



Title	Analysis of the Lactobacillus Metabolic Pathway
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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.

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13 **1. Supporting Materials and Methods.**

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

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

19 **Ortholog search.** The Kyoto encyclopedia of genes and genomes (KEGG)
20 pathway database (<http://www.genome.jp/kegg/pathway.html>) was used for the first
21 screening of the presence or absence of an ortholog that participates in the known
22 primary metabolic pathways in *Lactobacillus* strains. As for the genes that were absent
23 in *Lactobacillus* strains in the KEGG pathway, the detailed analyses using BLAST
24 searches (13) with the genes of *Escherichia coli* K-12 W3110 and *Lactobacillus*
25 *plantarum* WCFS1 as queries were performed and estimated values (e-values) were
26 calculated (we used e-value < 10⁻⁵ as a cutoff value).

27 **Construction of $\Delta folP$ and $\Delta folP/\Delta pabABC$ mutants.** To examine the presence of
28 an alternative pathway responsible for the formation of para-aminobenzoate (PABA),
29 two *E. coli* mutants were constructed. One was a *folP*-disrupted mutant ($\Delta folP$) and the
30 other was *folP* and *pabABC*-disrupted mutant ($\Delta folP/\Delta pabABC$). Both of the mutants
31 were constructed by the Lambda Red System (3).

32 In the first step, two DNA fragments containing a chloramphenicol resistance (*cat*)
33 and *sacB* gene encoding a sucrose from *Bacillus subtilis* were amplified with two sets of
34 primers. The L and L-CS primers were designed to contain sequences specific to the
35 upstream region of the target genes. The R and R-CS primers were designed for the
36 downstream region of the target gene. The *cat-sacB* cassette was constructed by fusion
37 PCR with L and R primers, and the thus amplified fragments were introduced into *E.*

38 *coli* BW25113 harboring the pKD46 plasmid. After induction of recombinant enzymes
39 with arabinose, chloramphenicol (Cm^r) resistant and sucrose sensitive colonies, in
40 which the targeted gene was replaced with the *cat-sacB* cassette, were selected.

41 In the second step, DNA fragments, in which the target genes were deleted in-frame,
42 were amplified. First, two DNA fragments carrying upstream and downstream regions
43 of the target gene were amplified with the L/RL and R/LR primers. Second, a target
44 gene-deleted fragment was amplified by fusion PCR with L and R primers. The fusion
45 PCR fragment was introduced into the strain constructed by the first step to replace the
46 *cat-sacB* cassette. Then, Cm^r sensitive and sucrose resistant colonies were selected and
47 gene-disruptions were confirmed by PCR. In the case of *folP* disruption, the *cat-sacB*
48 cassette was inserted between *folP* and the upstream gene, *ftsH*. A $\Delta folP/\Delta pabABC$
49 mutant was constructed from the $\Delta folP$ mutant by the same method as described above.
50 The *pabC*, *pabB*, and *pabA* genes were successively disrupted (Supporting Fig. 1). The
51 primers for their cassettes or fragments for disruption are shown in Supporting Table 1.

52 **Complementation of $\Delta folP$ and $\Delta folP/\Delta pabABC$ mutants by the plasmids**
53 **carrying *E. coli folP* and *LAF_1336*.** DNA fragments containing the *E. coli folP* gene
54 and *LAF_1336* gene were amplified by PCR with the primers 5'-
55 TTTGAATTCAGATGCTCTCATGAAATATGAGACTATCGACGC-3', 5'-
56 TTTAAGCTTTTACTCATAGCGTTTGTTCCTTTGCAGACAG-3' and 5'-
57 TTTGGATCCGGGAATTTACGATGGAAACCTGGGTCAGTC-3', 5'-
58 TTTAAGCTTTTACAGTTGCCAGTAACCACTGGTAATCGTGTC-3', respectively.
59 After sequence confirmation, each of the fragments was inserted into *EcoRI-HindIII* and
60 *BamHI-HindIII* digested pUC118 to construct pUC118-FolP and pUC118-1336, in
61 which the *folP* gene and *LAF_1336* could be expressed under the control of the native
62 promoter. The constructed plasmids were introduced into the $\Delta folP$ and $\Delta folP/\Delta pabABC$

63 mutants and the growth of the transformants on a minimal medium (M9 medium), in
64 which folic acid or PABA was added if necessary, was examined.

65 **A sequential enzymatic assay with the FolK and the LAF_1336 (FolP).** The *E. coli*
66 *folK* gene was amplified by PCR with the primers,
67 5'-TTTGGATCCACAGTGGCGTATATTGCCATAGG-3' and
68 5'-TTTAAGCTTTTACCATTTGTTTAATTTGTCAAATGCTCTTGTATG -3'. After
69 sequence confirmation, a 0.5 kb fragment was inserted into the same site of pQE30. A
70 plasmid, pQE30-FolK, in which a recombinant protein was expressed as an *N*-terminal
71 6 × His-tagged fusion protein, was selected. The *LAF_1336 (FolP)* gene was also
72 amplified by PCR with 5'-TTTGGATCCCCAACCACCACGGACGCCTTTAACCA
73 -3' and 5'-TTTAAGCTTTTACAGTTGCCAGTAACCACTGGTAATCGTGTC-3'.
74 After sequence confirmation, the fragment was inserted into the *Bam*HI and *Hind*III
75 sites of pMAL-c2X to construct pMAL-1336. *N*-Terminal His-tagged *E. coli* FolK and
76 maltose binding protein (MBP)-fused LAF_1336 were expressed in *E. coli* and purified
77 by a Ni-nitrilotriacetic acid column and amylose affinity column (Supporting Fig. 2)
78 These recombinant enzymes were used for an *in vitro* enzyme assay. The reaction
79 mixture (100 µl) contained 5 mM 6-hydroxymethyl-dihydropterin, 10 mM ATP, 1mM
80 MgCl₂, 0.5% (v/v) 2-mercaptoethanol, 1 mM PABA, 50 mM Tris-HCl (pH 8.0), and
81 220 µg/ml of the purified His-tagged FolK and 80µg/ml of the purified MBP-fused
82 LAF_1336 (FolP). The formation of 7,8-dihydropteroate was examined by liquid
83 chromatography (LC) and LC-mass-spectrometry (M) analysis. The HPLC conditions
84 involved a mobile phase where the concentration of acetonitrile in 20 mM potassium
85 phosphate (pH 2.5) was stepwise increased (0–5 min, 0%; 6 min, 8%; 15 min 20%; 16
86 min, 80%; 17–25 min, 0%) on a Mightisil RP-18 GP column (250 × 4.6 mm) with a
87 flow rate of 1.5 ml/min and detection at 210 nm.

Supporting Table 1. Growth of *Lactobacillus* strains in single-amino-acid-omitted medium.

Amino acids omitted	<i>L. fermentum</i>	<i>L. reuteri</i>	<i>L. brevis</i>
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	<i>L. fermentum</i>	<i>L. reuteri</i>	<i>L. brevis</i>
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	<i>L. fermentum</i>	<i>L. reuteri</i>	<i>L. brevis</i>
Thiamine	E (X) ^a	E (X) ^a	E (X) ^a
Nicotinate (NAD, NADP)	E (X)	E (X)	E (X)
Panθοthenate (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)

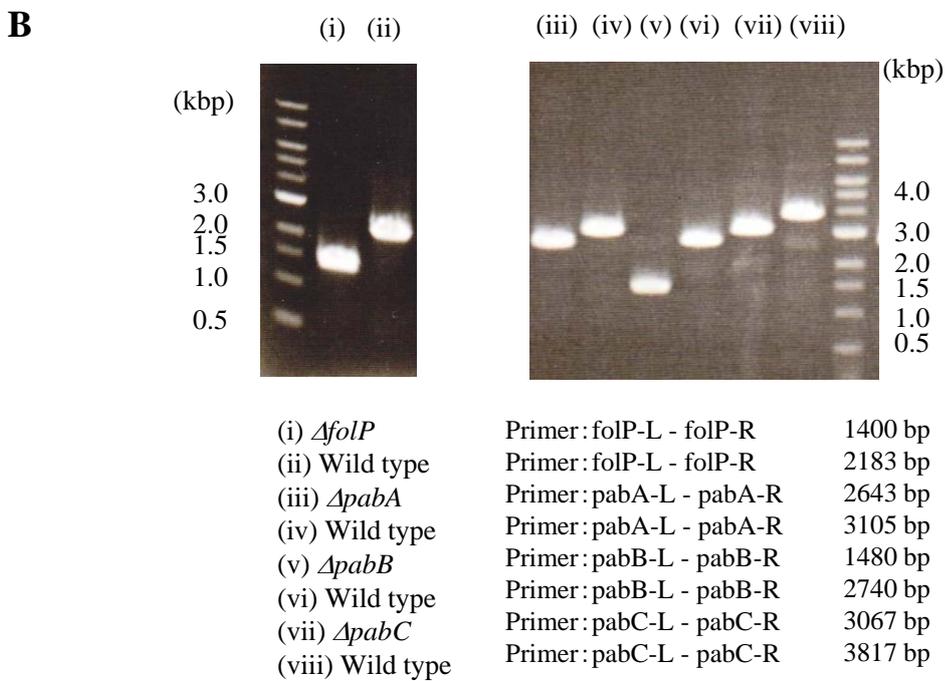
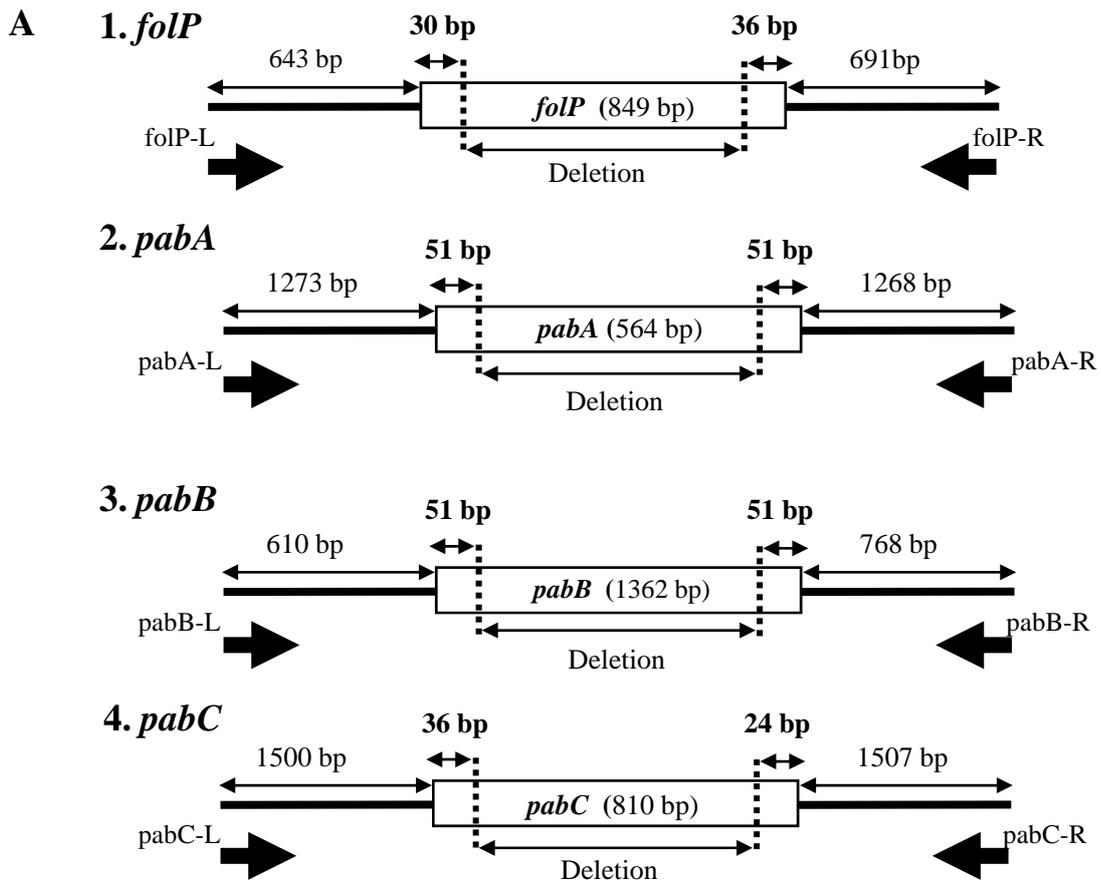
^a Presence of most of the orthologs but lacked orthologs of ThiC

^b S; growth stimulate effect.

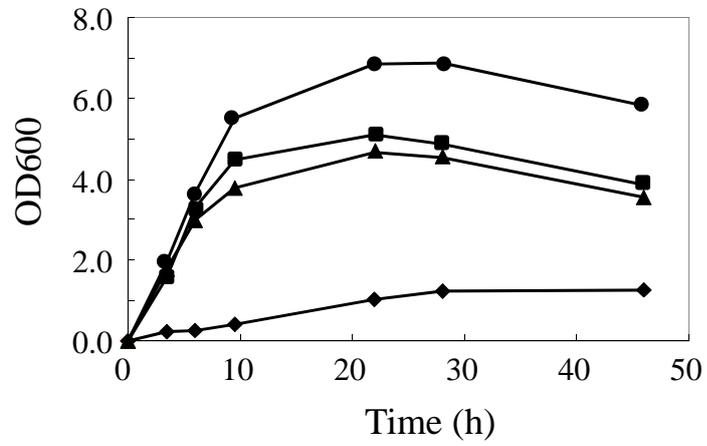
^c Absence of the genes responsible for the formation of *p*-aminobenzoate from chorismate

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

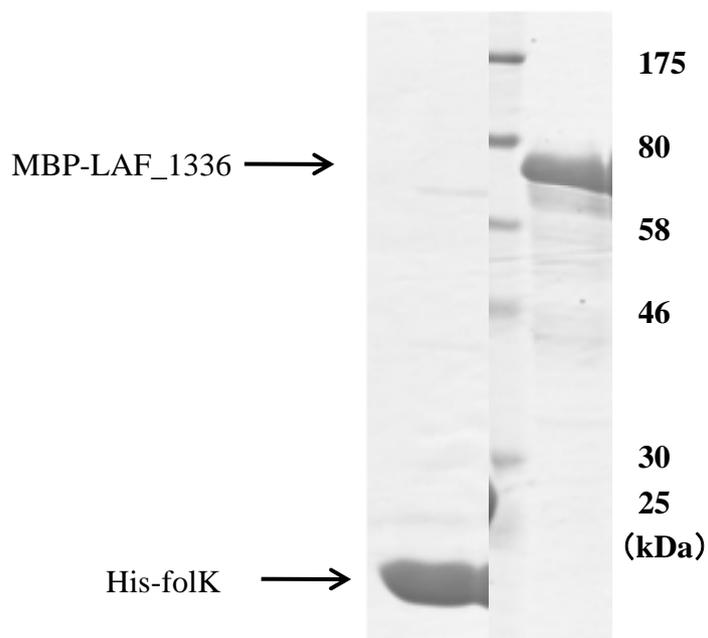
pabA (cat-sacB cassette)	1st step	L	AACAACACTACCTTTCACCGCGTCATTCCT
	L-CS	L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCctgtagaggtccaggtaaagaatcgta
		R-CS	CGCAAAGAAAATGCCGATATGCGGCCGCattcttagcgaacaaggacatcaactgctg
	R	R	GTTGCATCCCTTCATCGATATCCGCATCTT
(in-frame deletion)	2nd step	RL	cagcagttgatgtccttgttcgctaagaatCTGGTAGAGGTTCCAGGTAAGAATCGTA
	LR	LR	TACGATTCTTTTACCTGGAACCTCTACCAGattcttagcgaacaaggacatcaactgctg
pabB (cat-sacB cassette)	1st step	L	accaagttcgaatgaaagcagggcgaa
	L-CS	L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCagtaatcacagcgggagataacgtcttcat
		R-CS	R-CS
	R	R	cacgataatactcctgacaaggtgactgga
(in-frame deletion)	2nd step	RL	ttacttctccagttgctcaggatagattAGTAATCACAGCGGGAGATAACGTCTTCAT
	LR	LR	ATGAAGACGTTATCTCCCGCTGTGATTACTaatcgtatcctgaagcaactggagaagtaa
pabC (cat-sacB cassette)	1st step	L	aatcaccaccgttcaggctgccattga
	L-CS	L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCcagcgattcctgcttataaccgtaattaagaacat
		R-CS	R-CS
	R	R	cgaaagactcttgcataacgatccagct
(in-frame deletion)	2nd step	RL	TGCTGACGAAAAGGAGACATCGCCccgategcttactgccagcagttc
	LR	LR	gaatcgctggcagtaagcagatcggGGCGATGTCTCCTTTTCGTCAGCA
folP (cat-sacB cassette)	1st step	L	aactggaaagccagatttctacgtgtacg
	L-CS	L-CS	GTGCTACGCCTGAATAAGTGCGGCCGCttacttgtcgctaactgctctgacatggt
		R-CS	R-CS
	R	R	ttttcagccagcacagcagcctgga
(in-frame deletion)	2nd step	RL	ttactcatagcgtttgtttcctttgcagacagagtCAGTGAAGTACCCTGGGCAAAGAGTTTCAT
	LR	LR	ATGAAACTCTTTGCCAGGGTACTTCACTGactctgtctgcaaaggaaaacaacgctatgagtaa



Supporting Fig. 1.



Supporting Fig.2. Growth of the $\Delta folP$ mutant harboring pUC118 (diamond), wild type strain harboring pUC118 in the presence of folic acid (triangle), $\Delta folP$ mutant harboring pUC118 carrying the *folP* gene cloned from *E. coli* (square), and $\Delta folP$ 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