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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 ($\Delta pabABC$) as a host. A tracer experiment using [U-¹³C₆]glucose suggested that pABA was *de novo* synthesized in the transformant. An *E. coli* $\Delta pabABC\Delta aroB$ 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, *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 *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-dihydropterin with concomitant release of glycolaldehyde by 7,8-dihydroneopterin aldolase (FolB) and then pyrophosphorylated by 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (FolK). The resulting compound, 6-hydroxymethyl-7,8-dihydropterin 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 *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 *pabA*, *-B*, and *-C* (3) (Figure 1), suggesting that the strain utilizes an alternative pathway to synthesize pABA. Similarly, *Nitrosomonas europaea* NBRC14298 (ATCC19718) also has a complete set of folate biosynthetic gene orthologs, except for *pabA*, *-B*, and *-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 ($\Delta pabABC$) 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-¹³C₆]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 ($\Delta pabABC$).

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 MgSO₄, 0.1 mM CaCl₂]. 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 $\mu\text{g mL}^{-1}$ 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 *NdeI* and *BamHI* 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 *BamHI* and *HindIII* 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 *Bam*HI and *Hind*III 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 *Sau*3AI, 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_{lac}, Cm^r; Takara Bio Inc.) digested with *Bam*HI. pABA-auxotrophic *E. coli* Δ pabABC (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 pabABC Δ aroB, Δ pabABC Δ aroC, and Δ pabABC Δ aroD were transformed with appropriate plasmids. Each of the transformants (triplicate) grown on LB plates was dispersed in 200 μ L of sterilized distilled water and then a 50 μ L aliquot was inoculated into 5 mL of M9 medium supplemented with or without pABA. The growth was assessed at 30°C since *N. europaea*

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* $\Delta pabABC$

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 μ L) 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

by LC/MS.

Supernatants of the culture (5 μ L) 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 $\times$ 2.1 mm ID, 3 μ m; 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 $m/z = 138.056 \pm 0.010$ 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* Δ pabABC 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 (Δ pabABC). 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* $\Delta pabABC$ 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; dihydroneopterin aldolase, NE0223; 6-hydroxymethyl-7,8-dihydropterin 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 NH_4Cl 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, *NE1431* to *NE1434* (Figure 2A). To determine the essential gene(s) for complementation, the obtained plasmids were digested with *HindIII* and then self-ligated (Figure 2A). The constructed plasmid including *NE1434* gene again complemented the *E. coli* $\Delta pabABC$ mutant, showing that NE1434 was essential.

Cell growth of *E. coli* BW25113 (parental strain) and *E. coli* $\Delta pabABC$ mutant expressing *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 NE1434 gene would be involved in pABA biosynthesis.

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

Tracer experiment using [U-¹³C₆]glucose.

We next performed a tracer experiment using [U-¹³C₆]glucose to estimate the precursor compound of pABA. Since milligram quantities of labeled pABA are required for ¹³C-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* Δ *pabABC* 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*

213 gene product utilized a compound in the shikimate pathway by a genetic method. We
214 constructed *aroB* (3-dehydroquinate synthase), *aroC* (chorismate synthase), and *aroD*
215 (3-dehydroquinate dehydratase I)-knockout mutants from the *E. coli* Δ *pabABC* mutant. These
216 strains required aromatic amino acids, 4-hydroxy benzoate, and pABA for their growth.
217 However, all of the mutants harboring the *NE1434* gene were able to grow in M9 medium
218 without pABA (Figure S3), suggesting that none of the intermediates in the shikimate
219 pathway, except for 3-deoxy-D-arabinoheptulosonate-7-phosphate, could be the precursor for
220 NE1434-mediated pABA biosynthesis. To exclude the possibility that NE1434 utilized
221 aromatic amino acids and 4-hydroxy benzoate, which were added into the medium, as
222 precursors for pABA biosynthesis, we used these compounds as substrates for NE1434.
223 However, no products were formed. Taking these results together, we concluded that a new
224 enzyme/pathway for pABA biosynthesis is operating in *N. europaea*.

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

233

234 **Distribution of NE1434 orthologs.**

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

247 In the case of *Chlamydia*, *Chlamydia trachomatis* has a folate biosynthetic gene cluster
248 including *folA* (CT614), *-P* (CT613), and *-X* (CT612, *folB* ortholog) genes (5). However,
249 *pabA*, *-B*, and *-C* genes are missing from the genome as in *N. europaea*. However, the CT610
250 gene, an ortholog of *NE1434* (40% identity and 60% similarity), is located downstream of the
251 *folAPX* cluster in the same direction. To confirm whether CT610 participates in pABA
252 biosynthesis, the gene was cloned into a cloning vector (pUC-CT610H) and a pABA

auxotrophy complementation assay with the *E. coli* $\Delta pabABC$ 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* $\Delta pabABC$ (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. europaea*. 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* $\Delta pabABC$ 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.

273 Otherwise, the strain may biosynthesizes pABA in a totally different manner using a different
274 compound as the substrate.

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

282 We were not able to estimate the substrate of NE1434 by the tracer experiment, *in vitro*
283 analysis with recombinants, or genetic experiments. Since NE1434 has significant similarity
284 to PqqC, whose substrate and product are already known, studies of the detailed reaction
285 mechanism of PqqC would be a shortcut to identify the substrate of NE1434. Otherwise,
286 another approach such as analysis of the crystal structure of NE1434 might give us a clue.
287 The following aspects might also important in identifying it, though we cannot conceive an
288 experimental plan utilizing them. One is the fact that orthologs of NE1434 are specifically
289 distributed in the genomes of ammonia-oxidizing bacteria and archaea (Figure 4), which
290 possess no *pabA*, *-B*, and *-C* homologs in their genomes. The other is that CT610 was
291 reported to function as a modulator of host cell apoptosis via binding to the death domains of
292 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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FIGURE LEGENDS

Figure 1 Folate biosynthetic pathway.

373 FolE, GTP cyclohydrolase I (LAF1339, NE1163); FolB, 7,8-dihydroneopterin aldolase,
374 (LAF1341, NE0223); FolK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase
375 (LAF1340, NE0070); FolP, dihydropteroate synthase (LAF1336, NE0529); FolC,
376 dihydrofolate synthase (LAF1338, NE0696); FolA, dihydrofolate reductase (LAF0888,
377 NE0567).

378

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

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

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

391

392 **Figure 3** Tracer experiment using [U-¹³C₆]glucose as the sole carbon source.

393 The *E. coli* Δ *pabABC* mutant expressing the *NE1434* gene was cultivated with unlabeled (left

column) and [U-¹³C₆]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 ¹³C-labeled pABA. The upper line (blue) and lower line (red) in all four chromatograms indicate *E. coli* $\Delta pabABC$ harboring pUC-NE1434H and *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 *E. coli* $\Delta pabABC$ mutant using the *CT610* gene from *C. trachomatis* and the *pqqC* gene from *P. putida*.

For the pABA auxotrophy complementation assay, pUC-CT610H and pUC-pqqC were introduced into the *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 *E. coli* $\Delta pabABC$ harboring pUC-NE1434H.

Figure 1

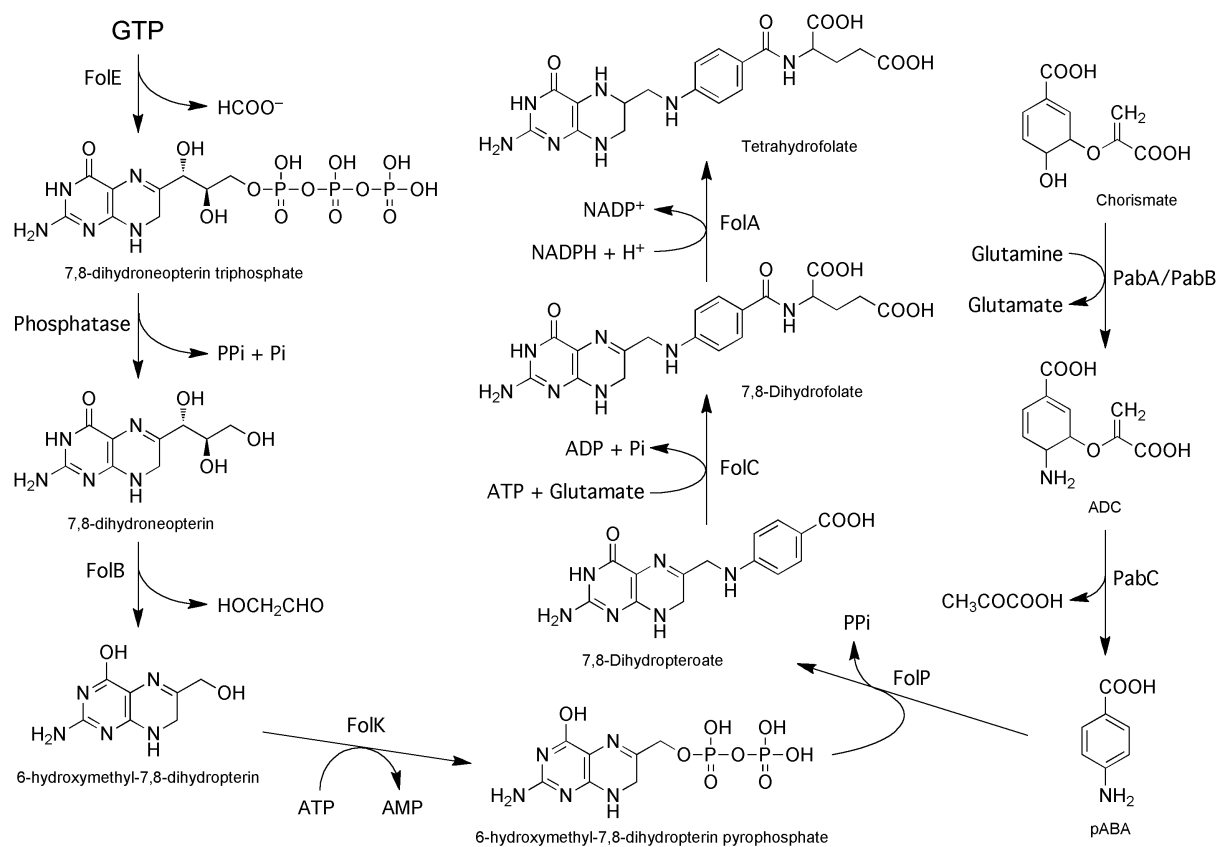


Figure 2

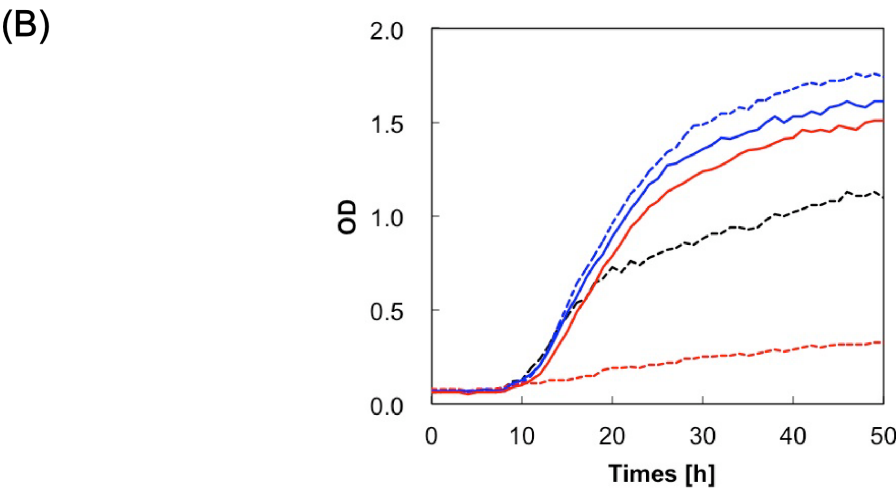
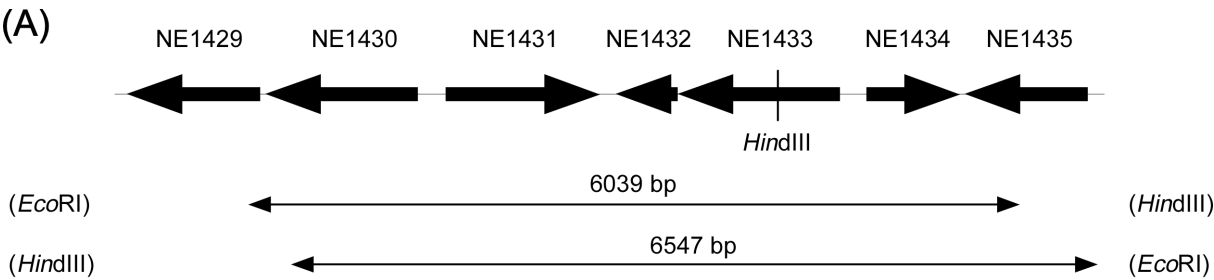


Figure 3

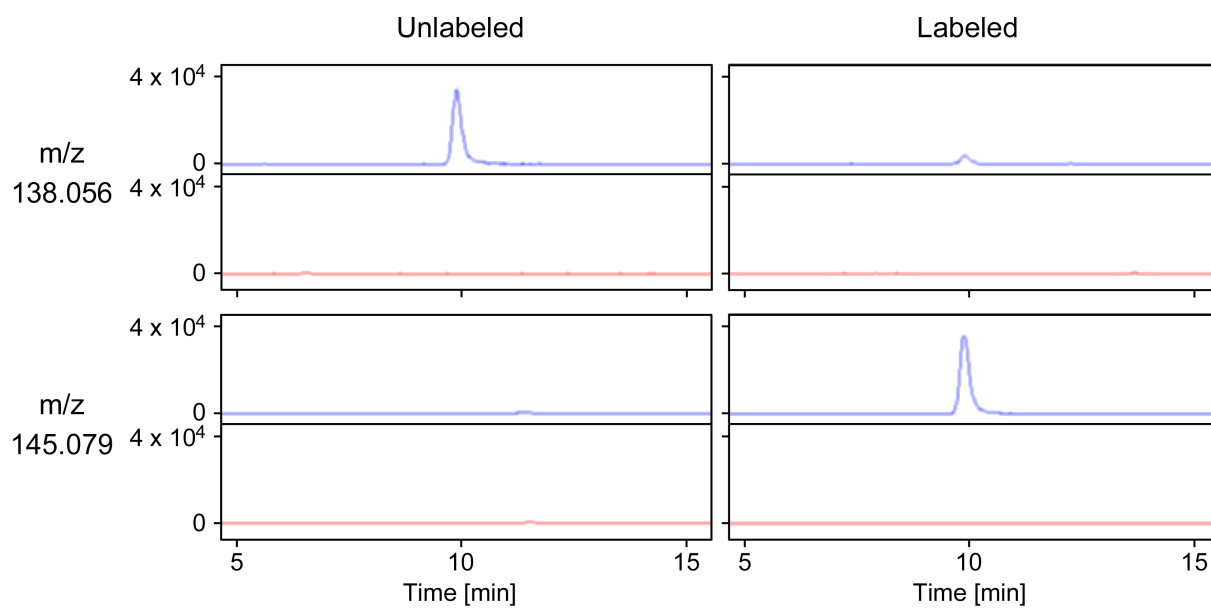
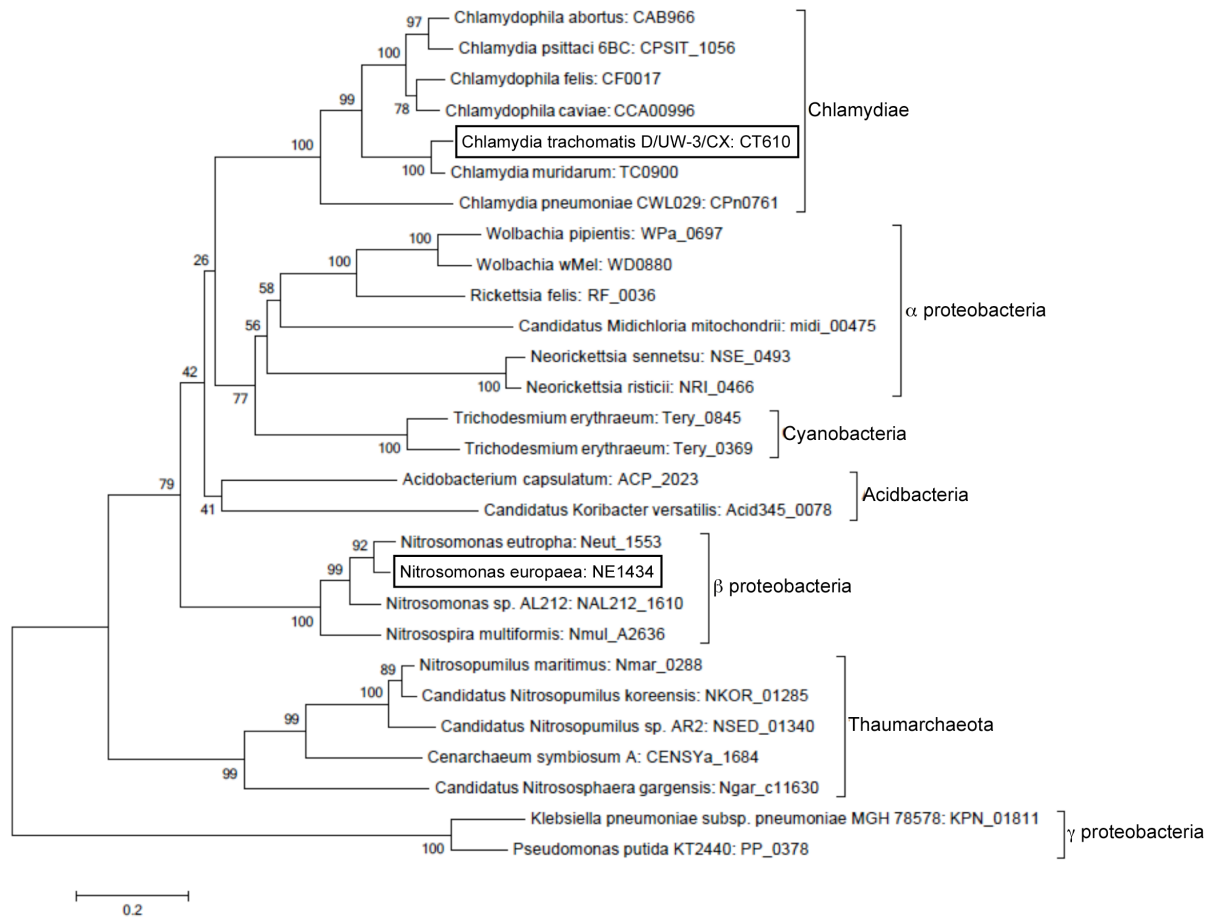
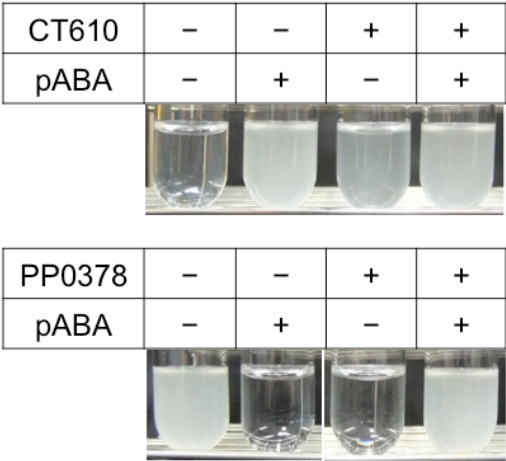


Figure 4



430 **Figure 5**
431



432
433

Table 1. Strains used in this study

Strains	Description	Source
<i>E. coli</i> JM109	<i>F'</i> [<i>traD36, proAB, lacI^f, lacZΔM15</i>], Δ (<i>lac-proAB</i>), <i>hsdR17</i> (<i>r_k⁻m_k⁺</i>), <i>recA1</i> , <i>endA1</i> , <i>relA1</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>e14⁻</i> (<i>mcrA⁻</i>)	Nippon Gene
<i>E. coli</i> BW25113	<i>rrmB</i> Δ <i>lacZ4787</i> <i>hsdR514</i> Δ (<i>araBAD</i>)567 Δ (<i>rhaBAD</i>)568 <i>rph-1</i>	NIG
<i>E. coli</i> Δ <i>pabABC</i>	BW25113 derivative, Δ <i>pabA</i> , - <i>B</i> , and - <i>C</i>	(2)
<i>L. fermentum</i> IFO 3956	wild type	NBRC
<i>N. europaea</i> NBRC 14298	wild type	NBRC
<i>C. trachomatis</i> UW-3/Cx	wild type	ATCC
ATCC VR-885		
<i>P. putida</i> NBRC 100650	KT2440 derivative; <i>rpoN::Km^r</i> ,	NBRC

NIG; National Institute of Genetics, Shizuoka, Japan

Table 2. Plasmids used in this study

Plasmids	Description	Source
pET28	N-terminus His-tag fused protein expression vector, Km ^r	Novagen
pET-NE2150	pET28 derivative; N-terminus His-tag fused NE2150	This study
pSTV29	cloning vector, Cm ^r	Takara Bio
pUC18	cloning vector, Ap ^r	Takara Bio
pUC-NE1434H	pUC18 derivative, C-terminus His-tag fused NE1434	This study
pUC-CT610H	pUC18 derivative, CT610 from <i>C. trachomatis</i>	This study
pUC-pqqC	pUC18 derivative, <i>pqqC</i> from <i>P. putida</i> KT2440	This study