High-Level Folate Production in Fermented Foods by the B\textsubscript{12} Producer \textit{Lactobacillus reuteri} JCM1112\textsuperscript{\textcopyright}

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We observed that \textit{Lactobacillus reuteri} JCM1112 produces \textit{B}_{12} and folate. However, the folate/\textit{B}_{12} mass ratio found was far below that desired for human consumption (~170:1). We used metabolic engineering applying genetic and physiological approaches to improve this ratio and developed a generic and natural process that significantly increases folate production.

Humans have an auxotrophic requirement for vitamin \textit{B}_{12} and folate, and the recommended intakes of these nutrients for healthy adults are 2.4 and 400 \textmu {g}/day, respectively (7). Suboptimal intake of either of these compounds has been linked to cardiovascular disease, neuropathy, birth defects, cancer, and different types of anemia, among other pathologies (4). Remarkably, the onset of vitamin \textit{B}_{12} deficiency symptoms is often delayed by an increased intake of folate (20). This masking of \textit{B}_{12} deficiency has resulted in the restriction of folate intake levels and prevented folate fortification in many countries (7). Strict vegetarian dietary regimens tend to be poor in intake levels and prevented folate fortification in many countries (20). This mask- ing of \textit{B}_{12} deficiency has resulted in the restriction of folate intake levels and prevented folate fortification in many countries (7).

Coenzyme \textit{B}_{12} is synthesized by a few members of the bacterial and archaeanal groups (13). In situ microbial \textit{B}_{12} production is a convenient strategy to achieve natural enrichment of fermented foods, notably from vegetable sources. \textit{Lactobacillus reuteri} is a gram-positive, heterofermentative lactic acid bacterium with a long history of safe use by the food industry (10). This microorganism ferments several sugars, and this flexibility leads to its capacity to thrive on several substrates of vegetable origin (14). Strain CRL1098 has been reported to produce different forms of \textit{B}_{12} (18, 25), and the draft genome sequence of strain JCM1112 (accession no. CP000705) (http://www.jgi.doe.gov/) suggests that it is able to produce folate, as well as \textit{B}_{12}. In this study, we investigated the possibility of using \textit{L. reuteri} for the combined production of both vitamins at a ratio desired for human consumption, ~170:1 (wt/wt).

\textbf{In silico analysis of the folate biosynthesis genes of \textit{L. reuteri} JCM1112}. Folate is a tripartite molecule assembled from GTP, \textit{para}-aminobenzoic acid (PABA), and one or more \textit{l}-glutamate moieties. The biosynthesis pathway has been extensively characterized in several lactic acid bacteria, including \textit{Lactobacillus plantarum} WCFS1 (Fig. 1). The predicted product of each folate biosynthesis gene of this bacterium was used to search the genome of \textit{L. reuteri} JCM1112 using the BLAST algorithm (2). The sequence identity of the bidirectional best hit was calculated on the nucleotide and amino acid levels based on separate Needleman-Wunsch global alignments (15) determined using the needle script included in EMBOSS (European Molecular Biology Open Software Suite) (17) with default settings. Gene order was analyzed using the ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) (16). The two clusters are very similar, as expected from the close phylogenetic relationship of their hosts (Table 1). Sequence identity is high at both the amino acid and nucleotide levels (on average, 43 and 51\%, respectively). Gene order is completely conserved throughout the entire length of the approximately 4.5-kb cluster composed of six genes.

\textbf{Characterization of \textit{B}_{12} and folate production in CDM by \textit{L. reuteri} JCM1112 and derivatives of this strain}. The \textit{human isolate} \textit{L. reuteri} JCM1112 (type strain) was obtained from the Japanese Collection of Microorganisms (Riken, Japan). It was cultured at 37°C in chemically defined medium (CDM) containing 10 mg/liter of PABA and lacking vitamin \textit{B}_{12} and folic acid (26). Folate in stationary-phase cultures was quantified as described previously (8) with a bioassay using \textit{Lactobacillus casei} ATCC 7469 as the indicator strain, which included enzymatic deconjugation of polyglutamate tails (23). The vitamin \textit{B}_{12} content was determined as described in the \textit{Official Methods of Analysis of AOAC International}, using the \textit{Lactobacillus delbrueckii} subsp. \textit{lactis} ATCC 7830 vitamin \textit{B}_{12} assay (9). Cell extracts of stationary-phase cultures used for \textit{B}_{12} analysis were prepared as previously described (18). In CDM \textit{L. reuteri} JCM1112 produces around 20 \textmu g of folate \textperiodcentered liter\textsuperscript{-1} \textperiodcentered unit of optical density at 600 nm (OD\textsubscript{600})\textsuperscript{-1} at an approximately 1:1 (wt/wt) ratio with \textit{B}_{12} (Fig. 2).

We used a metabolic engineering strategy as proof of principle for the possibility that the ratio of production of these two vitamins was influenced. We aimed at increasing folate production through the overexpression of the complete folate biosynthesis gene cluster, as described previously for other lactic acid bacteria (29, 30), ideally leaving the native \textit{B}_{12} production unchanged. The constructs used in this study cannot be directly used by the food industry, but the use of food-grade alternatives is possible. A wide variety of food-grade systems have been developed for lactic acid bacteria (namely,
and B12 production. The overproduction of folate was found to be 100-fold increase in folate levels (Fig. 2), while the control (L. reuteri JCM1112/pNZ7021) did not show any change in folate production.

Supplemented in the medium

- 6-hydroxymethyl-7,8-dihydropteroate
- dihydrofolate
- tetrahydrofolate (THF)
- pABA
- glutamate
- dihydroleotide
- GTP
- formate
- 7,8-dihydronopterin triphosphate
- PP
- Aspecific Ptse,
- 7,8-dihydronopterin glycolaldehyde
- FolB, E.C. 4.1.2.25
- FolK, E.C. 2.7.6.3
- FolP, E.C. 2.5.1.15
- FolA, E.C. 1.5.1.3
- folC2
- folK
- folP
- folE
- folC2
- pepN
- lapsus

**FIG. 1.** Folate biosynthesis pathway in *L. plantarum* WCFS1.

TABLE 1. Presence of folate biosynthesis genes in the genome of *L. reuteri* JCM1112 as determined by homology searches with *L. plantarum* WCFS1.

<table>
<thead>
<tr>
<th><em>L. plantarum</em> WCFS1 open reading frame</th>
<th>Gene</th>
<th>Length of protein (amino acids)*</th>
<th>Assigned function</th>
<th><em>L. reuteri</em> JCM1112 orthologue</th>
<th>Open reading frame</th>
<th>Length of protein (amino acids)*</th>
<th>Amino acid</th>
<th>Nucleotide</th>
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<tr>
<td>lp3292</td>
<td>folB</td>
<td>122</td>
<td>Dihydroleotide aldolase (EC 4.1.2.25)</td>
<td>Loue1280</td>
<td>111</td>
<td>48</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>lp3298</td>
<td>folK</td>
<td>170</td>
<td>2-Amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase (EC 2.7.6.3)</td>
<td>Loue1279</td>
<td>170</td>
<td>43</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>lp3297</td>
<td>folE</td>
<td>189</td>
<td>GTP cyclohydrolase I (EC 3.5.4.16)</td>
<td>Loue1278</td>
<td>192</td>
<td>57</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>lp3296</td>
<td>folC2</td>
<td>454</td>
<td>Folylpyrrolglutamate synthase (EC 6.3.2.17/dihydrofolate synthase (EC 6.3.2.12)</td>
<td>Loue1277</td>
<td>419</td>
<td>38</td>
<td>47</td>
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<tr>
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<td>xtp2</td>
<td>195</td>
<td>XTP pyrophosphatase (EC 3.6.1.-)</td>
<td>Loue1276</td>
<td>195</td>
<td>35</td>
<td>48</td>
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</tr>
<tr>
<td>lp3294</td>
<td>folP</td>
<td>263</td>
<td>Dihydroleotide synthase (EC 2.5.1.15)</td>
<td>Loue1275</td>
<td>387</td>
<td>37</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

* Length based on the number of amino acid residues predicted in the gene product.

Characterization of B12 and folate production in fruit fermentations. We assessed the applicability of the principle of improving folate/B12 ratios through genetic engineering to media other than CDM. Most (sub)tropical fruits are perishable and sensitive to chill damage, leading to losses of up to 40% in industrialized countries and far greater than 50% in less economically developed nations (6, 12). Fermentation is a secular process of food preservation, which in this case could increase the vitamin content of a raw material. Juice derived from two *Cucumis* spp. (melon and cucumber) was selected for natural enrichment, since this material is low in folate and deficient in B12 according to the USDA National Nutrient Database for Standard Reference (http://www.ars.usda.gov/ba/bhnrc/ndl/). Melon juice medium was made from *Cucumis melo var. reticulatus* after peeling and removal of seeds. The pulp was liquefied using a kitchen blender (Masterchef 370; Moulinex, France), and the resulting paste was squeezed through a cotton cloth. The flowthrough was centrifuged twice at 8,000 × g for 10 min using a Sorvall centrifuge (Newton, CT). The supernatant was stored at −20°C until it was used. Before inoculation, the melon juice was diluted at a 4:1 (vol/vol) ratio with potassium phosphate buffer (final concentration, 0.1 M; pH 5.8). Further dilution was found to result in growth impairment (data not shown). The final pH was adjusted to 6.0, and the melon juice medium was forced through a 0.22-μm filter to ensure sterility. Cucumber juice medium was prepared from intact cucumber (*Cucumis sativus*) and was sterilized using the procedure described above for melon medium with the following modifications: (i) an additional filtration step using a cellulose filter (0.15 mm) was used before centrifugation, and (ii) the cucumber juice was diluted in 1 volume of potassium phosphate buffer (final concentration, 0.1 M; pH 5.8). When mentioned below, both media were supplemented with 10 mg/liter PABA. This concentration of PABA does not conflict with existing food legislation as PABA is listed as a generally regarded as safe (GRAS) ingredient.

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compound with an upper intake limit of 30 mg/day (http://www.cfsan.fda.gov/~dms/opa-appa.html). If appropriate, 10 \( \mu \)g/ml chloramphenicol was also added. Biomass formation in the different growth media is indicated in Table 2.

Folate and B\(_12\) contents were determined for cultures of \( L.\) \( reuteri \) transformed with pNZ7026 and pNZ7021. The background folate levels in melon and cucumber media were found to be 22.5 \( \pm \) 0.9 and 10.0 \( \pm \) 0.4 \( \mu \)g/liter, respectively. As expected, B\(_12\) could not be detected in these media. The overexpression of the folate biosynthesis cluster of WCFS1 in \( L.\) \( reuteri \) JCM1112/pNZ7026 led to production of a high level of folate (2,518.2 \( \pm \) 182.1 \( \mu \)g liter\(^{-1} \cdot \) OD\(_{600}\) unit\(^{-1}\) and a folate/B\(_{12}\) ratio of \( \sim \) 250:1 (wt/wt), but only when PABA was added (Fig. 2). PABA availability has been shown to limit folate biosynthesis in several lactic acid bacteria (24, 30). The control experiment using \( L.\) \( reuteri \) JCM1112 with the empty vector pNZ7021 resulted in the production, in melon medium, of 131.7 \( \pm \) 5.5 \( \mu \)g \cdot liter\(^{-1} \cdot \) OD\(_{600}\) unit\(^{-1}\) of folate, which is more than five times greater than the production in CDM (\( P < 0.001, \) pairwise \( t \) test). In cucumber medium, folate production by JCM1112/pNZ7021 was negatively affected compared to the production in CDM, regardless of the addition of PABA (Fig. 2). The overexpression of the folate biosynthesis genes had an effect similar to that described for CDM, but the final folate/B\(_{12}\) ratios were 1 order of magnitude lower than desired. The twofold reduction in B\(_{12}\) production observed for the melon juice fermentation can be attributed to the amount of sugars present, \( \sim 1.5\% \) glucose and \( \sim 2\% \) fructose as determined by high-performance liquid chromatography analyses performed as described elsewhere (21). Such concentrations have been shown in previous studies to repress B\(_{12}\) biosynthesis at the transcriptional level (1, 19).

\[ \text{FIG. 2. Folate (open bars) and B}_{12}\text{ (shaded bars) production by } L.\text{ } reuteri \text{ wild-type and derivative strains and by } L.\text{ } plantarum \text{ WCFS1 in different media. Plasmid pNZ7021 is the empty plasmid, and plasmid pNZ7026 contains the folate biosynthesis gene cluster of } L.\text{ } plantarum. Each bar represents the average of three biological replicates, and the error bars show standard deviations. All experiments were repeated with at least two different batches of media with similar results.} \]

\[ \text{TABLE 2. Biomass formation in the different growth media} \]

<table>
<thead>
<tr>
<th>Strain</th>
<th>Final OD(_{600}) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDM</td>
<td>Melon juice</td>
</tr>
<tr>
<td></td>
<td>Melon juice enriched with PABA(^a)</td>
</tr>
<tr>
<td>( L.) ( reuteri ) JCM1112</td>
<td>2.8</td>
</tr>
<tr>
<td>( L.) ( reuteri ) JCM1112/pNZ7021</td>
<td>2.8</td>
</tr>
<tr>
<td>( L.) ( reuteri ) JCM1112/pNZ7026</td>
<td>2.5</td>
</tr>
<tr>
<td>( L.) ( plantarum ) WCFS1</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^a\) Supplemented with 10 \( \mu \)g/liter PABA.
\(^b\) ND, not determined.
The remarkable feature of melon fermentation in comparison to CDM and cucumber fermentation is the 5- to 10-fold-grater production of folate by the strain carrying the empty plasmid (pNZ7021). To establish the unique ability of melon juice to induce production of a high level of folate, we tested the parent strain, _L. reuteri_ JCM1112, and another lactic acid bacterium, _L. plantarum WCFS1_ (11). Both _L. reuteri_ and _L. plantarum_ showed a 5- to 10-fold increase in folate production in melon juice medium compared to CDM (Fig. 2). Folate biosynthesis relies on three building blocks (Fig. 1) whose availability does not seem to explain this unsuspected observation. We have experimentally ruled out PABA and L-glutamate since both of these compounds are present in excess in CDM. Regarding the other building block, it has been shown that GTP is not the rate-limiting substrate in folate biosynthesis (24), which can be explained by the small flux from GTP to folate in comparison to the total GTP pool. This implies that an increase in GTP availability for folate synthesis cannot reasonably explain the increase in folate production observed in melon juice. Folate production is tightly regulated on both the transcriptional and translational levels (22, 23, 28). We suspect that there might be an interaction between a compound present in melon juice and one of these regulatory factors. However, the nature of the postulated interaction is unclear and remains to be elucidated.

In this study, we demonstrated that it is possible to combine the production of folate and the production of _B12_ in _L. reuteri_. We used, as proof of principle, a metabolic engineering strategy to optimize the ratio of production of these two vitamins and assessed its applicability to fruit fermentations. This resulted in the development of a natural fermentation process to increase folate production by lactobacilli to levels substantially higher than those previously described (24). The findings reported here may lead to the development of (fermented) foods based on perishable fruits, such as melons, with extended durability and higher nutritional value. A good-tasting fermented melon juice or melon squash containing high folate and vitamin _B12_ levels could be the start of a product line with a longer shelf-life that especially targets vitamin-deficient populations.

**REFERENCES**


