Title: Analysis of the metabolic pathway in *Lactobacillus*.

Running title: An alternative para-aminobenzoate biosynthetic pathway.

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We performed analyses of the phenotypic and genotypic relationships focusing on biosyntheses of amino acids, purine/pyrimidines, and co-factors in three *Lactobacillus* strains. We found that *Lactobacillus fermentum* IFO 3956 perhaps synthesized para-aminobenzoate (PABA), an intermediate of folic acid biosynthesis, by an alternative pathway.

The biosynthetic pathways of primary metabolites have been established with model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. For a long time, the biosynthetic routes established were believed to be common among all microorganisms. However, we now realize that some microorganisms possess alternative biosynthetic pathways since genome data base has enabled us to examine the presence or absence of orthologs of the genes responsible for known biosynthetic pathways. These surveys were one of the triggers to find the 2-*C*-methyl-D-erythritol 4-phosphate pathway (6) for isopentenyl diphosphate biosynthesis and the futalosine pathway (4) for menaquinone biosynthesis. As exemplified by the discovery of these pathways, microorganisms are still expected to have additional alternative biosynthetic pathways for the primary metabolites.

Lactobacilli are Gram positive lactic acid-producing bacteria with low G+C contents, and are utilized in the food industry (7,15). These bacteria are known to have mutations in the many primary metabolic pathways and require rich media containing various amino acids and nucleobases for their growth. After the whole genome sequence of *Lactobacillus plantarum* WCFS1 was determined in 2003 (5), phenotypic and genotypic analysis of the primary metabolic pathway in *Lactobacillus* strains commenced (1, 2, 8, 11 12, 14). All of these analyses, however, were performed with a database of the known biosynthetic pathways. We are interested in an alternative

biosynthetic pathway for primary metabolites in microorganisms. Considering that some *Lactobacillus* strains do not possess a part of orthologs of the known biosynthetic pathways and that the genome size of *Lactobacillus* strains are relatively large (1.8 to 3.4 M) compared to those of the symbiotic bacteria, such as Mycoplasma strains (0.6 to 1.4 M; http://www.genome.jp/kegg/catalog/org_list.html), we expected the presence of an alternative primary metabolic pathway in *Lactobacillus* strains. In this paper, we examined the phenotypic and genotypic relationships in *Lactobacillus fermentum* IFO 3956 (genome size, 2.1 M) (10), *Lactobacillus reuteri* JCM 1112 (2.0 M) (10), and *Lactobacillus brevis* ATCC 367 (2.3 M) (9), all of which showed relatively good growth with the following synthetic media: LSP medium (patent JP2000-279166; 20 g/L glucose, 3.1 g/L KH_2PO_4, 1.5 g/L K_2HPO_4, 2 g/L diammonium hydrogen citrate, 10 g/L potassium acetate, 1 g/L calcium lactate, 0.02 g/L NaCl, 1 g/L tween 80, 0.5 g/L $MgSO_4 \bullet 7H_2O$, 0.05 g/L $MnSO_4 \bullet 5H_2O$, 0.5 g/L $CoSO_4$).

As for the biosynthesis of amino acid, purine/pyrimidines, and vitamin (thiamine, nicotinate, pantothenate, riboflavin, and vitamin B6) biosynthetic pathways, the phenotypes of the three strains were essentially in agreement with the genotype (supporting Table 1, 2, and 3) by the single-omission growth test, although we found several discrepancies, such as prototrophic phenotype despite the absence of orthlog genes and auxotrophic phenotype despite the presence of orthlog genes. However, these discrepancies were limited to one of the steps of the established biosynthetic pathway. In contrast, we observed a discrepancy between the phenotype and genotype for the biosynthesis of folic acid. Both *L. fermentum* IFO 3956 and *L. reuteri* JCM 1112 did not require folic acid for their growth in contrast to *L. brevis* ATCC 367, which was auxotrophic for folic acid. The former two strains did not possess orthologs of *pabA*, *B*, and *C*, which were involved in the conversion of chorismate into PABA, a intermediate

of folic acid biosynthesis. Therefore, we investigated the biosynthesis of PABA in *L. fermentum* IFO 3956 in more detail. In contrast to the absence of *pabA*, *B*, and *C* in the strain IFO 3956, we found an ortholog of FolP (LAF_1336, EC 2.5.1.15), which catalyzes the formation of 7,8-dihydropteroate from PABA and 6-hydroxymethyl-dihydropterin diphosphate. Therefore, we examined if LAF_1336 showed the expected enzyme activity. We constructed a Δ*folP E. coli* mutant by homologous recombination with the Lambda Red System (Supporting Table 4 and Supporting Fig. 1). The constructed Δ*folP E. coli* mutant required folic acid for its growth (Supporting Fig. 2) and was used in complementation experiments. The Δ*folP E. coli* mutant transformed with a plasmid carrying a *folP* gene cloned from *E. coli* was able to grow reasonably in the absence of folic acid. Moreover, the Δ*folP E. coli* mutant harboring a plasmid carrying *LAF_1336* was also able to grow without folic acid (Supporting Fig. 2), demonstrating that LAF_1336 complemented the *fol*P defect.

We examined LAF_1336 using PABA as the substrate through two strategies. First, we constructed a Δ*folP*/Δ*pabABC E. coli* mutant for *in vivo* analysis. The Δ*folP E. coli* mutant was used for the starting strain and *pabA*, *pabB*, and *pabC* were successively disrupted by homologous recombination. The growth of the constructed mutant, in which PABA was not supplied from chorismate, was completely dependent on the presence of folic acid. When pUC118-FolP, carrying the *E.coli folP* gene, was introduced into the Δ*folP*/Δ*pabABC E. coli* mutant, the transformant was able to grow in a medium containing PABA as expected (Table 1). The growth of the Δ*folP*/Δ*pabABC E. coli* mutant transformed with pUC118-1336 carrying *LAF_1336* was also completely dependent on the presence of PABA. These results clearly suggested that LAF_1336 used PABA as the substrate for the formation of folic acid *via* 7,8-dihydropteroate.

Next, we examined if LAF_1336 used PABA as a substrate in *in vitro* experiments. One of the substrate of LAF_1336 (FolP), 6-hydroxymethyl-dihydropterin diphosphate, was not commercially available, therefore we employed a sequential enzymatic assay with 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (FolK) [EC 2.7.6.3] and the LAF_1336 (FolP) as the catalysts and commercially available 6-hydroxymethyl-dihydropterin as the substrate. The *E coli* FolK and LAF_1336 (FolP) were expressed as His-tagged proteins and MBP-fused proteins, respectively (Supporting Fig. 3). The purified enzymes were incubated with 6-hydroxymethyl-dihydropterin in the presence of ATP and PABA and the formation of 7,8-dihydropteroate was examined. As shown in Fig. 1, several specific products were detected by HPLC analysis and one of them was confirmed to be 7,8-dihydropteroate by LC-MS analysis. These *in vivo* and *in vitro* experiments clearly showed that LAF_1336 (FolP) used PABA as the substrate. This result strongly suggested that the strain would possess an alternative pathway for PABA biosynthesis. We are now attempting to clarify the details of this new pathway.

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Fig. 1. HPLC and LC-MS analyses of the products formed from 6-hydroxymethyl-dihydropterin with recombinant FolK and LAF_1336 (FolP).

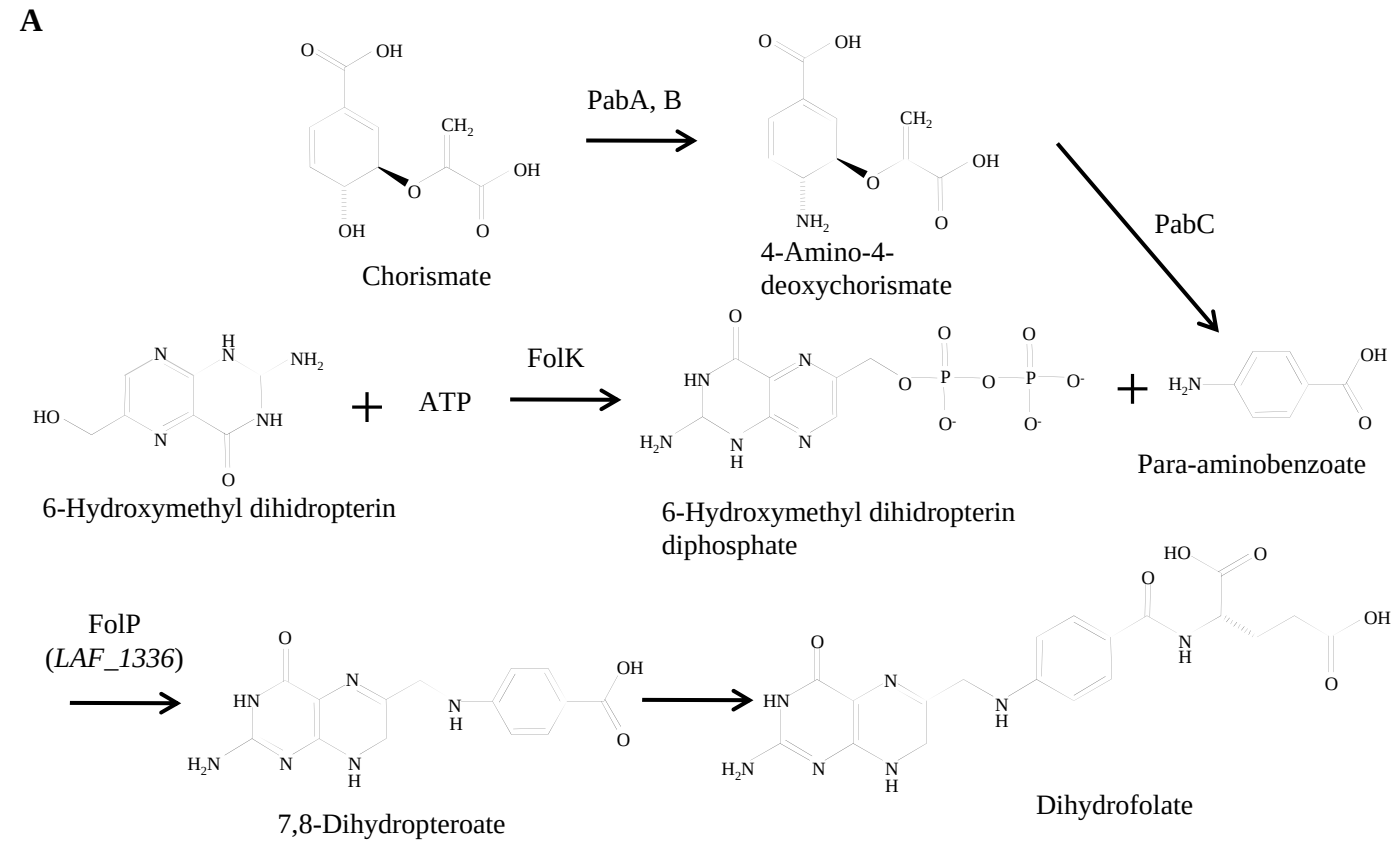
A: a schematic of dihydrofolate biosynthetic pathway from chorismate.

B: HPLC analysis of the reaction product without enzymes (**B-i**) and with both enzymes (**B-ii**). The peak of 7,8-dihydropteroate was subjected to LC-MS analysis (**B-iii**).

Table 1. Growth of the *ΔfolP*/*ΔpabABC* mutant and and its transformant harboring *E. coli folP* gene or *L. fermentum LAF_1336* gene.

Strain	- PABA	+ PABA
WT [pUC118: *folP*]	0.34	0.33
WT [pUC118: *LAF_1336*]	0.35	0.35
ΔfolP [pUC118: *folP*]	0.32	0.29
ΔfolP [pUC118: *LAF_1336*]	0.33	0.33
ΔpabA, ΔpabB, ΔpabC, ΔfolP [pUC118: *folP*]	0.00	0.50
ΔpabA, ΔpabB, ΔpabC, ΔfolP [pUC118: *LAF_1336*]	0.00	0.87

Growth of wild type (WT), *ΔfolP* mutant, and *ΔpabA, ΔpabB, ΔpabC, ΔfolP* mutant harboring pUC118 carrying *E. coli folP* gene or pUC118 carrying *LAF_1336* gene in M9 medium containing 1% glucose and ampicillin (0.1 mg/ml) was measured at OD_{600}.


A
PabA, B
PabC
Chorismate
4-Amino-4-deoxychorismate
FolK
ATP
6-Hydroxymethyl dihidropterin
6-Hydroxymethyl dihidropterin diphosphate
Para-aminobenzoate
FolP
(LAF_1336)
7,8-Dihydropteroate
Dihydrofolate

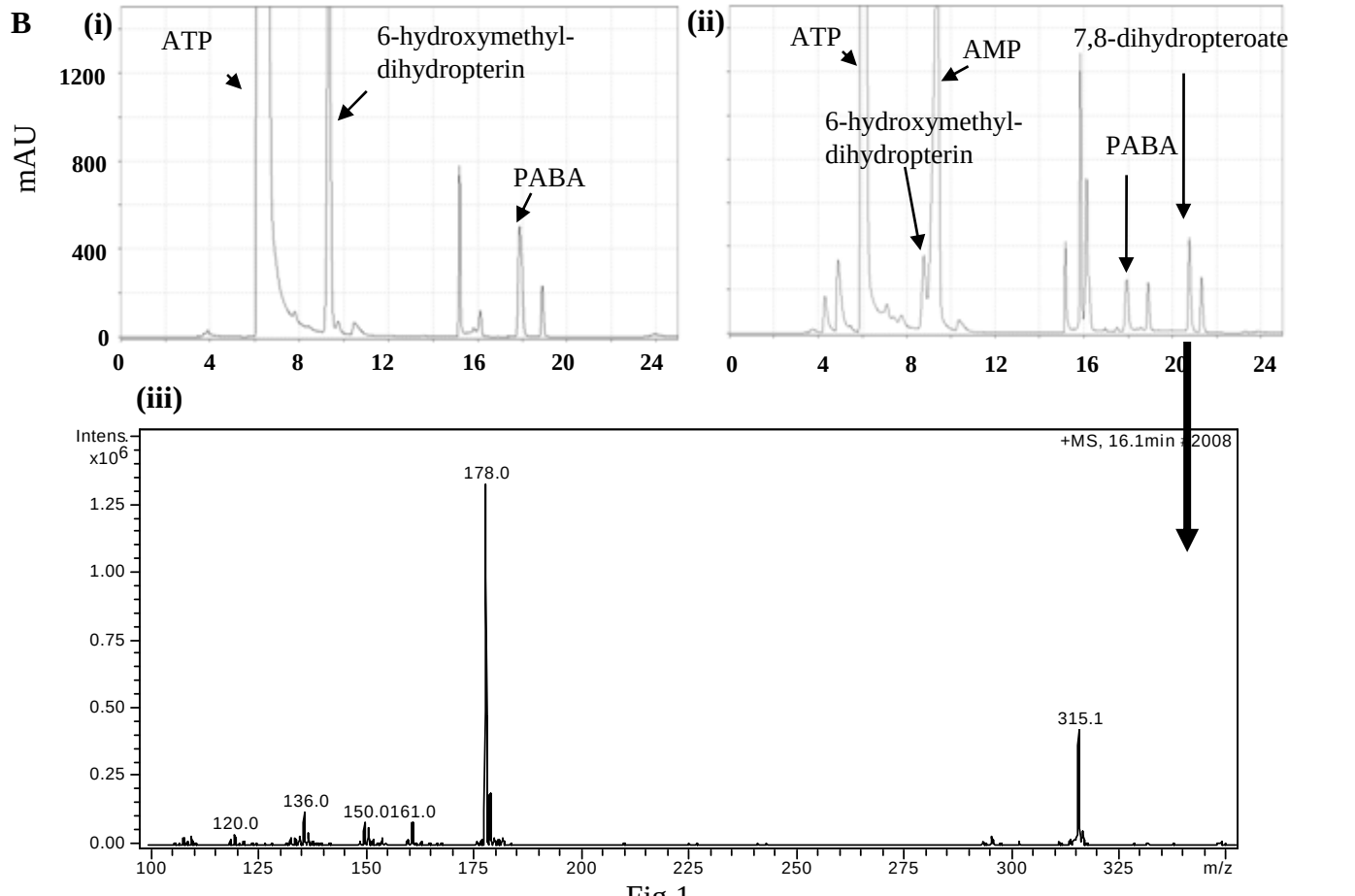

B
(i)
ATP
6-hydroxymethyl-dihydropterin
PABA
mAU
(ii)
ATP
AMP
7,8-dihydropteroate
6-hydroxymethyl-dihydropterin
PABA
(iii)
Intens.
x10^6
+MS, 16.1min #2008
178.0
315.1
120.0
136.0
150.0161.0
m/z

Fig.1.