A new gene responsible for para-aminobenzoate biosynthesis

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ABSTRACT

Folate is an essential cofactor in all living cells for one-carbon transfer reactions. *para*-Aminobenzoate (pABA), a building block of folate, is usually derived from chorismate in the shikimate pathway by reactions of aminodeoxychorismate synthase (PabA & B) and 4-amino-4-deoxychorismate lyase (PabC). We previously suggested that an alternative pathway for pABA biosynthesis would operate in some microorganisms such as *Lactobacillus fermentum* and *Nitrosomonas europaea* since these bacteria showed a prototrophic phenotype to pABA despite the fact that there are no orthologs of *pabA*, *B*, and *C* in their genome databases. In this study, a gene of unknown function, *NE1434*, was obtained from *N. europaea* by shotgun cloning using a pABA-auxotrophic *Escherichia coli* mutant (*ΔpabABC*) as a host. A tracer experiment using [U-13C6]glucose suggested that pABA was *de novo* synthesized in the transformant. An *E. coli* *ΔpabABCΔaroB* mutant carrying the *NE1434* gene exhibited a prototrophic phenotype to pABA, suggesting that compounds in the shikimate pathway including chorismate were not utilized as substrates by NE1434. Moreover, the *CT610* gene, an ortholog of *NE1434* located in the folate biosynthetic gene cluster in *Chlamydia trachomatis*, also complemented pABA-auxotrophic *E. coli* mutants. Taken together, these results suggest that NE1434 and CT610 participate in pABA biosynthesis.
Folate, composed of a pterin moiety, \textit{para}-aminobenzoate (pABA) and glutamic acid(s), is an essential cofactor for all living cells and plays critical roles in a diverse range of metabolic pathways, mainly in one-carbon transfer reactions such as amino acid interconversions, and purine and pyrimidine biosynthesis. Most bacteria and plants have the pathway for \textit{de novo} synthesis of folate (Figure 1) (1). The first step in forming the pterin moiety is conversion of GTP into 7,8-dihydroneopterin triphosphate by GTP cyclohydrolase I (FolE). After dephosphorylation by phosphatases, the resulting 7,8-dihydroneopterin is converted to 6-hydroxymethyl-7,8-dihydropteroxin with concomitant release of glycolaldehyde by 7,8-dihydroneopterin aldolase (FolB) and then pyrophosphorylated by 6-hydroxymethyl-7,8-dihydropteroxin pyrophosphokinase (FolK). The resulting compound, 6-hydroxymethyl-7,8-dihydropteroxin pyrophosphate, is attached to pABA—which is supplied from chorismate by two enzymes, aminodeoxychorismate (ADC) synthase (PabA/B) and ADC lyase (PabC)—by dihydropteroate synthase (FolP) to yield dihydropteroate, which is glutamylated by dihydrofolate synthase (FolC) and then reduced by dihydrofolate reductase (FolA) to give tetrahydrofolate.

We previously showed that \textit{Lactobacillus fermentum} IFO 3956 exhibited a prototrophic phenotype to pABA in a single-omission growth test (2), despite the fact that the strain lacks orthologs of \textit{pabA}, \textit{-B}, and \textit{-C} (3) (Figure 1), suggesting that the strain utilizes an alternative pathway to synthesize pABA. Similarly, \textit{Nitrosomonas europaea} NBRC14298 (ATCC19718) also has a complete set of folate biosynthetic gene orthologs, except for \textit{pabA}, \textit{-B}, and \textit{-C}, in
its genome (KEGG, http://www.genome.jp/kegg/; (4)), despite its chemoautotrophic phenotype. In this paper, we performed shotgun cloning experiments with a pABA-auxotrophic E. coli mutant (ΔpabABC) as a host and L. fermentum IFO 3956 and N. europaea NBRC14298 as DNA donors. We successfully obtained a complementary gene, NE1434, from N. europaea. A tracer experiment using [U-^{13}C_6]glucose suggested that NE1434 was involved in de novo pABA biosynthesis. We found that Chlamydia trachomatis possessed a NE1434 ortholog (CT610) in its folate biosynthetic gene cluster and confirmed that the CT610 gene also complemented the pABA-auxotrophic E. coli mutant (ΔpabABC).

MATERIALS AND METHODS

Bacterial strains and cultures

Strains used in this study are summarized in Table 1. Lactobacillus fermentum IFO 3956 and Nitrosomonas europaea NBRC 14298 (ATCC 19718) were obtained from the Biological Resource Center, National Institute of Technology and Evaluation (NITE), Tokyo, Japan. Escherichia coli JM109 (Nippon Gene Co., Ltd, Tokyo, Japan) was routinely used for plasmid construction. For pABA auxotrophy complementation assays, a pabA, -B, and -C gene deleted mutant derived from E. coli BW25113 was used (2). The media used were LB broth medium (Lennox; Life Technologies Corp., Carlsbad, CA, USA) and M9 minimal medium [M9 minimal salts (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.4 % (w/v) glucose, 5 mM MgSO_4, 0.1 mM CaCl_2]. For growth
on plates, 1.5% (w/v) agar was added into the media. Ampicillin (Ap), chloramphenicol (Cm), and kanamycin (Km) were added to the media at concentrations of 100, 30, and 25 µg mL⁻¹ if necessary.

**Plasmids construction**

Plasmids used in this study are listed in Table 2. All primers were designed using the genomic DNA sequence. A *pabB* homolog, *NE2150* (4), was amplified by PCR using PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Shiga, Japan), genomic DNA of *N. europaea* as a template, and the primers PB1 and PB2, whose sequences are shown in Table S1. The PCR reaction was carried out as recommended by the manufacturer. The amplified DNA fragment was treated with *Nde*I and *Bam*HI and cloned into the same sites of the pET28 vector (Merck Ltd., Tokyo, Japan) to express NE2150 as an *N*-terminal His-tag fused protein. The plasmid was designated pET-NE2150.

For expression of *C*-terminal His-tag fused NE1434 (4), the DNA fragment was amplified by PCR using the method described above with genomic DNA of *N. europaea* and the primers PB3 and PB4 (Table S1). The amplified DNA fragment was cloned into the *Bam*HI and *Hind*III sites of the pUC18 vector (Takara Bio Inc.) to construct pUC-NE1434H.

For *in vivo* expression of the *CT610* (5) and *pqqC* genes (6), DNA fragments were amplified by PCR with genomic DNA of *C. trachomatis* strain UW-3/Cx (ATCC VR-885D) and *P. putida* KT2440 using appropriate sets of primers (PB5 to PB8, Table S1). Each of the
amplified DNA fragments was cloned into the BamHI and HindIII sites of the pUC18 vector (Takara Bio Inc.) to construct pUC-CT610H and pUC-pqqC, respectively. The DNA sequences of all amplified fragments were analyzed with the BigDye Terminator v3.1 Cycle Sequencing Kit using the ABI PRISM 3130 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan).

**Shotgun cloning of a gene related to pABA biosynthesis from L. fermentum and N. europaea**

Genomic DNA from *L. fermentum* and *N. europaea* was partially digested with Sau3AI, and approximately 7-kbp DNA fragments were separated by agarose gel electrophoresis and then purified. The DNA fragments were ligated into the pSTV29 cloning vector (pACYC184 ori, *P*<sub>lac</sub>, Cm<sup>r</sup>; Takara Bio Inc.) digested with BamHI. pABA-auxotrophic *E. coli ΔpabABC* (2) was transformed with the ligation mixture and plated onto M9 minimal media plates.

**pABA auxotrophy complementation assay**

For the pABA auxotrophy complementation assay, the *E. coli* mutants Δ*pabABC* (2), Δ*pabABCAroB*, Δ*pabABCAroC*, and Δ*pabABCAroD* were transformed with appropriate plasmids. Each of the transformants (triplicate) grown on LB plates was dispersed in 200 µL of sterilized distilled water and then a 50 µL aliquot was inoculated into 5 mL of M9 medium supplemented with or without pABA. The growth was assessed at 30°C since *N. europea*
prefers a cultivation temperature at 20 to 30°C. Cell growth of the transformants was monitored at one-hour intervals using a TAITEC OD-monitorC&T system (Saitama, Japan) and compared to that of parental strain (Figure 2B).

Construction of aroB, -C, and -D disrupted mutants of E. coli ΔpabABC

To construct the disruptants, the Quick & Easy E. coli Gene Deletion Kit (Gene Bridges GmbH, Heidelberg, Germany) was used. Briefly, the genes encoding aroB (b3389), aroC (b2329), or aroD (b1693) (7) were replaced with a kanamycin-resistance gene cassette by homologous recombination and then the cassette was removed from the chromosome by a FLP-recombinase according to the manufacturer’s protocol (Figure S3). The sequences of the primers are shown in Table S1.

Analysis of pABA productivity

pABA production was examined under the cultivation condition as previously described (8). An aliquot (60 µL) of overnight culture was inoculated into 3 mL of fresh LB medium and cultured at 30°C for 3 h. Then, IPTG was added at a final concentration of 0.5 mM and cultivation was continued for an additional 3 h. After the cells were harvested and washed with the same amount of M9 medium, the collected cells were suspended in 3 mL of M9 medium including Ap and 0.5 mM IPTG, followed by cultivation at 30°C. Culture samples were collected at appropriate time intervals (24, 48, and 72 h) to analyze pABA productivity.
Supernatants of the culture (5 µL) were analyzed using an Agilent Technologies 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Bruker Daltonics micrOTOF-HS (Bruker Daltonics Inc., Billerica, MA, USA). The analytical conditions were as follows: InertSustain C18 column (150 mm × 2.1 mm ID, 3 µm; GL Sciences Inc., Tokyo, Japan); column temperature, 35°C; 5% (v/v) acetonitrile solution with 0.1% (v/v) formic acid by isocratic flow; flow rate, 0.2 mL/min. pABA was eluted at approximately 10 min and detected as $m/z = 138.056 ± 0.010$ and $145.079 ± 0.010$, which correspond to the [M+H]$^+$ ions for non-labeled and $^{13}$C-labeled pABA.

**RESULTS**

**Shotgun cloning experiment with the *E. coli* ΔpabABC mutant.**

We previously showed that *L. fermentum* IFO 3956 was prototrophic to pABA and that LAF1336, an ortholog of FolP in *L. fermentum* IFO 3956, utilized 6-hydroxymethyl-7,8-dihydropterin pyrophosphate and pABA to form dihydropteroate (2), despite the fact that the strain has no orthologs of PabA, -B, and -C (3). These facts strongly suggested that the strain biosynthesizes pABA via a new enzyme/pathway. We therefore used genomic DNA of *L. fermentum* IFO 3956 as donor DNA to obtain a DNA fragment that could complement the pABA-auxotrophic *E. coli* mutant (ΔpabABC). Approximately 7-kbp DNA fragments partially digested with *Sau*3AI were used for shotgun cloning and screening.
was carried out on the basis of complementation of pABA auxotrophy of the *E. coli* Δ*pabABC* mutant on M9 minimal plates without supplementation of pABA. However, no transformants were obtained even though we performed several trials. A reason for the failure in complementation using *L. fermentum* genome is discussed later.

We then used *N. europaea* NBRC 14298 as the DNA donor. The strain also has a complete set of orthologs of the folate biosynthetic genes, except for pABA-biosynthesis related genes, in its genome (GTP cyclohydrolase I, NE1163; dihydronopterin aldolase, NE0223; 6-hydroxymethyl-7,8-dihydropteropt pyrophosphokinase, NE0070; dihydropteroate synthase, NE0529; dihydrofolate synthase, NE0696; dihydrofolate reductase, NE0567) (4). As for pABA biosynthetic genes, only one ortholog, NE2150, which was annotated as TrpE, was hit by a BLAST search (KEGG) when *E. coli* PabB was used as a query sequence. Microorganisms generally possess two orthologs of PabB; one is PabB, which is responsible for pABA biosynthesis, and the other is an anthranilate synthase subunit (TrpE), which is responsible for tryptophan biosynthesis (9). To examine whether *NE2150* participates in pABA biosynthesis, we prepared recombinant NE2150 and used it for *in vitro* assays (Figure S1A). When chorismate and NH₄Cl were used as substrates, the formation of anthranilate was confirmed (Figure S1B); in contrast, no product was observed when NE2150 was omitted, demonstrating that *NE2150* actually encodes anthranilate synthase (TrpE) and that *N. europaea* synthesizes pABA via a new enzyme/pathway.

To obtain a gene related to pABA biosynthesis from *N. europaea*, we performed
shotgun cloning using the method described above. We obtained dozens of colonies on M9 minimal plates without pABA. The DNA sequences of plasmids randomly prepared from the transformants revealed that they had a common region containing four genes, \textit{NE1431} to \textit{NE1434} (Figure 2A). To determine the essential gene(s) for complementation, the obtained plasmids were digested with \textit{HindIII} and then self-ligated (Figure 2A). The constructed plasmid including \textit{NE1434} gene again complemented the \textit{E. coli ΔpabABC} mutant, showing that \textit{NE1434} was essential.

Cell growth of \textit{E. coli BW25113} (parental strain) and \textit{E. coli ΔpabABC} mutant expressing \textit{NE1434} gene in M9 minimum medium was compared. As shown in Figure 2B, the transformant showed the almost the same growth curve as that of the parental strain, showing that \textit{NE1434} gene would be involved in pABA biosynthesis.

\textit{NE1434} was previously annotated as pyrroloquinoline-quinone (PQQ) synthase (\textit{pqqC}). However, the low similarity between \textit{NE1434} and PqqC (24\% identity and 40\% similarity for \textit{Klebsiella pneumoniae} KPN01811, 25\% identity and 39\% similarity for \textit{Pseudomonas putida} PP0378 (10)) and the absence of other genes related to PQQ biosynthesis in the flanking region suggested that \textit{NE1434} was a new enzyme responsible for pABA biosynthesis.

\textbf{Tracer experiment using [U-$^{13}$C$_6$]glucose.}

We next performed a tracer experiment using [U-$^{13}$C$_6$]glucose to estimate the precursor compound of pABA. Since milligram quantities of labeled pABA are required for $^{13}$C-NMR
analysis and the concentration of coenzymes and vitamins including folate in cells is generally quite low, we first examined pABA productivity in the *E. coli* ΔpabABC mutant harboring pUC-NE1434H. After the transformant was cultured in M9 medium, the culture broth and cell extracts were analyzed by HPLC and LC/MS as described in the Material and Methods. pABA was detected only with LC/MS ([M+H]^+ = 138.056, Figure 3, left column) and the productivity was too low to purify labeled pABA. Then, we examined whether pABA was *de novo* synthesized in the transformant using [U-^{13}C_{6}] glucose as the sole carbon source. As shown in Figure 3 (right column), one peak corresponding to ^{13}C-labeled pABA ([M+H]^+ = 145.079) was specifically detected, confirming that the NE1434 gene product participates in *de novo* pABA production.

**Elucidation of the substrate of NE1434.**

We tried to elucidate the substrate of NE1434 by several methods. In the known pathway, pABA is derived from chorismate in two steps catalyzed by ADC synthase (PabAB) and ADC lyase (PabC). We therefore prepared recombinant NE1434 fused with a His-tag at the C-terminus (Figure S2) and incubated it with chorismate. However, no product was formed under the various conditions tested such as addition of co-factors and metals and different pH conditions.

Besides chorismate, 3-dehydroquinate, an intermediate in the shikimate pathway (11), is also used as a precursor for pABA biosynthesis. We therefore examined whether the NE1434...
gene product utilized a compound in the shikimate pathway by a genetic method. We constructed *aroB* (3-dehydroquinate synthase), *aroC* (chorismate synthase), and *aroD* (3-dehydroquinate dehydratase I)-knockout mutants from the *E. coli ΔpabABC* mutant. These strains required aromatic amino acids, 4-hydroxy benzoate, and pABA for their growth. However, all of the mutants harboring the *NE1434* gene were able to grow in M9 medium without pABA (Figure S3), suggesting that none of the intermediates in the shikimate pathway, except for 3-deoxy-D-arabinoheptulosonate-7-phosphate, could be the precursor for *NE1434*-mediated pABA biosynthesis. To exclude the possibility that *NE1434* utilized aromatic amino acids and 4-hydroxy benzoate, which were added into the medium, as precursors for pABA biosynthesis, we used these compounds as substrates for *NE1434*. However, no products were formed. Taking these results together, we concluded that a new enzyme/pathway for pABA biosynthesis is operating in *N. europaea*.

We also tried to isolate a pABA auxotrophic mutant from *E. coli ΔpabABC* expressing the *NE1434* gene because a gene complimenting the mutant is thought to participate in biosynthesis of the substrate of *NE1434*. Therefore, the *NE1434* gene was integrated into the *tnaA* locus (tryptophanase gene) in *E. coli ΔpabABC* (Figure S4). The strain was treated with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and pABA auxotroph were screened. We screened approximately 30,000 colonies including penicillin-concentrated auxotrophs. However, no mutants exhibiting a pABA auxotrophic phenotype were obtained, suggesting that an essential gene might be responsible for biosynthesis of the substrate of *NE1434*.
**Distribution of NE1434 orthologs.**

The *NE1434* gene was previously annotated as *pqqC*, which is a member of the TenA/Thi-4/PqqC family of Pfam (http://pfam.sanger.ac.uk/). We investigated the distribution of NE1434 orthologs using BLASTP and MEGA5 (12). Orthologs were collected from the genome databases of prokaryotes that have no gene clusters for PQQ biosynthesis (10), and therefore could be responsible for pABA biosynthesis like NE1434, using the BLASTP program with NE1434 as a query sequence and a cutoff e-value of 10^{-24}. The orthologs were aligned together with PqqC from *K. pneumoniae* and *P. putida*, both of which were confirmed to participate in PQQ biosynthesis, to construct a phylogenetic tree (Figure 4). The orthologs were distributed among two groups of prokaryotes. One was ammonia-oxidizing microorganisms such as *Nitrosomonas*, *Nitrosopumilus*, *Thaumarchaeota*, and the nitrogen-fixing *Trichodesmium erythraeum*. The other included obligate intracellular bacteria such as *Chlamydia*, *Chlamydophila*, *Wolbachia*, *Rickettsia*, and *Neorickettsia*.

In the case of *Chlamydia*, *Chlamydia trachomatis* has a folate biosynthetic gene cluster including *folA* (*CT614*), -*P* (*CT613*), and -*X* (*CT612, folB* ortholog) genes (5). However, *pabA*, -*B*, and -*C* genes are missing from the genome as in *N. europaea*. However, the *CT610* gene, an ortholog of *NE1434* (40% identity and 60% similarity), is located downstream of the *folAPX* cluster in the same direction. To confirm whether *CT610* participates in pABA biosynthesis, the gene was cloned into a cloning vector (pUC-CT610H) and a pABA
auxotrophy complementation assay with the *E. coli* Δ*pabABC* mutant was carried out. As shown in Figure 5, the *CT610* gene complemented pABA auxotrophy in the same manner as *NE1434*, suggesting that *CT610* is also involved in pABA biosynthesis in *C. trachomatis*. In contrast, the *PP0378* gene (pUC-pqqC), which is an ortholog of the *pqqC* gene located in the PQQ biosynthetic gene cluster in *P. putida* KT2440 (6) and has low similarity to NE1434 (25% identity and 39% similarity), was unable to complement pABA-auxotrophic *E. coli* Δ*pabABC* (Figure 5) suggesting that *PP0378* is involved in PQQ biosynthesis and that only orthologs with a high e-value to NE1434 are responsible for pABA biosynthesis.

**DISCUSSION**

We previously showed that some bacteria lacked orthologs of *pabA*, -B, and -C, which are essential for pABA biosynthesis, though they exhibited a prototrophic phenotype to pABA (2). In this study, we showed that *NE1434*, which is annotated as *pqqC* but has low similarity, was responsible for *de novo* pABA biosynthesis in *N. europae*a. The *CT610* gene in *C. trachomatis*, which is an ortholog of *NE1434* located in the folate biosynthetic gene cluster, also complemented the pABA-auxotrophy of the *E. coli* Δ*pabABC* mutant (Figure S5A). In contrast, we could not obtain any genes that complemented the pABA auxotrophic phenotype when the genome of *L. fermentum* IFO 3956 was used as a DNA source. Considering that it has no orthologs of the *NE1434* gene, two or more genes located at separated loci might be responsible for biosynthesis of pABA in *L. fermentum* IFO 3956.
Otherwise, the strain may biosynthesizes pABA in a totally different manner using a different compound as the substrate.

We tried to identify the substrate of NE1434 by several approaches. Since intermediate compounds in the shikimate pathway such as chorismate and 3-dehydroquinate are used as substrates for pABA biosynthesis (11), we first examined this possibility. However, recombinant NE1434 did not react with chorismate and none of the aroB, -C, and -D knockout mutants of E. coli ΔpabABC harboring the NE1434 gene required pABA for their growth, suggesting that intermediate compounds in shikimate pathway are not used as the substrate of NE1434.

We were not able to estimate the substrate of NE1434 by the tracer experiment, *in vitro* analysis with recombinants, or genetic experiments. Since NE1434 has significant similarity to PqqC, whose substrate and product are already known, studies of the detailed reaction mechanism of PqqC would be a shortcut to identify the substrate of NE1434. Otherwise, another approach such as analysis of the crystal structure of NE1434 might give us a clue.

The following aspects might also important in identifying it, though we cannot conceive an experimental plan utilizing them. One is the fact that orthologs of NE1434 are specifically distributed in the genomes of ammonia-oxidizing bacteria and archaea (Figure 4), which possess no *pabA*, -B, and -C homologs in their genomes. The other is that CT610 was reported to function as a modulator of host cell apoptosis via binding to the death domains of tumor necrosis factor family receptors (13).
The folate biosynthetic pathway has been a target for the development of antibacterial and antiparasitic drugs (1). Sulfonamide and diaminopyrimidine antibiotics are successful examples. Phylogenetic analysis (Figure 4) showed that obligate intracellular pathogenic bacteria such as *C. trachomatis* and *Neorickettsia sennetsu* possess NE1434 orthologs. Since an NE1434 ortholog is absent in mammalian cells, NE1434 orthologs would be an attractive target for development of antibiotics with high specificity to human pathogens.

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**Author contributions**

Y.S., M.K., and T.D. designed the experiments. Y.S. M.K., and D.K. performed the experiments. Y.S. and T.D. wrote the manuscript.

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pabB indicates a common evolutionary origin of p-aminobenzoate synthetase and


FIGURE LEGENDS

Figure 1 Folate biosynthetic pathway.
FolE, GTP cyclohydrolase I (LAF1339, NE1163); FolB, 7,8-dihydroneopterin aldolase (LAF1341, NE0223); FolK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (LAF1340, NE0070); FolP, dihydropteroate synthase (LAF1336, NE0529); FolC, dihydrofolate synthase (LAF1338, NE0696); FolA, dihydrofolate reductase (LAF0888, NE0567).

**Figure 2** Shotgun cloning experiment with the *E. coli* Δ*pabABC* mutant and genomic DNA of *N. europaea* NBRC 14298.

(A) DNA fragments obtained by shotgun cloning are shown schematically. The predicted function of each gene is as follows: NE1429, anhydro-N-acetylmuramic acid kinase; NE1430, M23/M37 family peptidase; NE1431, tyrosyl-tRNA synthetase; NE1432, transcriptional regulator; NE1433, serine hydroxymethyltransferase; NE1434, pyrroloquinoline-quinone synthase; NE1435, transcription regulator. Parentheses indicate restriction sites in pSTV29 cloning vector.

(B) Growth curves of *E. coli* BW25113 (parent strain) harboring pUC19 (blue dashed line), *E. coli* BW25113 harboring pUC-NE1434H (blue line), *E. coli* Δ*pabABC* mutant harboring pUC-NE1434H (red line), *E. coli* Δ*pabABC* mutant harboring pUC19 (red dashed line), and *E. coli* Δ*pabABC* mutant supplemented pABA in M9 minimum medium are shown.

**Figure 3** Tracer experiment using [U-$^{13}$C$_6$]glucose as the sole carbon source.

The *E. coli* Δ*pabABC* mutant expressing the *NE1434* gene was cultivated with unlabeled (left
column) and [U-\textsuperscript{13}C\textsubscript{6}]glucose (right column). After cultivation for 72 h, the culture broth was analyzed by LC/MS. The upper two chromatograms were selected for \( m/z = 138.056 \pm 0.010 \), which corresponds to the [M+H]\(^+\) ion for pABA with all unlabeled carbon. The lower two chromatograms were selected for \( m/z = 145.079 \pm 0.010 \) corresponding to the [M+H]\(^+\) ion for \( ^{13}\text{C} \)-labeled pABA. The upper line (blue) and lower line (red) in all four chromatograms indicate \textit{E. coli} \( \Delta pabABC \) harboring pUC-NE1434H and \textit{E. coli} \( \Delta pabABC \) harboring pUC18 (control), respectively.

**Figure 4** Phylogenetic tree of NE1434 and its orthologs.

The sequences of the NE1434 gene product and its orthologs were aligned using MEGA5 (8). A phylogenetic tree was constructed using the neighbor-joining method. The numbers on the tree indicate percentages from bootstrap sampling with 1000 replications.

**Figure 5** Complementation assay of the \textit{E. coli} \( \Delta pabABC \) mutant using the \textit{CT610} gene from \textit{C. trachomatis} and the \textit{pqqC} gene from \textit{P. putida}.

For the pABA auxotrophy complementation assay, pUC-CT610H and pUC-pqqC were introduced into the \textit{E. coli} \( \Delta pabABC \) mutant and the growth of the transformants was examined. Experimental procedures were the same as those used for the same experiment with \textit{E. coli} \( \Delta pabABC \) harboring pUC-NE1434H.
Figure 1

7,8-dihydropterin triphosphate

GTP

FolE

HCOO−

Phosphatase

PPI + Pi

7,8-dihydropterin

FolB

HOCH₂CHO

6-hydroxymethyl-7,8-dihydropterin

ATP

AMP

FolK

6-hydroxymethyl-7,8-dihydropterin pyrophosphate

Tetrahydrofolate

NADP⁺

NADPH + H⁺

FolA

7,8-Dihydrofolate

FolC

ATP + Glutamate

7,8-Dihydropteroate

FolP

PPI

Chorismate

Glutamine

Glutamate

PabA/PabB

ADC

PabC

COOH

COOH

NH₂

NH₂

pABA
Figure 2

(A) NE1429  NE1430  NE1431  NE1432  NE1433  NE1434  NE1435

\[\text{HindIII}\]

(EcoRI)

(HindIII)

6039 bp

6547 bp

(EcoRI)

(B)

\begin{align*}
\text{OD} & \quad \text{Times [h]} \\
0.0 & \quad 0 \quad 10 \quad 20 \quad 30 \quad 40 \quad 50 \quad 2.0
\end{align*}
Figure 3

![Graphs showing unlabeled and labeled data with m/z values 138.056 and 145.079 over time.](image)
Figure 4
**Figure 5**

<table>
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<th>CT610</th>
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<th>PP0378</th>
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<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pABA</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

![Image of test results]
### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> JM109</td>
<td>( F'[traD36, proAB, lacF, lacZΔM15], ) Δ(lac-proAB), hisG17 (pT17), recA1, endA1, relA1, supE44, thi-1, gyrA196, ala4 (merA1) )</td>
<td>Nippon Gene</td>
</tr>
<tr>
<td><em>E. coli</em> BW25113</td>
<td>( rnpA Δ.lacZ47387 hisR514 Δ(araBAD)567 Δ(araBAD)568 rph-1 )</td>
<td>NIG</td>
</tr>
<tr>
<td><em>E. coli</em> (ΔpolABC)</td>
<td>BW25113 derivative, (ΔpolA), (-B), and (-C)</td>
<td>(2)</td>
</tr>
<tr>
<td><em>L. fermentum</em> IFO 3936</td>
<td>wild type</td>
<td>NBRC</td>
</tr>
<tr>
<td><em>N. europaea</em> NBRC 14298</td>
<td>wild type</td>
<td>NBRC</td>
</tr>
<tr>
<td><em>C. trachomatis</em> UW-3/Cx</td>
<td>wild type</td>
<td>ATCC</td>
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<td>ATCC VR-885</td>
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<td></td>
</tr>
<tr>
<td><em>P. putida</em> NBRC 100650</td>
<td>KT2440 derivative, (rpoN)-Km'</td>
<td>NBRC</td>
</tr>
</tbody>
</table>

NIG: National Institute of Genetics, Shizuoka, Japan

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435
Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
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<tr>
<td>pET28</td>
<td>N-terminus His-tag fused protein expression vector, Km&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Novagen</td>
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<td>pET-NE2150</td>
<td>pET28 derivative; N-terminus His-tag fused NE2150</td>
<td>This study</td>
</tr>
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<td>pSTV29</td>
<td>cloning vector, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Takara Bio</td>
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<tr>
<td>pUC18</td>
<td>cloning vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Takara Bio</td>
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<tr>
<td>pUC-NE1434H</td>
<td>pUC18 derivative, C-terminus His-tag fused NE1434</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-CT610H</td>
<td>pUC18 derivative, CT610 from <em>C. trachomatis</em></td>
<td>This study</td>
</tr>
<tr>
<td>pUC-pqqC</td>
<td>pUC18 derivative, pqqC from <em>P. putida</em> KT2440</td>
<td>This study</td>
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