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New gene responsible for *para*-aminobenzoate biosynthesis

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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 and -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-$^{13}C_6$]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.

[**Key words:** *para*-Aminobenzoate; Folate; *Nitrosomonas europaea*; *Lactobacillus fermentum*; *Chlamydia trachomatis*]

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 (Fig. 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) (Fig. 1), suggesting that the strain utilizes an alternative pathway to synthesize pABA. Similarly, *Nitrosomonas europaea* NBRC 14298 (ATCC 19718) also has a complete set of folate biosynthetic gene orthologs, except for *pabA*, *-B*, and *-C*, in its genome [Kyoto Encyclopedia of Genes and Genomes, KEGG, http://www.genome.jp/kegg/; (4)], despite its chemoautotrophic phenotype. In this paper, we performed shotgun cloning experiments with a pABA-auxotrophic *Escherichia coli* mutant (*ΔpabABC*) as a host and *L. fermentum* IFO 3956 and *N. europaea* NBRC 14298 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 an *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. *L. fermentum* IFO 3956 and *N. europaea* NBRC 14298 (ATCC 19718) were obtained from the Biological Resource Center, National Institute of Technology and Evaluation (NITE), Tokyo, Japan. *E. 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.

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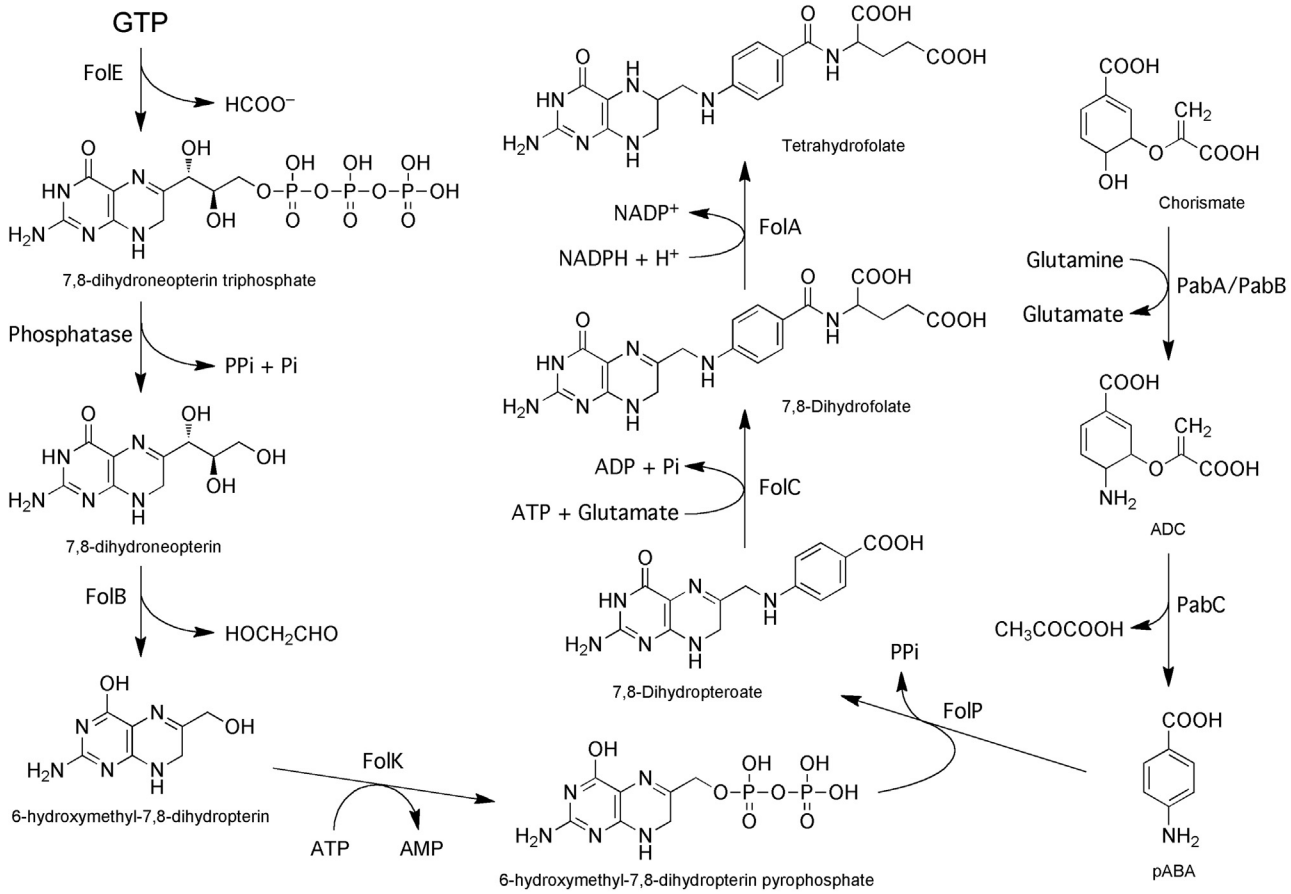

FIG. 1. Folate biosynthetic pathway. FolE, GTP cyclohydrolase I (LAF1339, NE1163); FolB, 7,8-dihydroneopterin aldolase, (LAF1341, NE0223); FolK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (LAF1340, NE0070); FolP, dihydropteroate synthase (LAF1336, NE0529); FolC, dihydrofolate synthase (LAF1338,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

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 *Hin*dIII 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 *Pseudomonas 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 *Hin*dIII 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

TABLE 1. Strains used in this study.

Strains	Description	Source
E. coli JM109	*F'[traD36, proAB, lacI^q, lacZ△M15], △(lac-proAB), hsdR17 ($r_K^- m_K^+$), recA1 endA1, relA1, supE44, thi-1, gyrA96, e14$^-$(mcrA$^-$)*	Nippon gene
E. coli BW25113	*rrnB △lacZ4787 hsdR514 △(araBAD)567△(rhaBAD)568 rph-1*	NIG
E. coli△pabABC	BW25113 derivative, △*pabA, -B,* and *-C*	2
L. fermentum IFO 3956	Wild type	NITE
N. europaea NBRC 14298	Wild type	NITE
C. trachomatis UW-3/Cx ATCC VR-885	Wild type	ATCC
P. putida NBRC 100650	KT2440 derivative; *rpoN*::Km^r	NITE

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 *C. trachomatis*	This study
pUC-pqqC	pUC18 derivative, *pqqC* from *P. putida* KT2440	This study