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Analysis of the *Lactobacillus* Metabolic Pathway▽†

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We performed analyses of the phenotypic and genotypic relationships focusing on biosyntheses of amino acids, purine/pyrimidines, and cofactors 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 to all microorganisms. However, we now realize that some microorganisms possess alternative biosynthetic pathways since the genome database has enabled us to determine 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 expected to have additional alternative pathways for the biosynthesis of 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 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 pathway for biosynthesis of primary metabolites in microorganisms. Considering that some *Lactobacillus* strains do not possess some of the orthologs of the known biosynthetic pathways and that the genome sizes of *Lactobacillus* strains are relatively large (1.8 to 3.4 Mb) compared to those of the symbiotic bacteria, such as *Mycoplasma* strains (0.6 to 1.4 Mb) (http://www.genome.jp/kegg/catalog/org_list.html), we suspected 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 Mb) (10), *Lactobacillus reuteri* JCM 1112 (2.0 Mb) (10), and *Lactobacillus brevis* ATCC 367 (2.3 Mb) (9), all of which showed relatively good growth in LSP medium (3a) (20 g/liter glucose, 3.1 g/liter KH_2PO_4, 1.5 g/liter K_2HPO_4, 2 g/liter diammonium hydrogen citrate, 10 g/liter potassium acetate, 1 g/liter calcium lactate, 0.02 g/liter NaCl, 1 g/liter Tween 80, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 0.05 g/liter $MnSO_4 \cdot 5H_2O$, 0.5 g/liter $CoSO_4$).

As for the amino acid, purine/pyrimidine, and vitamin (thiamine, nicotinate, pantothenate, riboflavin, and vitamin B_6) biosynthetic pathways, the phenotypes of the three strains were essentially in agreement with the genotype (see Tables S1, S2, and S3 in the supplemental material) by the single-omission growth test, although we found several discrepancies, such as a prototrophic phenotype despite the absence of ortholog genes and an auxotrophic phenotype despite the presence of ortholog 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. Neither *L. fermentum* IFO 3956 nor *L. reuteri* JCM 1112 required 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* (13), which were involved in the conversion of chorismate into *para*-aminobenzoate (PABA), an intermediate of folic acid biosynthesis. Therefore, we investigated the biosynthesis of PABA in *L. fermentum* IFO 3956 in more detail. Although *pabA*, *-B*, and *-C* were absent in

TABLE 1. Growth of the Δ*folP* Δ*pabABC* mutant and its transformant harboring the *E. coli folP* gene or *L. fermentum LAF_1336* gene[a]

Strain genotype	OD_{600}	
	Without PABA	With PABA
WT [pUC118: *folP*]	0.34	0.33
WT [pUC118: *LAF_1336*]	0.35	0.35
Δ*folP* [pUC118: *folP*]	0.32	0.29
Δ*folP* [pUC118: *LAF_1336*]	0.33	0.33
Δ*pabA* Δ*pabB* Δ*pabC* Δ*folP* [pUC118: *folP*]	0.00	0.50
Δ*pabA* Δ*pabB* Δ*pabC* Δ*folP* [pUC118: *LAF_1336*]	0.00	0.87

[a] Growth of the wild type (WT), Δ*folP* mutant, and Δ*pabA* Δ*pabB* Δ*pabC* Δ*folP* mutant harboring pUC118 carrying the *E. coli folP* gene or carrying the *LAF_1336* gene in M9 medium containing 1% glucose and ampicillin (0.1 mg/ml) was determined by measuring the optical density at 600 nm (OD_{600}).

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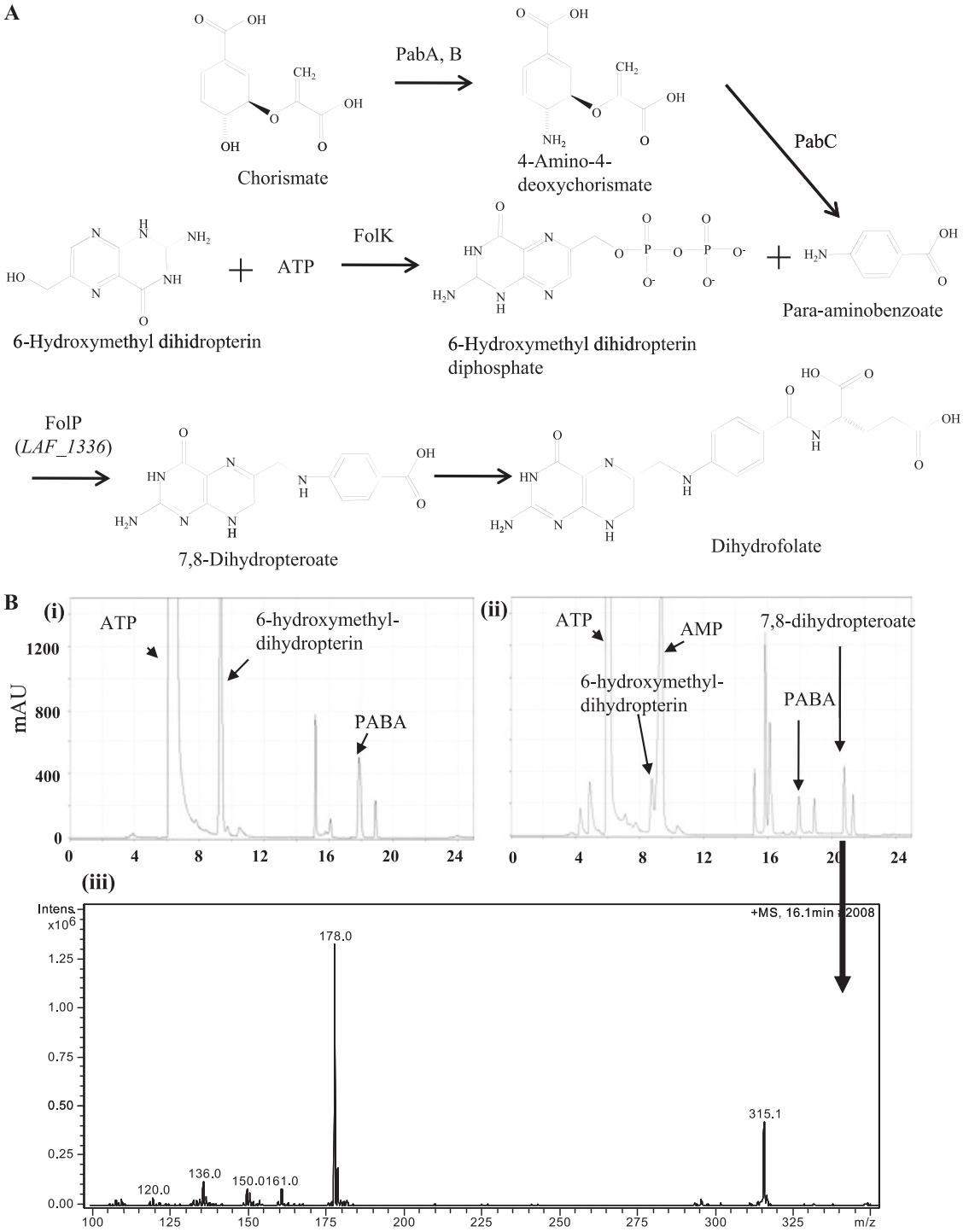

FIG. 1. HPLC and LC-MS analyses of the products formed from 6-hydroxymethyl-dihydropterin with recombinant FolK and LAF_1336 (FolP). (A) Schematic of the dihydrofolate biosynthetic pathway from chorismate. (B) HPLC analysis of the reaction product without enzymes (i) and with both enzymes (ii). The peak of 7,8-dihydropteroate was subjected to LC-MS analysis (iii).

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 (3) (see Table S4 and Fig. S1 in the supplemental material). The constructed Δ*folP E. coli* mutant required folic acid for its growth (see Fig. S2 in the supplemental material) 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 (see Fig. S2 in the supplemental material), demonstrating that LAF_1336 complemented the *folP* defect.

We examined LAF_1336 using PABA as the substrate by 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 as 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 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 substrates 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 LAF_1336 (FolP) as the catalysts and commercially available 6-hydroxymethyl-dihydropterin as the substrate. *E. coli* FolK and LAF_1336 (FolP) were expressed as His-tagged proteins and maltose-binding protein (MBP)-fused proteins, respectively (see Fig. S3 in the supplemental material). 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 high-pressure liquid chromatography (HPLC) analysis, and one of them was confirmed to be 7,8-dihydropteroate by liquid chromatography-mass spectrometry (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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