



Title	Analysis of the Lactobacillus Metabolic Pathway
Author(s)	Kuratsu, Masahiro; Hamano, Yoshimitsu; Dairi, Tohru
Citation	Applied and Environmental Microbiology, 76(21), 7299-7301 <a href="https://doi.org/10.1128/AEM.01514-10">https://doi.org/10.1128/AEM.01514-10</a>
Issue Date	2010-11
Doc URL	<a href="http://hdl.handle.net/2115/45374">http://hdl.handle.net/2115/45374</a>
Rights	Copyright © 2010, American Society for Microbiology
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	AEM76-21_7299-7301.pdf



[Instructions for use](#)

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

2 Running title: An alternative para-aminobenzoate biosynthetic pathway.

3

4 Authors: Masahiro Kuratsu<sup>1</sup>, Yoshimitsu Hamano,<sup>2</sup> and Tohru Dairi<sup>3</sup>

5

6 *Kyowa Hakko Bio Co. Ltd., Chiyoda-ku, Tokyo 100-8185, Japan,* <sup>1</sup> *Department of*

7 *Bioscience, Fukui Prefectural University, Fukui 910-1195, Japan,* <sup>2</sup> *Graduate School of*

8 *Engineering, Hokkaido University, Hokkaido 060-8628, Japan.* <sup>3</sup>

9

10 Footnote:

11 \* Corresponding author. Mailing address: Graduate School of Engineering, Hokkaido

12 University, Hokkaido 060-8628, Japan. Tel. +81-11-706-7815; Fax. +81-11-706-7118;

13 E-mail: dairi@eng.hokudai.ac.jp

14

15

16

17

18

19

20

21

22

23

24

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

30

31       The biosynthetic pathways of primary metabolites have been established with  
32 model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. For a  
33 long time, the biosynthetic routes established were believed to be common among all  
34 microorganisms. However, we now realize that some microorganisms possess  
35 alternative biosynthetic pathways since genome data base has enabled us to examine the  
36 presence or absence of orthologs of the genes responsible for known biosynthetic  
37 pathways. These surveys were one of the triggers to find the 2-C-methyl-D-erythritol  
38 4-phosphate pathway (6) for isopentenyl diphosphate biosynthesis and the futasine  
39 pathway (4) for menaquinone biosynthesis. As exemplified by the discovery of these  
40 pathways, microorganisms are still expected to have additional alternative biosynthetic  
41 pathways for the primary metabolites.

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

50 biosynthetic pathway for primary metabolites in microorganisms. Considering that  
51 some *Lactobacillus* strains do not possess a part of orthologs of the known biosynthetic  
52 pathways and that the genome size of *Lactobacillus* strains are relatively large (1.8 to  
53 3.4 M) compared to those of the symbiotic bacteria, such as *Mycoplasma* strains (0.6 to  
54 1.4 M; [http://www.genome.jp/kegg/catalog/org\\_list.html](http://www.genome.jp/kegg/catalog/org_list.html)), we expected the presence of  
55 an alternative primary metabolic pathway in *Lactobacillus* strains. In this paper, we  
56 examined the phenotypic and genotypic relationships in *Lactobacillus fermentum* IFO  
57 3956 (genome size, 2.1 M) (10), *Lactobacillus reuteri* JCM 1112 (2.0 M) (10), and  
58 *Lactobacillus brevis* ATCC 367 (2.3 M) (9), all of which showed relatively good  
59 growth with the following synthetic media: LSP medium (patent JP2000-279166; 20  
60 g/L glucose, 3.1 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.5 g/L K<sub>2</sub>HPO<sub>4</sub>, 2 g/L diammonium hydrogen citrate, 10  
61 g/L potassium acetate, 1 g/L calcium lactate, 0.02 g/L NaCl, 1 g/L tween 80, 0.5 g/L  
62 MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.05 g/L MnSO<sub>4</sub>•5H<sub>2</sub>O, 0.5 g/L CoSO<sub>4</sub>).

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

75 of folic acid biosynthesis. Therefore, we investigated the biosynthesis of PABA in *L.*  
76 *fermentum* IFO 3956 in more detail. In contrast to the absence of *pabA*, *B*, and *C* in the  
77 strain IFO 3956, we found an ortholog of FolP (LAF\_1336, EC 2.5.1.15), which  
78 catalyzes the formation of 7,8-dihydropteroate from PABA and  
79 6-hydroxymethyl-dihydropterin diphosphate. Therefore, we examined if LAF\_1336  
80 showed the expected enzyme activity. We constructed a  $\Delta folP$  *E. coli* mutant by  
81 homologous recombination with the Lambda Red System (Supporting Table 4 and  
82 Supporting Fig. 1). The constructed  $\Delta folP$  *E. coli* mutant required folic acid for its  
83 growth (Supporting Fig. 2) and was used in complementation experiments. The  $\Delta folP$  *E.*  
84 *coli* mutant transformed with a plasmid carrying a *folP* gene cloned from *E. coli* was  
85 able to grow reasonably in the absence of folic acid. Moreover, the  $\Delta folP$  *E. coli* mutant  
86 harboring a plasmid carrying LAF\_1336 was also able to grow without folic acid  
87 (Supporting Fig. 2), demonstrating that LAF\_1336 complemented the *folP* defect.

88 We examined LAF\_1336 using PABA as the substrate through two strategies. First,  
89 we constructed a  $\Delta folP/\Delta pabABC$  *E. coli* mutant for *in vivo* analysis. The  $\Delta folP$  *E. coli*  
90 mutant was used for the starting strain and *pabA*, *pabB*, and *pabC* were successively  
91 disrupted by homologous recombination. The growth of the constructed mutant, in  
92 which PABA was not supplied from chorismate, was completely dependent on the  
93 presence of folic acid. When pUC118-FolP, carrying the *E. coli folP* gene, was  
94 introduced into the  $\Delta folP/\Delta pabABC$  *E. coli* mutant, the transformant was able to grow in  
95 a medium containing PABA as expected (Table 1). The growth of the  $\Delta folP/\Delta pabABC$   
96 *E. coli* mutant transformed with pUC118-1336 carrying LAF\_1336 was also completely  
97 dependent on the presence of PABA. These results clearly suggested that LAF\_1336  
98 used PABA as the substrate for the formation of folic acid *via* 7,8-dihydropteroate.



- 123 2. **Christiansen, J. K., J. E. Hughes, D. L. Welker, B. T. Rodríguez, J. L. Steele,**  
124 **and J. R. Broadbent.** 2008. Phenotypic and genotypic analysis of amino acid  
125 auxotrophy in *Lactobacillus helveticus* CNRZ 32. Appl. Environ. Microbiol.  
126 **74**:416-423.
- 127
- 128 3. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal  
129 genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA  
130 **97**:6640-6645.
- 131
- 132 4. **Hiratsuka, T., K. Furihata, J. Ishikawa, H. Yamashita, N. Itoh, H. Seto, and T.**  
133 **Dairi.** 2008. An alternative menaquinone biosynthetic pathway operating in  
134 microorganisms. Science. **321**:1670-1673.
- 135
- 136 5. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P.**  
137 **Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers,**  
138 **W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R.**  
139 **Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003.  
140 Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad.  
141 Sci. USA **100**:1990-1995.
- 142
- 143 6. **Kuzuyama, T., and H. Seto H.** 2003. Diversity of the biosynthesis of the isoprene  
144 units. Nat. Prod. Rep. **20**:171-183.
- 145
- 146 7. **London, J.** 1976. The ecology and taxonomic status of the lactobacilli. Annu. Rev.  
147 Microbiol. **30**:279-301.

148

149 8. **Makarova, K. S., and E. V. Koonin.** 2007. Evolutionary genomics of lactic acid  
150 bacteria. *J. Bacteriol.* **189**:1199-1208.

151

152 9. **Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A.**  
153 **Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev,**  
154 **Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V.**  
155 **Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J.-H. Lee,**  
156 **I. Di´az-Mun˜ iz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E.**  
157 **Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C.**  
158 **Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O’Sullivan, J. Steele, G. Unlu,**  
159 **M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D.**  
160 **Mills.** 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci.*  
161 *USA* **103**:15611-15616.

162

163 10. **Morita, H., H. Toh, S. Fukuda, H. Horikawa, K. Oshima, T. Suzuki, M.**  
164 **Murakami, S. Hisamatsu, Y. Kato, T. Takizawa, H. Fukuoka, T. Yoshimura,**  
165 **K. Itoh, D. J. O’Sullivan, L. L. McKay, H. Ohno, J. Kikuchi, T. Masaoka, and**  
166 **M. Hattori.** 2008. Comparative genome analysis of *Lactobacillus reuteri* and  
167 *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin  
168 production. *DNA Res.* **15**:151-161.

169

170 11. **O’Sullivan, O., J. O’Callaghan, A. Sangrador-Vegas, O. McAuliffe, L. Slattery,**  
171 **P. Kaleta, M. Callanan, G. F. Fitzgerald, R. P. Ross, and T. Beresford.** 2009.  
172 Comparative genomics of lactic acid bacteria reveals a niche-specific gene set.



173 BMC Microbiol. **9**:50.

174

175 12. **Pastink, M. I., B. Teusink, P. Hols, S. Visser, W. M. de Vos, and J. Hugenholtz.**

176 2009. Genome-scale model of *Streptococcus thermophilus* LMG18311 for

177 metabolic comparison of lactic acid bacteria. Appl. Environ.

178 Microbiol. **75**:3627-3633.

179

180 13. **Pearson, W.R., and D. J. Lipman.** 1988. Improved tools for biological sequence

181 comparison. Proc. Natl. Acad. Sci. USA **85**:2444-2448.

182

183 14. **Teusink, B., F. H. van Enkevort, C. Francke, A. Wiersma, A. Wegkamp, E. J.**

184 **Smid, and R. J. Siezen.** 2005. In silico reconstruction of the metabolic pathways of

185 *Lactobacillus plantarum*: comparing predictions of nutrient requirements with those

186 from growth experiments. Appl. Environ. Microbiol. **71**:7253-7262.

187

188 15. **Wood, B. J., and W. H. Holzapfel.** 1995. The genera of lactic acid bacteria, 1<sup>st</sup> ed.

189 Blackie Academic and Professional, Glasgow, United Kingdom.

190

191

192

193

194

195

196

197

198 **Fig. 1.** HPLC and LC-MS analyses of the products formed from  
199 6-hydroxymethyl-dihydropterin with recombinant FolK and LAF\_1336 (FolP).  
200 **A:** a schematic of dihydrofolate biosynthetic pathway from chorismate.  
201 **B:** HPLC analysis of the reaction product without enzymes (**B-i**) and with both enzymes  
202 (**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 its transformant harboring *E. coli folP* gene or *L. fermentum LAF\_1336* gene.

Strain	- PABA	+ PABA
WT [pUC118: <i>folP</i> ]	0.34	0.33
WT [pUC118: <i>LAF_1336</i> ]	0.35	0.35
<i>ΔfolP</i> [pUC118: <i>folP</i> ]	0.32	0.29
<i>ΔfolP</i> [pUC118: <i>LAF_1336</i> ]	0.33	0.33
<i>ΔpabA, ΔpabB, ΔpabC, ΔfolP</i> [pUC118: <i>folP</i> ]	0.00	0.50
<i>ΔpabA, ΔpabB, ΔpabC, ΔfolP</i> [pUC118: <i>LAF_1336</i> ]	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<sub>600</sub>.

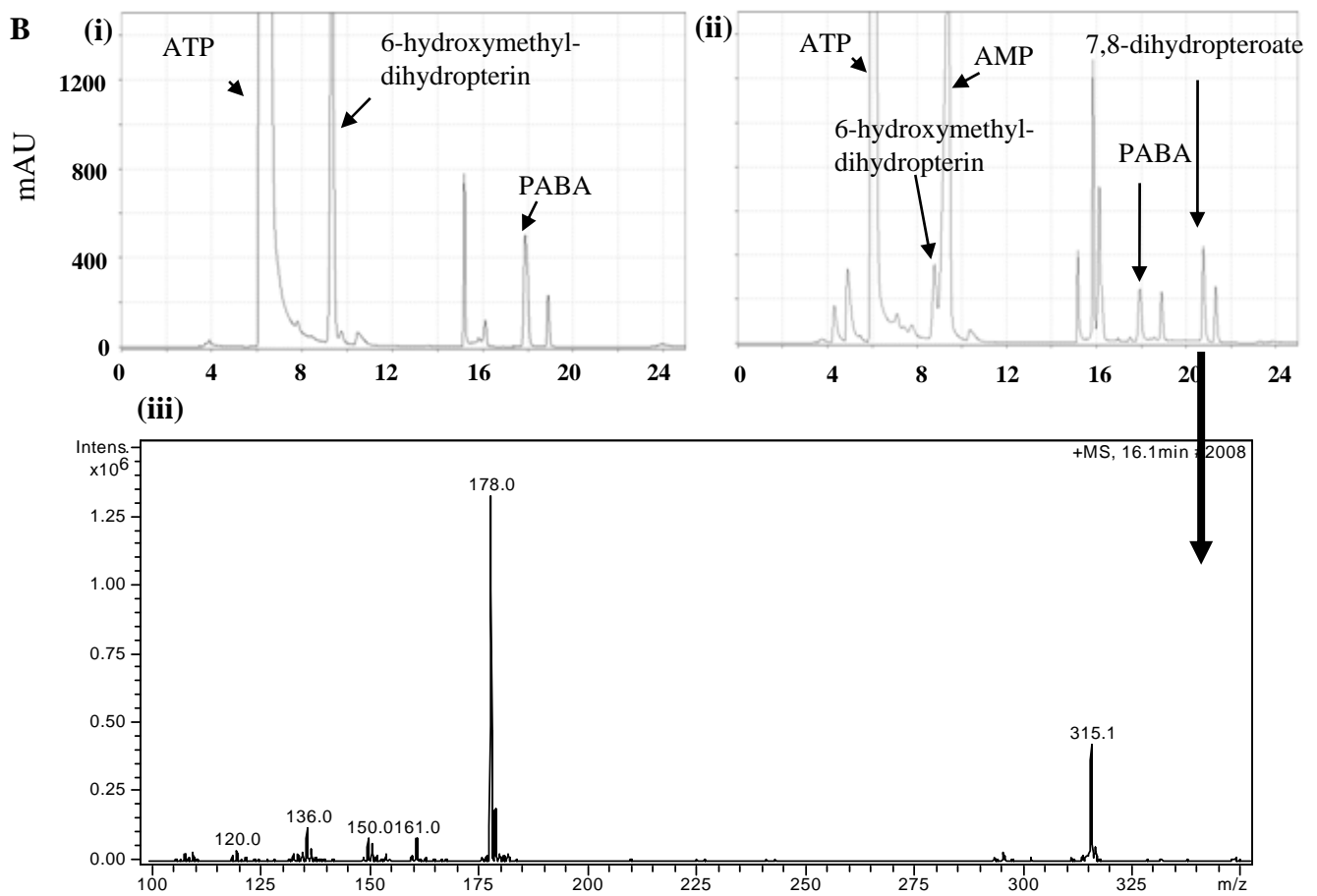
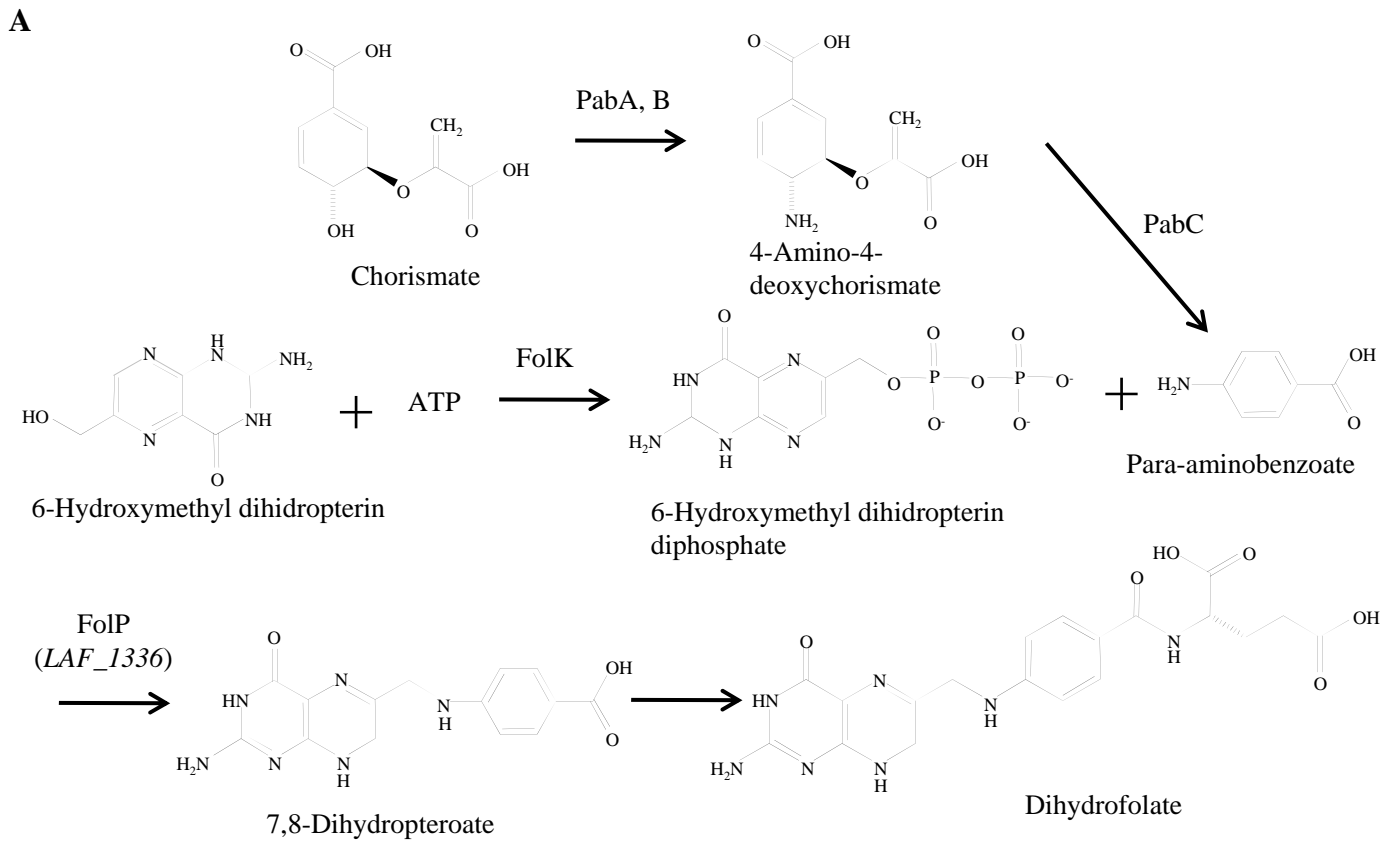


Fig.1.  
11