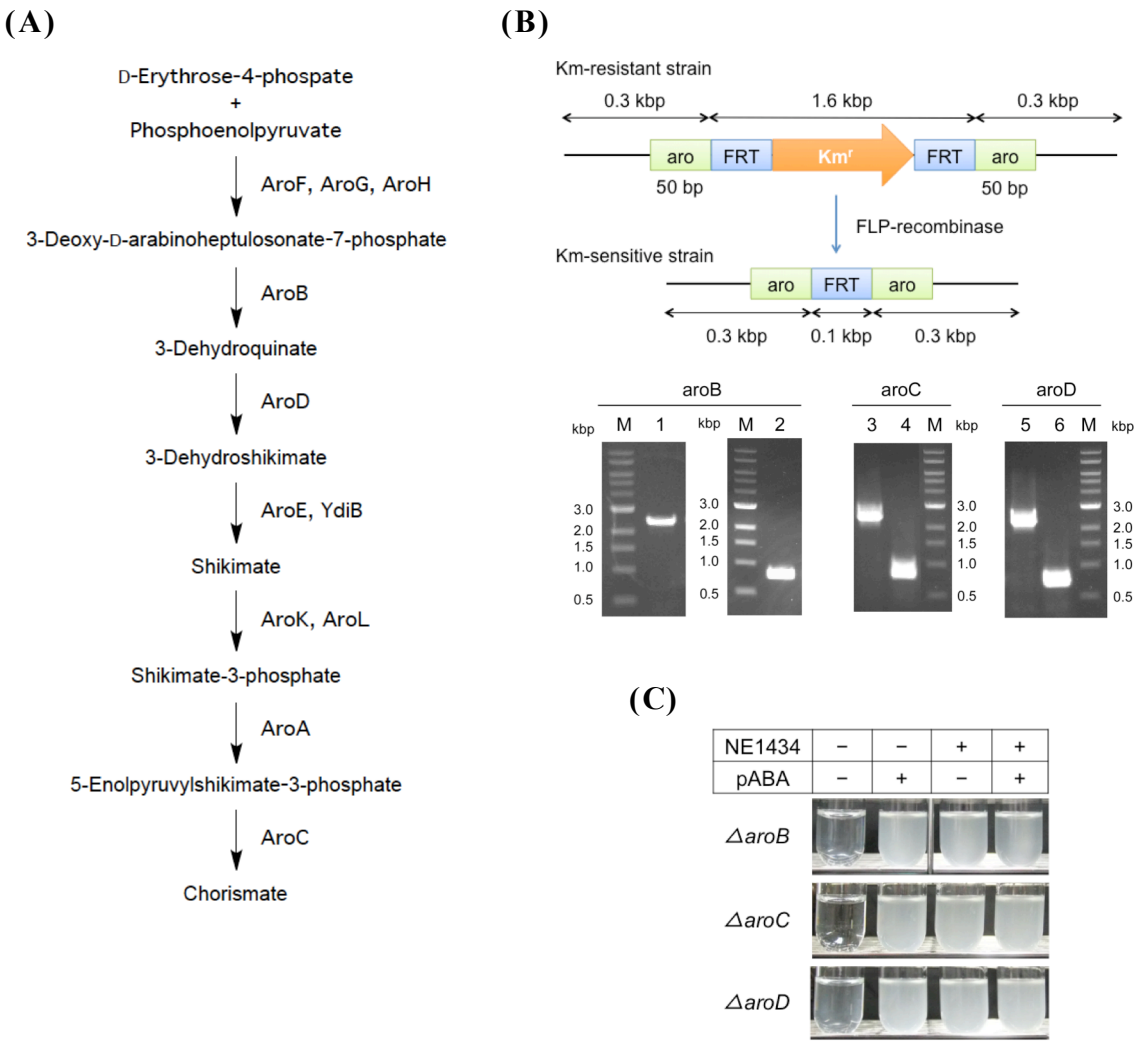


Figure S2. Purification of *C*-terminal His-tagged NE1434 protein and in vitro assay using the purified protein.

C-terminal His-tagged NE1434 protein (28.9 kDa) was purified from the cell lysate of *E. coli* JM109 harboring pUC-NE1434H by the same method used for NE2150 purification and analyzed by SDS-PAGE. C, crude lysate (20 μg); P, purified protein (10 μg).

In vitro assay was carried out in 100 mM of sodium phosphate (pH7.5) or bicine (pH8.5) at 30°C for 1 or 20 h. Chorismate was used as substrates. Glutamine, glycine, alanine, NH_4Cl were used as donors of amino group. NAD(P)H, $NAD(P)^+$, PLP, FAD, ascorbate, $MgCl_2$, $FeCl_3$ were added into the reaction mixture as cofactors. Reaction mixtures (10 μL) were analyzed using a Shimadzu LC-20 system (Shimadzu Co., Kyoto, Japan). The analytical conditions were as follows: InertSustain C18 column (150 mm × 4.6 mm ID, 5 μm; GL Sciences Inc., Tokyo, Japan); column temperature, 35°C; isocratic flow, 5% (v/v) acetic acid:acetonitrile = 95:5; flow rate, 1 mL/min. Elutied compounds were detected by a diode array spectrophotometer. However, no product was detected in the all samples.



112

113

114 **Figure S3.** Construction of *aroB, aroC,* and *aroD*-knockout mutants from the *E. coli*

115 *ΔpabABC* mutant.

116 **(A)** The shikimate pathway.

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118 **(B)** To construct *aroB, aroC,* and *aroD* disruptants, the Quick & Easy *E. coli* Gene Deletion

119 Kit (Gene Bridges GmbH, Heidelberg, Germany) was used according to the manufacturer's

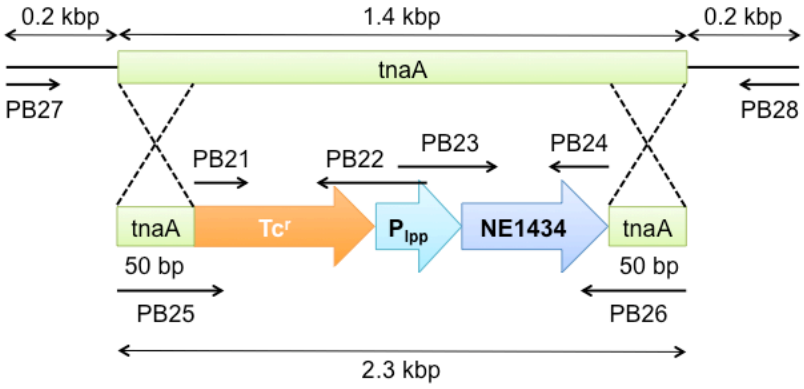
120 protocol. In brief, DNA fragments containing a Km-resistance gene cassette (Gene Bridges

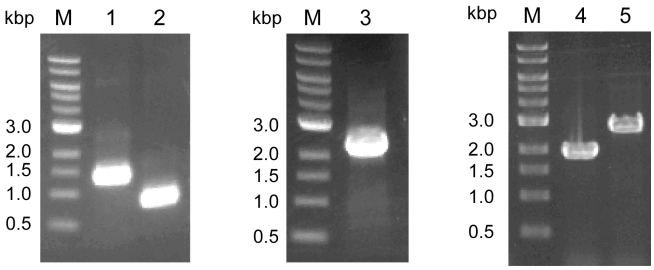
121 GmbH) flanked with FRT sites and 50-bp homologous arms whose sequences were identical

to the target regions were amplified by PCR with appropriate sets of primers (Table S1). The amplified DNA fragments were used to transform the *E. coli ΔpabABC* mutant (2) harboring the pRedET plasmid. Gene-disruption in Km-resistant colonies was checked by PCR (lane 1, 3, and 5) using appropriate sets of two primers that hybridized approximately 300 bp up- and downstream of the target genes (Table S1). Furthermore, the sequences of the amplicons were analyzed to confirm the deletion. The selection marker in the obtained mutants was removed with FLP-recombinase and the gene deletions were confirmed by PCR (lane 2, 4, and 6) and direct sequencing of the amplicons.

(C) Disruption of *aroB*, *aroC*, and *aroD* in *E. coli ΔpabABC* (2) harboring pUC-NE1434H had no effect on its growth. Each of the three types of transformants (triplicate) was grown on LB plates, dispersed in 200 μL of sterilized distilled water and then a 30 μL aliquot was inoculated into 3 mL of M9 media containing Phe, Tyr, Trp, and 4-hydroxy benzoate with/without pABA (10 μg mL^{-1}). Their growth was assessed after 2–3 days cultivation at 30°C.

(A)





(B)

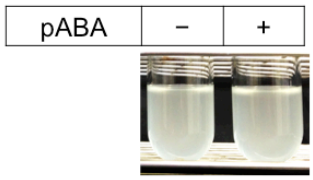


Figure S4. Construction of a host strain to isolate a pABA auxotrophic mutant from *E. coli* *ΔpabABC* expressing the *NE1434* gene.

(A) To express the *NE1434* gene constitutively in the *E. coli* chromosome, the gene was integrated into the *tnaA* locus (tryptophanase gene) in *E. coli ΔpabABC* (2) using the Red/ET recombination system (Gene Bridges GmbH). The DNA fragment used for integration was constructed in three steps; first, the tetracycline resistance gene (Tc^r) and part of the lipoprotein promoter (P_{lpp}, (3)) were amplified by PCR with the primers PB21 and PB22 (lane

1), which target the *N*-terminus of the Tc^r gene and the 5′-half of P_{lpp}, respectively; second, the other part of P_{lpp} and the *NE1434* gene were amplified with PB23 and PB24 (lane 2), which target the 3′-half of *lpp* and the *C*-terminus of *NE1434*, respectively; third, to assemble the two amplicons with arms for the *tnaA* gene, PCR was carried out with both of the amplicons and the primers PB25 and PB26 (lane 3). The former and the latter carry 50-bp *N*-terminal and *C*-terminal sequences of *tnaA*. To obtain an *NE1434*-integrated strain, the *E. coli ΔpabABC* mutant harboring pRedET was transformed with the amplified DNA fragment and selected on LB plates containing Tc. The gene-integration of Tc-resistant colonies was checked by PCR using two primers, PB27 and PB28, which hybridize approximately 200 bp up- and downstream of the *tnaA* gene (Table S1), respectively (lane 4, parental strain; lane 5, the NE1434-integrated strain). Furthermore, the sequence of the amplicon was confirmed.

(B) The *NE1434*-integrated *E. coli ΔpabABC* strain thus obtained was confirmed to be able to grow in M9 media without pABA in the same way as *E. coli ΔpabABC* (2) harboring *NE1434* gene on the plasmid.

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