1 Supporting Material for "Analysis of the metabolic pathway in *Lactobacillus*."

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9 1. Supporting Materials and Methods.

10 2. Supporting Table 1 to 4.

11 3. Supporting Figures 1–3.

12 4. References.

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1. Supporting Materials and Methods.

Chemicals. All chemicals added into the medium were analytical grade.

Bacterial strains. *L. fermentum* IFO 3956 was obtained from the Biological Resource Center, National Institute of Technology and Evaluation, Japan. *L. reuteri* JCM 1112 and *L. brevis* ATCC 367 were obtained from the Japan Collection of Microorganisms (RIKEN BioResource Center).

Ortholog search. The Kyoto encyclopedia of genes and genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html) was used for the first screening of the presence or absence of an ortholog that participates in the known primary metabolic pathways in *Lactobacillus* strains. As for the genes that were absent in *Lactobacillus* strains in the KEGG pathway, the detailed analyses using BLAST searches (1) with the genes of *Escherichia coli* K-12 W3110 and *Lactobacillus* plantarum WCFS1 as queries were performed and estimated values (e-values) were calculated (we used e-value < 10-5 as a cutoff value).

Construction of Δ*folP* and Δ*folP*/Δ*pabABC* mutants. To examine the presence of an alternative pathway responsible for the formation of para-aminobenzoate (PABA), two *E. coli* mutants were constructed. One was a *folP*-disrupted mutant (Δ*folP*) and the other was *folP* and *pabABC*-disrupted mutant (Δ*folP*/Δ*pabABC*). Both of the mutants were constructed by the Lambda Red System (2).

In the first step, two DNA fragments containing a chloramphenicol resistance (*cat*) and *sacB* gene encoding a sucrase from *Bacillus subtilis* were amplified with two sets of primers. The L and L-CS primers were designed to contain sequences specific to the upstream region of the target genes. The R and R-CS primers were designed for the downstream region of the target gene. The *cat*-*sacB* casssette was constructed by fusion PCR with L and R primers, and the thus amplified fragments were introduced into *E. coli* BW25113 harboring the pKD46 plasmid. After induction of recombinant enzymes

40 with arabinose, chloramphenicol (Cm^{r}) resistant and sucrose sensitive colonies, in
41 which the targeted gene was replaced with the *cat*-*sacB* cassette, were selected.
42 In the second step, DNA fragments, in which the target genes were deleted in-frame,
43 were amplified. First, two DNA fragments carrying upstream and downstream regions
44 of the target gene were amplified with the L/RL and R/LR primers. Second, a target
45 gene-deleted fragment was amplified by fusion PCR with L and R primers. The fusion
46 PCR fragment was introduced into the strain constructed by the first step to replace the
47 *cat*-*sacB* casssette. Then, Cm^{r} sensitive and sucrose resistant colonies were selected and
48 gene-disruptions were confirmed by PCR. In the case of *folP* disruption, the *cat*-*sacB*
49 cassette was inserted between *folP* and the upstream gene, *ftsH*. A Δ*folP*/Δ*pabABC*
50 mutant was constructed from the Δ*folP* mutant by the same method as described above.
51 The *pabC*, *pabB*, and *pabA* genes were successively disrupted (Supporting Fig. 1). The
52 primers for their cassettes or fragments for disruption are shown in Supporting Table 4.
53 **Complementation of Δ*folP* and Δ*folP*/Δ*pabABC* mutants by the plasmids
54 carrying *E. coli folP* and *LAF_1336***. DNA fragments containing the *E. coli folP* gene
55 and *LAF_1336* gene were amplified by PCR with the primers 5′-
56 TTTGAATTCAGATGCTCTCATGAAATATGAGACTATCGACGC-3′, 5′-
57 TTTAAGCTTTTACTCATAGCGTTTGTTTTCCTTTGCAGACAG-3′ and 5′-
58 TTTGGATCCGGGAATTTACGATGGAAACCTGGGTCAGTC-3′, 5′-
59 TTTAAGCTTTTACAGTTGCCAGTAACCACTGGTAATCGTGTC-3′, respectively.
60 After sequence confirmation, each of the fragments was inserted into *EcoR*I-*Hind*III and
61 *BamH*I-*Hind*III digested pUC118 to construct pUC118-FolP and pUC118-1336, in
62 which the *folP* gene and *LAF_1336* could be expressed under the control of the native
63 promoter. The constructed plasmids were introduced into the Δ*folP* and Δ*folP*/Δ*pabABC*
64 mutants and the growth of the transformants on a minimal medium (M9 medium), in
65 which folic acid or PABA was added if necessary, was examined.

66 **A sequential enzymatic assay with the FolK and the LAF_1336 (FolP).** The *E. coli*
67 *folK* gene was amplified by PCR with the primers,
68 5′-TTTGGATCCACAGTGGCGTATATTGCCATAGG-3′ and
69 5′-TTTAAGCTTTTACCATTTGTTTAATTTGTCAAATGCTCTTGTATG -3′. After
70 sequence confirmation, a 0.5 kb fragment was inserted into the same site of pQE30. A
71 plasmid, pQE30-FolK, in which a recombinant protein was expressed as an *N*-terminal
72 6 × His-tagged fusion protein, was selected. The *LAF_1336 (FolP)* gene was also
73 amplified by PCR with 5′-TTTGGATCCCCAACCACCACGGACGCCTTTAACCA
74 -3′ and 5′-TTTAAGCTTTTACAGTTGCCAGTAACCACTGGTAATCGTGTC-3′.
75 After sequence confirmation, the fragment was inserted into the *BamH*I and *Hind*III
76 sites of pMAL-c2X to construct pMAL-1336. *N*-Terminal His-tagged *E. coli* FolK and
77 maltose binding protein (MBP)-fused LAF_1336 were expressed in *E. coli* and purified
78 by a Ni-nitrilotriacetic acid column and amylose affinity colum (Supporting Fig. 2)
79 These recombinant enzymes were used for an *in vitro* enzyme assay. The reaction
80 mixture (100 μl) contained 5 mM 6-hydroxymethyl-dihydropterin, 10 mM ATP, 1mM
81 $MgCl_2$, 0.5% (v/v) 2-mercaptoethanol, 1 mM PABA, 50 mM Tris-HCl (pH 8.0), and
82 220 μg/ml of the purified His-tagged FolK and 80μg/ml of the purified MBP-fused
83 LAF_1336 (FolP). The formation of 7,8-dihydropteroate was examined by liquid
84 chromatography (LC) and LC-mass-spectrometry analysis. The HPLC conditions
85 involved a mobile phase where the concentration of acetonitrile in 20 mM potassium
86 phosphate (pH 2.5) was stepwise increased (0–5 min, 0%; 6 min, 8%; 15 min 20%; 16
87 min, 80%; 17–25 min, 0%) on a Mightisil RP-18 GP column (250 × 4.6 mm) with a
88 flow rate of 1.5 ml/min and detection at 210 nm.
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Supporting Table 1. Growth of *Lactobacillus* strains in single-amino-acid-omitted medium.

Amino acids omitted	*L. fermentum*	*L. reuteri*	*L. brevis*
His	N (O) [a]	E (X)	E (X)
Ser	N (O ← Cys) [b]	E (X)	E (X)
Cys	N (O ← Ser) [b]	N (O ← Ser) [b]	E (X)
Gly	N (O)	N (O ← Ser) [b]	E (O) [f]
Ala	N (O)	N (O)	N (O)
Val	E (X) [c]	E (X)	E (X)
Leu	E (X)	E (X)	E (X)
Asp	N (O)	N (O)	N (O ← Asn) [g]
Asn	N (O)	N (O)	N (O ← Asp) [g]
Lys	N (O)	N (O)	E (X)
Met	E (O) [d]	E (O) [d]	E (X)
Thr	E (O) [d]	E (X)	E (X)
Iso	E (X)	E (X)	E (X)
Try	E (X)	E (X)	E (X)
Tyr	E (X)	E (X)	E (X)
Phe	E (X)	E (X)	E (X)
Glu	N (X ← Gln) [e]	N (O ← Gln) [e]	E (X)
Gln	N (O ← Glu) [e]	N (O ← Glu) [e]	N (O ← Glu) [e]
Pro	N (O)	N (O)	E (X)
Arg	E (X)	E (X)	E (X)

[a] N; Non-essential (grow without the amino acid) , O; presence of whole set of biosynthetic genes.
[b] Ser, Cys, and Gly would be synthesized from Cys, Ser, and Ser by inter-conversions, respectively.
[c] E; Essential (not grow without the amino acid) , X; absence of at least one biosynthetic gene.
[d] Some genes responsible for the formation of Thr /Met from homoserine would be non-functional or pseudo genes.
[e] Glu (Gln) would be synthesized from Gln (Glu) by inter-conversion.
[f] Glycine hydroxymethyltransferase (EC 2.1.2.1, *glyA*) gene would be non-functional.
[g] Asn (Asp) would be synthesized from Asp (Asn) by inter-conversion.

Supporting Table 2. Growth of *Lactobacillus* strains in single-purine/pyrimidine-omitted medium.

Purine/Pyrimidine	*L. fermentum*	*L. reuteri*	*L. brevis*
Purine	N (O)	E (O) [a]	E (X)
Pyrimidine	N (O)	N (O)	E (X)

[a] some genes responsible for the formation of IMP from PRPP would be nonfunctional or pseudogene (require IMP for the growth).

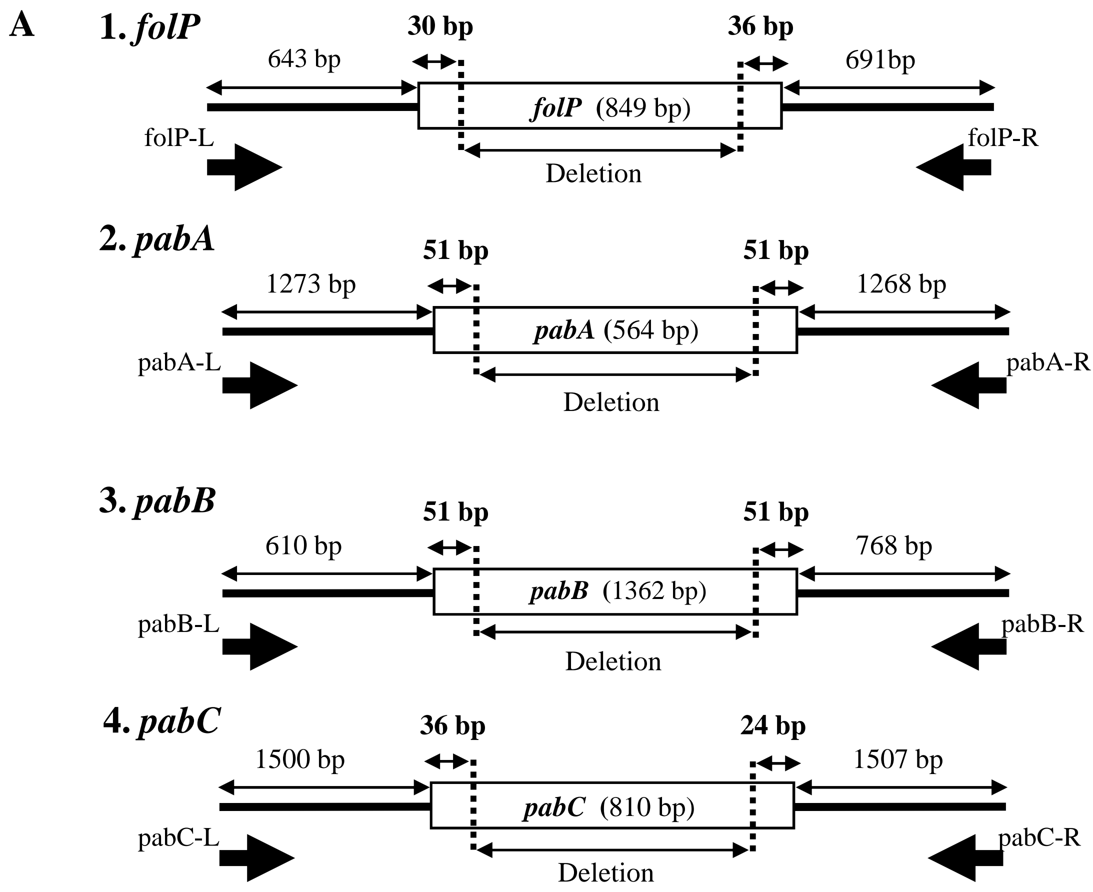
Supporting Table 3. Growth of *Lactobacillus* strains in single-vitamin-omitted medium.

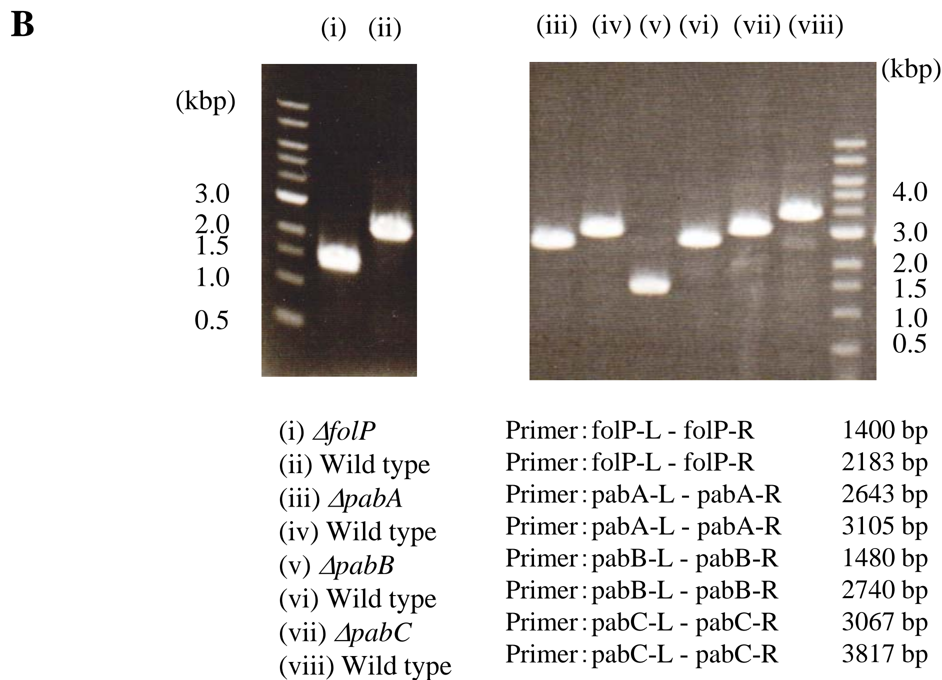
Vitamine	*L. fermentum*	*L. reuteri*	*L. brevis*
Thiamine	E (X)[a]	E (X)[a]	E (X)[a]
Nicotinate (NAD, NADP)	E (X)	E (X)	E (X)
Panthothenate (CoA)	E (X)	E (X)	E (X)
Riboflavin (FMN, FAD)	N (O)	N (O)	N (O)
Vitamin B6 (PLP etc.)	S (X)[b]	E (X)	E (X)
Folic acid (THF)	N (X)[c]	N (X)[b]	E (X)

[a] Presence of most of the orthologs but lacked orthologs of ThiC
[b] S; growth stimulate effect.
[c] Absence of the genes responsible for the formation of *p*-aminobenzoate from chorismate

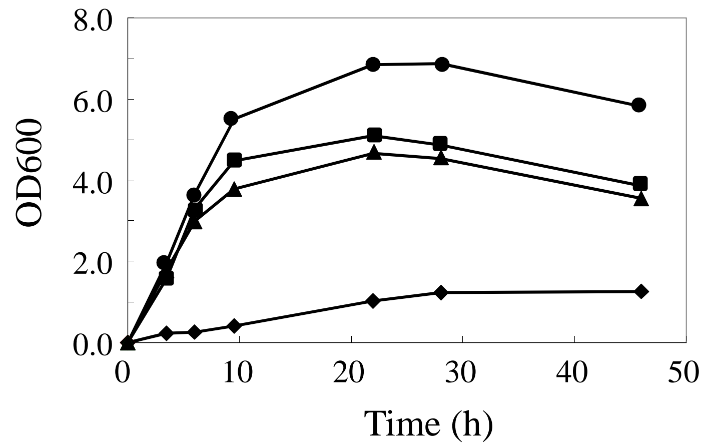
Supporting Table 4. Primers used for *ΔfolP and ΔfolP/ΔpabABC mutants*

Gene	Step	Primer	Sequence
pabA	1st step	L	AACAACACTACCTTTCACCGCGTCATTCCT
(cat-sacB cassete)		L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCctggtagaggttccaggtaaaagaatcgta
		R-CS	CGCAAAAGAAAATGCCGATATGCGGCCGCattcttagcgaacaaggacatcaactgctg
		R	GTTGCATCCCTTCATCGATATCCGCATCTT
	2nd step	RL	cagcagttgatgtccttgttcgctaagaatCTGGTAGAGGTTCCAGGTAAAAGAATCGTA
(in-frame deletion)		LR	TACGATTCTTTTACCTGGAACCTCTACCAGattcttagcgaacaaggacatcaactgctg
pabB	1st step	L	accaagttcgatgaatgaaagcacggcgaa
(cat-sacB cassete)		L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCagtaatcacagcgggagataacgtcttcat
		R-CS	CGCAAAAGAAAATGCCGATATGCGGCCGCaatcgtatcctgaagcaactggagaagtaa
		R	cacgataatactcctgacaaggtgactgga
	2nd step	RL	ttacttctccagttgcttcaggatacgattAGTAATCACAGCGGGAGATAACGTCTTCAT
(in-frame deletion)		LR	ATGAAGACGTTATCTCCCGCTGTGATTACTaatcgtatcctgaagcaactggagaagtaa
pabC	1st step	L	aatcaccaccgttcaggctgccattga
(cat-sacB cassete)		L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCcagcgattcctgcttataaccgttaattaagaacat
		R-CS	CGCAAAAGAAAATGCCGATATGCGGCCGCccactttgtgagcgcccgaattag
		R	cgaaagactcttgctcaatacgatccagct
	2nd step	RL	TGCTGACGAAAAGGAGACATCGCCccgatcgcttactgccagcgattc
(in-frame deletion)		LR	gaatcgctggcagtaagcgatcggGGCGATGTCTCCTTTTCGTCAGCA
folP	1st step	L	aactggaaagccagatttctacgctgtacg
(cat-sacB cassete)		L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCttacttgtcgcctaactgctctgacatggt
		R-CS	CGCAAAAGAAAATGCCGATATGCGGCCGCgttcccgcatcagatgactgtatttgtacc
		R	tttttcagccagcacacgagcctgga
	2nd step	RL	ttactcatagcgtttgttttcctttgcagacagagtCAGTGAAGTACCCTGGGCAAAGAGTTTCAT
(in-frame deletion)		LR	ATGAAACTCTTTGCCCAGGGTACTTCACTGactctgtctgcaaaggaaaacaaacgctatgagtaa

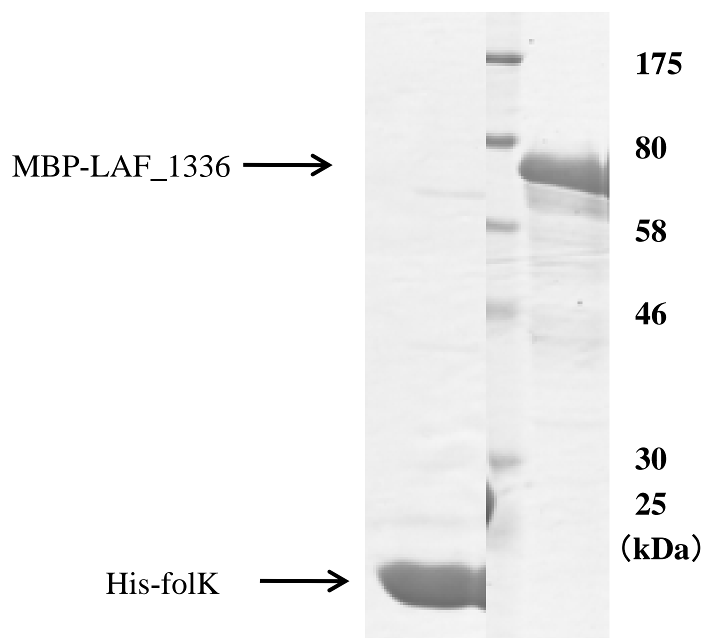




Supporting Fig. 1. The *folP*, *pabA, pabB,* and *pabC* genes and their flanking regions are shown (A). PCR analysis of the Genomic DNA of the constructed mutants (B).



Supporting Fig.2. Growth of the *ΔfolP* mutant harboring pUC118 (diamond), wild type strain harboring pUC118 in the presence of folic acid (triangle), *ΔfolP* mutant harboring pUC118 carrying the *folP* gene cloned from *E. coli* (square), and *ΔfolP* mutant harboring pUC118 carrying the *folP* gene cloned from *L. fermentum* IFO 3956 (*LAF_1336*) (circle) in Luria broth containing ampicillin was measured.



Supporting Fig. 3. Electrophoresis of the overproduced and purified *E coli* FolK and LAF_1336 (FolP)

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