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Characterization of the Role of *para*-Aminobenzoic Acid Biosynthesis in Folate Production by *Lactococcus lactis*▽

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The *pab* genes for *para*-aminobenzoic acid (*p*ABA) biosynthesis in *Lactococcus lactis* were identified and characterized. In *L. lactis* NZ9000, only two of the three genes needed for *p*ABA production were initially found. No gene coding for 4-amino-4-deoxychorismate lyase (*pabC*) was initially annotated, but detailed analysis revealed that *pabC* was fused with the 3′ end of the gene coding for chorismate synthetase component II (*pabB*). Therefore, we hypothesize that all three enzyme activities needed for *p*ABA production are present in *L. lactis*, allowing for the production of *p*ABA. Indeed, the overexpression of the *p*ABA gene cluster in *L. lactis* resulted in elevated *p*ABA pools, demonstrating that the genes are involved in the biosynthesis of *p*ABA. Moreover, a *p*ABA knockout (KO) strain lacking *pabA* and *pabBC* was constructed and shown to be unable to produce folate when cultivated in the absence of *p*ABA. This KO strain was unable to grow in chemically defined medium lacking glycine, serine, nucleobases/nucleosides, and *p*ABA. The addition of the purine guanine, adenine, xanthine, or inosine restored growth but not the production of folate. This suggests that, in the presence of purines, folate is not essential for the growth of *L. lactis*. It also shows that folate is not strictly required for the pyrimidine biosynthesis pathway. *L. lactis* strain NZ7024, overexpressing both the folate and *p*ABA gene clusters, was found to produce 2.7 mg of folate/liter per optical density unit at 600 nm when the strain was grown on chemically defined medium without *p*ABA. This is in sharp contrast to *L. lactis* strains overexpressing only one of the two gene clusters. Therefore, we conclude that elevated folate levels can be obtained only by the overexpression of folate combined with the overexpression of the *p*ABA biosynthesis gene cluster, suggesting the need for a balanced carbon flux through the folate and *p*ABA biosynthesis pathway in the wild-type strain.

Many plants and bacteria have the ability to produce folate. Folate is essential for most animals, including humans, and insufficient intake of folate may lead to physiological disorders, such as anemia and neural tube defects in newborns (20). Among elderly people, folate deficiency may also lead to mental disorders such as psychiatric syndromes and decreased cognitive performance (6, 13). It is also assumed that folate has protective properties against cardiovascular diseases and a few types of cancer (4, 5, 20). Metabolic engineering of fermentative microbes can be used to produce food products with elevated folate contents.

Folate biosynthesis proceeds via the conversion of GTP in seven consecutive steps to the biologically active cofactor tetrahydrofolate (THF). Two condensation reactions take place in the biosynthesis pathway of THF. The first is the condensation of *para*-aminobenzoic acid (*p*ABA) with 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine to produce dihydropteroate. The second is the reaction of glutamate with dihydropteroate to form dihydrofolate (8) (Fig. 1). *p*ABA itself is synthesized from the pentose phosphate pathway; in this pathway, D-erythrose 4-phosphate is condensed with phosphoenolpyruvate to ultimately lead to chorismate (Fig. 1). Chorismate serves as a branching point for the synthesis of the aromatic amino acids (tryptophan, phenylalanine, tyrosine) and *p*ABA (21). In *Escherichia coli*, chorismate is converted via chorismate synthetase components I and II (PabB and PabA, EC 6.3.5.8) into 4-amino-4-deoxychorismate. Subsequently, pyruvate is cleaved by 4-amino-4-deoxychorismate lyase (PabC, EC 4.1.3.38), to result in *p*ABA (10, 26) (Fig. 1). *p*ABA knockout (KO) strains of *E. coli* are unable to grow in the absence of *p*ABA or folate in a minimal medium (9, 15). Without *p*ABA, no THF can be produced, and THF is essential as the donor and acceptor of one-carbon groups (i.e., methyl, formyl, methenyl, and methylene) in the biosynthesis of purines and pyrimidines, formyl-methionyl $tRNA^{fMet}$, and some amino acids (24, 37). Interestingly, it was found that *Streptococcus faecalis* R, incapable of producing folate, was able to grow on medium without folate. Growth was achieved in a chemically defined medium (CDM) supplemented with medium components which require folate for biosynthesis, i.e., methionine, serine, thymine, adenine, and guanine (33).

An extensive genetic metabolic engineering approach has been applied to the folate biosynthesis genes in *Lactococcus lactis* (38). The overexpression of the folate gene cluster (*folB folKE folP folQ folC*) resulted in a folate-overproducing strain, but only when *p*ABA was added to the medium.

In this paper, we describe the involvement of the *p*ABA genes in the production of folate in *L. lactis*. First, the genes coding for *p*ABA biosynthesis were characterized. Second, a *p*ABA KO strain was constructed; in this strain, folate production and growth performance were analyzed in the presence and absence of *p*ABA, glycine, serine, and nucleobases/nucleosides (guanine, adenine, uracil, xanthine, inosine, thymidine, and orotic acid). Finally, we analyzed the effect of overexpres-

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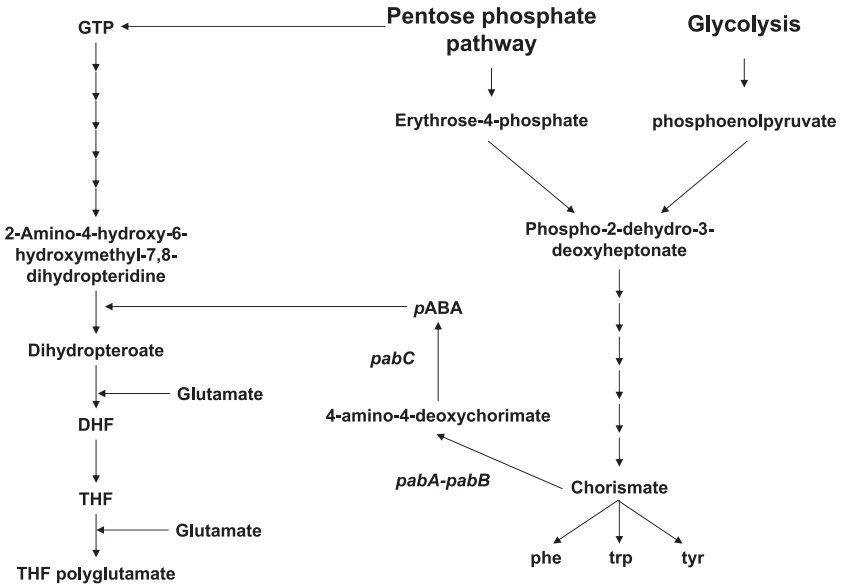

FIG. 1. Biosynthesis pathway of *p*ABA and folate, showing the conversion of GTP into THF, the route for *p*ABA biosynthesis from chorismate, and the incorporation of *p*ABA into THF.

sion of the folate biosynthesis genes combined with overexpression of the *p*ABA biosynthesis genes on folate pools.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and cultivations. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. The *Lactococcus lactis* NZ9000-derived strains were grown at 30°C on CDM (25, 27, 29) and M17 broth and agar (43). CDM contains the following medium components: 3 g/liter K_2HPO_4, 3 g/liter KH_2PO_4, 1 g/liter sodium acetate, 0.6 g/liter ammonium citrate, 11 g/liter glucose · $1H_2O$, 0.5 g/liter ascorbic acid, 0.25 g/liter L-tyrosine, 0.001 g/liter Ca-(D)-(+)-pantothenate, 0.0025 g/liter D-biotin, 0.001 g/liter 6,8-thiotic acid, 0.001 g/liter nicotinic acid, 0.005 g/liter pyridoxamine HCl, 0.002 g/liter pyridoxine HCl, 0.001 g/liter riboflavin, 0.001 g/liter thiamine HCl, 0.001 g/liter vitamin B_{12}, 0.005 g/liter inosine, 0.005 g/liter thymidine, 0.005 g/liter orotic acid, 0.2 g/liter $MgCl_2$ · $6H_2O$, 0.05 g/liter $CaCl_2$ · $2H_2O$, 0.016 g/liter $MnCl_2 \cdot 4H_2O$, 0.003 g/liter $FeCl_3 \cdot 6H_2O$, 0.005 g/liter $FeCl_2 \cdot 4H_2O$, 0.005 g/liter $ZnSO_4 \cdot 7H_2O$, 0.0025 g/liter $CoSO_4 \cdot 7H_2O$, 0.0025 g/liter $CuSO_4 \cdot 5H_2O$, 0.0025 g/liter $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.24 g/liter alanine, 0.125 g/liter arginine, 0.42 g/liter aspartic acid, 0.13 g/liter cysteine-HCl, 0.50 g/liter glutamic acid, 0.175 g/liter glycine, 0.15 g/liter histidine, 0.21 g/liter isoleucine, 0.475 g/liter leucine, 0.44 g/liter lysine, 0.125 g/liter methionine, 0.275 g/liter phenylalanine, 0.675 g/liter proline, 0.34 g/liter serine, 0.225 g/liter threonine, 0.05 g/liter tryptophan, 0.325 g/liter valine, 0.01 g/liter guanine, 0.01 g/liter adenine, 0.01 g/liter uracil, and 0.01 g/liter xanthine. CDM was used to evaluate the growth rate and the pools of *p*ABA and folate. M17 broth and M17 agar were used for the construction of the engineered strains. The CDM described here may lack nucleobases, nucleosides, glycine, serine, and *p*ABA. When these components were added separately, the concentrations as described above were used. Spent medium was made by growth of the NZ9000Δ*p*ABA strain on CDM lacking nucleobases, nucleosides, glycine, serine, and *p*ABA, and the spent medium was filter sterilized with a 0.22-μm filter. The pH of the spent medium was adjusted to the original pH of 6.5, and 0.5% additional glucose was added; afterwards, this medium was filter sterilized again. The spent medium was subsequently used as the growth medium to test specific nutrient requirements of *L. lactis* NZ9000Δ*p*ABA. The *E. coli* DH5α strain (47), used for the construction of the *p*ABA KO strain, was grown at 37°C in TY medium plates (30). Growth rates for the *L. lactis* strains were determined in 96-well microtiter plates by measuring turbidity at 600 nm using the Spectra Max384 spectrophotometer (Molecular Devices). To select and maintain the plasmid, we used the following antibiotics: chloramphenicol at 10 mg/liter, tetracycline at 10 mg/liter, and erythromycin at 10 mg/liter for *L. lactis* and at 50 mg/liter for *E. coli*.

Genetic constructs and DNA methods. *E. coli* DH5α was transformed by the $CaCl_2$ procedure (32) and *L. lactis* strains by electroporation (7). PCRs for the construction of KO and overproduction vectors were performed with *Pfx* polymerase (Invitrogen, Breda, The Netherlands). *Taq* polymerase (Promega Biotech, Roosendaal, The Netherlands) was used for the PCRs to check the lengths of inserts. *L. lactis* genomic DNA was isolated using established procedures (18, 45), and plasmid DNA was isolated using Jetstar columns (Genomed GmbH, Bad Deynhausen, Germany). All restriction enzymes but Ecl136II (Fermentas UAB, Vilnius, Lithuania) were purchased from Invitrogen. The preintegration vector for the inactivation of the *pabA-pabBC* gene cluster was constructed by using the Cre-*lox* system (17). The sequence with the up- and downstream regions of the *p*ABA gene cluster of *L. lactis* MG1363 was used for amplification by PCR. This sequence appears in the GenBank nucleotide sequence library under accession number AM406671. The first amplified linear fragment of DNA is 1,359 base pairs in length (the *pabA* fragment); the second fragment, with a length of 550 base pairs, is designated the *pabBC* fragment. The *pabA* fragment contains the upstream region of *pabA*, annotated as a surface protein gene, and ends with the first 15 base pairs of *pabA*. For the PCR, the forward primer FLLSURFPRxho-F (the sequence was modified to introduce an XhoI digestion sequence [Table 1]) and the reverse primer RLLpabA15nucl-R were used (Table 1). The second fragment, the *pabBC* fragment, contains the final 15 base pairs of *pabBC*, the intergenic regions, and 346 base pairs of the neighboring open reading frame *yneH*. The forward primer FLLpabBC15nucl-F and reverse primer RLLyneHBgl2-R (the sequence was modified to introduce a BglII digestion sequence [Table 1]) were used for the PCR. After amplification of the two PCR fragments, plasmid pNZ5319 was digested with PmeI and XhoI and the *pabA* fragment was digested with XhoI. Subsequently, the digested *pabA* fragment was ligated into the PmeI/XhoI-digested pNZ5319 vector with T4 DNA ligase (Invitrogen) and was then cloned in the *E. coli* DH5α strain. Transformants with the expected insert length were selected on TY agar plates containing erythromycin. The selected colonies were checked for the proper digestion profile. The resulting plasmid, designated pNZ7027, was isolated and used for Ecl136II and BglII digestion, and the *pabBC* fragment was digested by BglII and subsequently ligated into the Ecl136II/BglII-digested pNZ7027 vector with T4 ligase. *E. coli* DH5α was subsequently transformed with the ligation mix. After 2 days of growth, a few colonies were picked and cultivated. From these strains, the *p*ABA KO integration vector (pNZ7028) was isolated, checked for the presence of the *pabBC* fragment by PCR, and digested to check for restriction profiles. The pNZ7028 *p*ABA KO vector was transferred to competent cells of the *L. lactis* NZ9000 strain. The transformed cells were selected on M17 plates containing chloramphenicol. To select colonies with the double-crossover genotype, erythromycin-sensitive colonies were selected by colony streaking on plates containing

TABLE 1. List of strains, constructed plasmids, and primers used

Strain, plasmid, or primer	Relevant feature(s)[a]	Source or reference
Strains		
E. coli DH5α	Cloning host	47
L. lactis NZ9000	MG1363 *pepN*::*nisRK*, cloning host	16
L. lactis NZ7024	*L. lactis* NZ9000 containing pNZ7019 and pNZ7023	This study
L. lactis NZ9000Δ*p*ABA	*L. lactis* NZ9000 lacking *pabA* and *pabBC*	This study
L. casei ATCC 7469	*L. casei*, the indicator strain for the folate microbiological assay	11
Plasmids[b]		
pNZ8148	Cm^r; empty vector, nisin promoter	16
pNZ7020	Cm^r pNZ8148 derivative, containing the *pabA* and *pabBC* genes of *L. lactis* NZ9000	This study
pNZ7021	Cm^r pNZ8148 derivative, nisin promoter replaced by *pepN* promoter	This study
pNZ7022	Cm^r RpNZ7021 derivative, containing the *pabA* and *pabBC* genes of *L. lactis* NZ9000	This study
pNZ7017	Cm^r, pNZ8148 derivative containing *folKE* of *L. lactis*	39
pNZ7019	Cm^r, pNZ8148 derivative containing the *folB*, *folP*, *folKE*, *folQ*, and *folC* gene cluster of *L. lactis*	46
pNZ5319	Cm^r Ery^r, double-crossover deletion vector	17
pNZ7027	Cm^r Ery^r, pNZ5319 vector containing a linear PCR product; surface protein gene upstream of *pabA*, including the final 15 bases pairs of *pabA*	This study
pNZ7028	Cm^r Ery^r, pNZ7028 containing a linear PCR product and the final 15 base pairs of *pabBC* up to 346 bp in *yneH*	This study
pIL253	Ery^r	35
pNZ7023	Ery^r, pIL253 derivative, containing the nisin promoter along with *pabA* and the *pabBC* gene cluster of *L. lactis*	This study
pNZ5327	Tet^r pGHOST derivative, containing the resolvase gene for recombination of *lox* sites	This study
pNZ5348	Tet^r NZ7110 derivative, containing the resolvase gene for recombination of *lox* sites	17
pGHOST8	Tet^r *ori*(Ts)	2
Primers		
FLLSURFPRxho-F	CGTTCTCGAGTGATCATATATC	
RLLpabA15nucl-R	TAAAAGTAATCGCATTTTATC	
FLLpabB/C15nucl-F	GATGGTATTTCTTAATTATAAAG	
RLLyneHbgl2-R	GTACGAAGATCTTCATTATTG	
FpRB36catF3	CATTACCGAAGTAATCGTTAAAC	
RLLyneHupstream	CTTTTAAATGTAGCAAAAAC	
66doRCS85	GTTTTTTTCTAGTCCAAGCTCACA	
FLLSURFPRupstream	GTTTTATAAAACATGAAAG	
lclpabAsphI2	AGTCGCATGCGAGGAGGATAAA*ATG*CGATTAC	
lclpabB/CXbaI	CAAGGCTTTTCTAGATTAAGAAATACCATCG	
pnisF	TAGTCTTATAACTATACTGAC	
pabB/CrevR	CTAGATTAAGAAATACCATCG	

[a] The XhoI digestion sequence is underlined in the forward primer FLLSURFPRxho-F, as are the BglII digestion sequence in the reverse primer RLLyneHBgl2-R, the SphI site in the lclpabAsph primer, and the XbaI site in the reverse primer lclpabBCxbaI.
[b] Where indicated, all pNZ plasmids contain the pSH71 or PWV01 origin of replication.

chloramphenicol with or without erythromycin. Erythromycin-sensitive *L. lactis* colonies were then analyzed by PCR to confirm the double-crossover genotype. The analysis was performed by using the forward primer FLLsurfpRupstream and the reverse primer 66doRCS85 to amplify the upstream region of the surface protein gene up to the chloramphenicol resistance gene. The downstream region of *yneH* was also checked by PCR. For this purpose, the forward primer FpRB36catF3 and the reverse primer RLLyneHupstream were used to amplify the chloramphenicol resistance gene in the downstream region of *yneH*. A clean deletion mutant was obtained by the addition of a plasmid which contained the resolvase gene to recombine the two *lox* loci on both sides of the chloramphenicol resistance gene. The plasmid for the recombination of the two *lox* sites in *L. lactis* was constructed as follows. The resolvase gene was obtained by digestion of plasmid pNZ5348 (17) with HindIII and KpnI. Subsequently, plasmid pGHOST8 (2) was digested with SmaI. Afterwards, both digested DNA fragments were mixed and used for T4 DNA ligation. After the ligation, SmaI digestion was performed to prevent self-ligation. Then the ligated DNA fragment was transferred to *L. lactis* NZ9000 and tetracycline-resistant strains were selected and checked for the correct restriction profile. The vector with the proper orientation was named pNZ5327. Growth experiments with the transformants containing pNZ5327 were performed at 20°C. The clean KO was made by transferring pNZ5327 into competent *L. lactis* NZ9000Δ*p*ABA. Tetracycline- and chloramphenicol-resistant colonies were picked up and grown on M17 at 42°C for 1 h; thereafter, the cells were further cultivated at 30°C. This temperature shift leads to the instability of the maintenance of pNZ5327. After prolonged incubation for approximately 24 h, the vector is lost. The clean KO genotype of strain NZ9000Δ*p*ABA was tested by PCR using the forward primer FLLsurfpRupstream and the reverse primer RLLyneHupstream. A single *L. lactis* colony lacking the *p*ABA gene cluster was selected, cultivated, and stored as a glycerol stock. This strain was designated NZ9000Δ*p*ABA.

Overproduction of *p*ABA by using the pNZ8148 and the pIL253 vector. For the overproduction of *p*ABA and complementation of strain NZ9000Δ*p*ABA, two nisin-inducible vectors were constructed. One vector was based on pNZ8148 (16) and the other one on pIL253 (35). First, the pNZ8148-derived vector was constructed; the *L. lactis p*ABA biosynthesis gene cluster *pabA-pabBC* was amplified by PCR using the forward primer lclpabAsphI2 and the reverse primer lclpabB/CxbaI. The lclpabAsphI2 and lclpabB/CxbaI primers were modified in their sequences to introduce two restriction sites. The forward primer contained an SphI site, and the reverse primer contained an XbaI restriction site (Table 1). The vector pNZ8148 was digested with XbaI and SphI, and the same digestion was performed on the amplified DNA of the *p*ABA gene cluster. Subsequently, both fragments were mixed, ligated with T4 DNA ligase, and transferred to the *L. lactis* NZ9000 wild-type strain and to the NZ9000Δ*p*ABA strain. The resulting vector was named pNZ7020. For the second overproduction vector based on pIL253, plasmid pNZ7020 (containing the *p*ABA gene cluster behind the nisin promoter) was used as a template for a PCR using forward primer pnisF and reverse primer pabBCrevR. The PCR product, containing the nisin promoter and the *p*ABA gene cluster, was mixed with a SmaI-digested pIL253 vector and subsequently ligated using T4 DNA ligase. After ligation, the mixture was again

digested with SmaI to prevent the self-ligation of pIL253. The ligation mix was transferred to competent cells of *L. lactis* NZ9000, NZ9000Δ*p*ABA, and *L. lactis* harboring the folate overexpression vector pNZ7019 (46). Transformed cells capable of growing on erythromycin were selected, checked by PCR for proper orientation, and further checked by restriction profiling. The resulting vector was named pNZ7023. *L. lactis* harboring pNZ7019 and pNZ7023 was designated *L. lactis* NZ7024. Cultivation of this strain requires the presence of both chloramphenicol and erythromycin.

A pNZ8148 derivative was used for the constitutive overexpression of *p*ABA. The pNZ8148 vector was first digested using BglII and SphI. The same digestion was also performed on the pNZ7017 vector (39), thereby excising the constitutive *pepN* promoter. The digested pNZ8148 vector and *pepN* promoter were mixed, and both fragments were ligated using T4 ligase. Subsequently, the ligation mixture was added to competent cells of *L. lactis* NZ9000 for transformation by electroporation. Transformants capable of growing on M17 plates containing chloramphenicol were selected and checked for the presence of the vector. The resulting vector was named pNZ7021. Plasmid pNZ7021 was then isolated from *L. lactis*. To construct the vector for constitutive *p*ABA overproduction, first the *p*ABA gene cluster of *L. lactis* was amplified using the forward primer lclpabAsphI2 and the reverse primer lclpabB/CxbaI (whose sequences were modified to introduce an SphI and XbaI digestion sequence [Table 1]). Plasmid pNZ7021 and the PCR product were digested with SphI and XbaI. These two DNA fragments were ligated and transferred into electrocompetent cells of *L. lactis* NZ9000 and NZ9000Δ*p*ABA. The constructed vector was designated pNZ7022. In addition, the *L. lactis* wild-type and NZ9000Δ*p*ABA strains were transformed with pNZ8148 to deliver experimental control strains.

Folate and *p*ABA analyses. Folate was quantified using a microbiological assay with *Lactobacillus casei* ATCC 7469 as the indicator strain. Enzymatic deconjugation of polyglutamate tails was part of the sample preparation (11, 40). The detection limit of the microbiological folate assay was determined to be 2 μg/liter. To analyze folate production in the *p*ABA KO strain grown on CDM lacking *p*ABA and/or nucleobases/nucleosides, the following concentration step was employed to detect low folate concentrations. A culture (250 ml) was centrifuged and washed twice with 50 ml wash buffer (100 mM sodium acetate and 1% vitamin C) to prevent oxidation of the folate molecules. The pellet was resuspended in 5 ml wash buffer and subsequently extracted by bead beating (three times for 30 s at speed 4 using a FastprepFP120 apparatus [Qbiogene Inc., France]). The broken cells were boiled for 3 min. Subsequently, the supernatant was collected by centrifugation and the centrifugation step was repeated several times to remove all cell debris. The clear supernatant was finally lyophilized, and the resulting cell extract was dissolved in 0.5 ml 0.1 M NaOH and was further used for folate analyses in the microbiological assay.

The *p*ABA production levels were determined using a procedure based on the high-performance liquid chromatography (HPLC) method described previously (41, 42). Instead of using an HPLC separation gradient of 75% elution liquid B (MilliQ plus 1.5% formic acid) and 25% elution liquid C (80% MilliQ–20% methanol plus 1.5% formic acid), a ratio of 60% elution liquid B to 40% elution liquid C was used for the proper separation of *p*ABA on the column. For the *p*ABA standard, 10 mg/liter of *p*ABA was used.

Chemicals. All chemicals were reagent grade and obtained from commercial sources.

RESULTS

Identification of the *p*ABA genes in *L. lactis*. The *pabC* gene, essential for the conversion of 4-amino-4-deoxychorismate to *pabA*, was initially not identified in the genomes of *Lactococcus lactis* SK11 and IL-1403 (ERGO database). The *pabC* gene could also not be identified in the *p*ABA gene cluster of MG1363. Nevertheless, strains SK11 and MG1363 were able to produce folate (41), suggesting that the *p*ABA biosynthesis pathway is complete in these strains. Interestingly, all three *L. lactis* strains contain the *pabA* and *pabB* genes, which are essential for the conversion of chorismate into 4-amino deoxychorismate. To identify the missing *pabC* gene, the *pabB* gene of IL-1403 was compared to all genomes present in the ERGO database by BLAST P (31) analysis. This revealed that *pabB* in *Clostridium difficile* 630 was very similar to the first part of *pabB* in *L. lactis* IL-1403. However, *pabB* of *L. lactis* contains an additional sequence with a length of 741 base pairs, which is homologous to *pabC* of *C. difficile* 630 (Fig. 2). The *pabC* of *C. difficile* 630 shares homology to known 4-amino deoxychorismate lyases of both *E. coli* and *Bacillus subtilis* (10, 36). Based on this in silico analysis, we conclude that *L. lactis* contains a *pabBC* fusion gene. The BLAST search also revealed that such *pabBC* fusion genes are present in both gram-positive and gram-negative species, such as *Streptococcus pyogenes* M5 and *Helicobacter pylori* J99, respectively (Fig. 2). Based on the *L. lactis* MG1363 sequence, we hypothesize that the three *L. lactis* strains possess all genes needed for *p*ABA production, which would be in agreement the observed folate production in the *L. lactis* NZ9000 strain, when cultivated in the absence of *p*ABA (41).

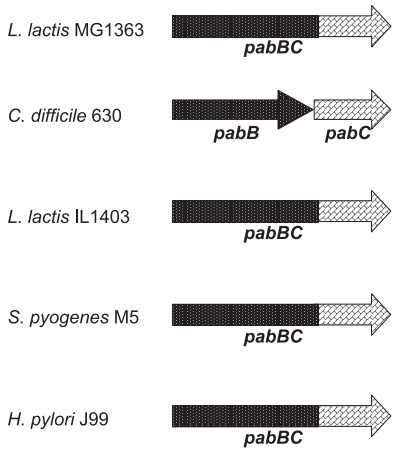

FIG. 2. Organization of the *pabBC* fusion gene in *Lactococcus lactis* MG1363 compared to those of the *pabB* (*p*ABA synthetase component I) and *pabC* (4-amino-4-deoxychorismate lyase) genes of *Clostridium difficile* 630, *Lactococcus lactis* IL-1403, *Streptococcus pyogenes* M5, and *Helicobacter pylori* J99. The E score for the similarity between the *pabC* gene of *C. difficile* 630 and the *pabC* part of the *L. lactis* IL-1403 *pabBC* gene upon BLAST P analysis was $1.e^{-7}$, suggesting similar functions.

Inactivation of the *pabA-pabBC* gene cluster. The presence, activity, and physiological role of the *p*ABA biosynthesis pathway in *L. lactis* was evaluated by inactivating the *p*ABA gene cluster through the construction a double-crossover mutant. Strain *L. lactis* NZ9000Δ*p*ABA lacks 98.8% of the *p*ABA gene cluster. The growth rate, final optical density at 600 nm (OD_{600}), and total folate pools were determined for the wild-type strain and strain NZ9000Δ*p*ABA, grown on CDM in the presence or absence of *p*ABA and nucleobases/nucleosides (Table 2). The growth rate and final OD_{600} of strain NZ9000Δ*p*ABA on medium lacking *p*ABA and nucleobases/nucleosides are severely impaired, reaching a maximum growth rate of only 0.02 h^{-1} and a final OD_{600} of 0.5, which are much lower than the growth rate of 0.31 h^{-1} and final OD_{600} of 3.2 for the wild type. The fact that the NZ9000Δ*p*ABA strain was able to grow poorly but consistently on medium lacking *p*ABA and nucleobases/nucleosides is somewhat unexpected. Therefore, we checked whether the CDM contained trace amounts *p*ABA, folate, glycine, serine, or nucleobases/nucleosides. To test this, the following experiment was conducted. Strain NZ9000Δ*p*ABA was grown on CDM lacking *p*ABA

TABLE 2. Growth rates, final OD_{600} values, and total folate production levels of the wild-type and NZ9000Δ*p*ABA strains grown on CDM with and without *p*ABA and nucleobases/nucleosides

Presence of *p*ABA	Presence of nucleobase/ nucleoside	Wild type		NZ9000Δ*p*ABA		Mean concn (μg/liter) of folate (SD) produced in:	
		Mean growth rate (h^{-1}) (SD)	OD_{600}	Mean growth rate (h^{-1}) (SD)	OD_{600}	Wild type	NZ9000Δ*p*ABA
−	−	0.31 (0.03)	3.2	0.02 (0.001)	0.5	91.7 (10.5)	0.05 (0.01)[a]
+	−	0.31 (0.01)	3.3	0.29 (0.03)	3.1	129.9 (7.9)	92.0 (12.3)
−	+	0.32 (0.05)	3.5	0.26 (0.04)	3.3	103.7 (9.6)	0.05 (0.01)[a]
+	+	0.32 (0.03)	3.5	0.35 (0.06)	3.3	90.3 (16.0)	109.7 (10.3)

[a] This sample was concentrated 500-fold prior to its addition in the microbiological assay. The actual concentration given in this table is the calculated value based on analysis of the concentrated sample in the assay.

glycine, serine, and nucleobases/nucleosides until no further increase in optical density was observed (OD_{600} of 0.5). The spent medium of strain NZ9000Δ*p*ABA was filter sterilized and adjusted to the initial pH, and finally, 0.5% additional glucose was added. The freshly inoculated NZ9000Δ*p*ABA strain was unable to grow on the spent medium, even though glucose and other nutrients were still present in sufficient amounts. The addition of *p*ABA to the spent medium resulted in good growth of the KO mutant, showing that the medium still supports growth. The spent CDM was subsequently supplemented with either adenine, guanine, inosine, or xanthine (purines); orotic acid, thymidine, or uracil (pyrimidines); glycine/serine; or *p*ABA. Interestingly, the growth of the *p*ABA KO strain was restored in the presence of the purine nucleobases (adenine, guanine, and xanthine) as well as the purine nucleoside inosine. The addition of pyrimidines (uracil, thymidine, and orotic acid) as well as the mixture of glycine and serine did not support the growth of the mutant in the spent medium (Table 3). The results of this experiment, together with the data supplied in Table 2, show that, in *L. lactis*, folate is essential for purine biosynthesis. The experiments also show that folate is not strictly required for the pyrimidine biosynthesis pathway. Finally, we conclude that the folate-dependent interconversion of glycine and serine is not essential for growth (24, 37).

Folate pools were determined in the wild type and in strain NZ9000Δ*p*ABA on CDM containing or lacking *p*ABA and nucleobases/nucleosides (Table 2). Regardless of whether *p*ABA and nucleobases/nucleosides were added to the medium, wild-type *L. lactis* accumulated folate pools ranging from 90.3 to 129.9 μg/liter. This is consistent with folate pools in *L. lactis* strains measured in another study (41). However, the *p*ABA KO strain was unable to accumulate folate pools when cultivated on CDM lacking *p*ABA, thereby reaching folate levels of only 0.05 μg/liter. This low level of accumulated folate is possibly a consequence of the uptake of trace amounts of folate or folate precursors from the medium (see above). Interestingly, the growth rate of strain NZ9000Δ*p*ABA was restored by the addition of nucleobases/nucleosides to the medium, although folate pools remained very low (0.05 μg/liter). The addition of *p*ABA to a culture of the NZ9000Δ*p*ABA strain boosted folate pools back to wild-type levels, i.e., approximately 100 μg/liter (Table 2). These experiments suggest that folate production in *L. lactis* is essential for growth only when no nucleobases/nucleosides are present in the medium. In the presence of purines, folate is not essential for growth.

TABLE 3. Growth of the NZ9000Δ*p*ABA strain on spent medium without and with supplementation

Medium component(s) added to spent medium	Growth[a]
None	−
Adenine	+
Guanine	+
Inosine	+
Xanthine	+
Orotic acid	−
Thymidine	−
Uracil	−
Glycine and serine	−
*p*ABA	+
All nucleobases/nucleosides	+

[a] No growth is expressed as −, and growth is expressed as +.

Effect of *p*ABA overproduction on the folate and *p*ABA pools in the wild-type and NZ9000Δ*p*ABA strains. The *pabA-pabBC* genes for the biosynthesis of *p*ABA were cloned on different plasmids to determine the impact of *p*ABA overproduction on the production of folate in the wild-type and NZ9000Δ*p*ABA strains. Strain NZ9000 and NZ9000Δ*p*ABA were complemented with two nisin-inducible *p*ABA overexpression vectors (pNZ7020 and pNZ7023) and one constitutive *p*ABA overexpression vector (pNZ7022). Folate analysis, performed on strain NZ9000Δ*p*ABA harboring pNZ7020, showed that pools were restored to wild-type levels, regardless of whether nisin was added for induction (data not shown). The folate analyses performed on the wild-type strain harboring pNZ7020 displayed a shift in folate distribution across the intracellular and extracellular compartment upon induction of expression with nisin (Fig. 3). The *L. lactis* wild-type strain harboring the empty vector pNZ8148 showed no shift in folate distribution upon induction with nisin, indicating that this shift was caused by the overproduction of *p*ABA and not by the nisin itself. The shift in folate distribution was also observed in strain NZ9000 (wild type) harboring pNZ7022 (constitutive overexpression) and pNZ7023 (nisin-induced overexpression) as well as in the NZ9000Δ*p*ABA strain harboring the same *p*ABA overproduction vectors, pNZ7020, pNZ7022, and pNZ7023 (data not shown). In none of the strains were increased folate pools observed. In conclusion, overproduction of *p*ABA did not increase folate production levels, suggesting the need for a bal-

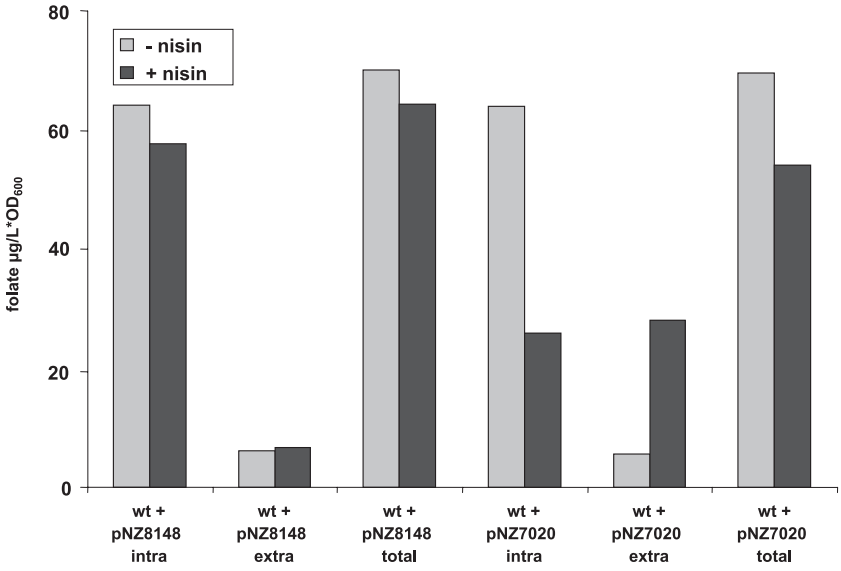

FIG. 3. Folate production (μg/ml per OD_{600} unit) of the *L. lactis* wild-type strain (wt) harboring pNZ8148 (empty vector) and pNZ7020 (nisin-inducible *p*ABA overexpression vector). The folate pool quantities of intracellular, extracellular, and total fractions are shown. The uninduced folate levels are depicted in gray bars, and the induced folate levels are shown in black.

anced carbon flux through the folate and the *p*ABA biosynthesis pathway.

The levels of *p*ABA production in the *L. lactis* wild type, the NZ9000Δ*p*ABA strain, and the NZ9000Δ*p*ABA strain harboring pNZ7020, pNZ7022, and pNZ7023 were quantified. All strains were cultivated on CDM with nucleobases/nucleosides in the absence of *p*ABA. The levels of *p*ABA production were determined by HPLC. The accumulation of *p*ABA could not be detected in the *L. lactis* wild-type or NZ9000Δ*p*ABA strain or in the uninduced NZ9000Δ*p*ABA strain carrying pNZ7020 or pNZ7023. However, after nisin induction, the NZ9000Δ*p*ABA strains harboring pNZ7020 and pNZ7023 were found to accumulate pools of 7.8 and 5.3 mg of *p*ABA/liter, respectively, per OD_{600} unit in the cell extract. An equivalent amount of *p*ABA was found to be produced by strain NZ9000Δ*p*ABA containing pNZ7022 (5.3 mg/liter per OD_{600} unit). Assuming complete retention of *p*ABA and an intracellular volume of 3.6 μl/mg cell protein (an OD_{600} value of 1 corresponds with 0.2 mg cell protein per ml) (28), the intracellular *p*ABA pool reaches a size of 57 to 84 mM. In conclusion, overexpression of the *pabA-pabBC* genes in *L. lactis* resulted in a high intracellular accumulation of *p*ABA, proving their involvement in the biosynthesis of *p*ABA.

Overexpression of folate and *p*ABA. Overexpression of the *p*ABA gene cluster in strain NZ9000Δ*p*ABA boosted the *p*ABA production but did not lead to elevated folate pools. Sybesma (38) has shown that *p*ABA biosynthesis controls the overproduction of folate in a strain which overexpresses the entire folate gene cluster. We hypothesize that the expression of the pteridine pathway and that of the *p*ABA pathway are tightly coupled. In previous work (38), an *L. lactis* strain (NZ9000 containing the pNZ7019 vector) that produces high folate levels was constructed; the folate biosynthesis genes (*folB*, *folKE*, *folP*, *folQ*, and *folC*) were cloned on a high-copy-number vector (pNZ8148 derivative) under the control of the *pepN* promoter. This strain was cultivated in CDM in the presence and absence of *p*ABA. Only when *p*ABA was supplied to the medium were high folate levels (2.3 mg/liter per OD_{600} unit) found to be produced. In the absence of *p*ABA, no elevated folate levels were observed (Fig. 4.). Vector pNZ7023 (pIL253 derivative), containing the *pabA-pabBC* gene cluster under the control of the nisin promoter, was transferred to competent cells of *L. lactis* NZ9000 harboring pNZ7019. The resulting strain, named *L. lactis* NZ7024, was cultivated on CDM without *p*ABA. Interestingly, after nisin induction, this

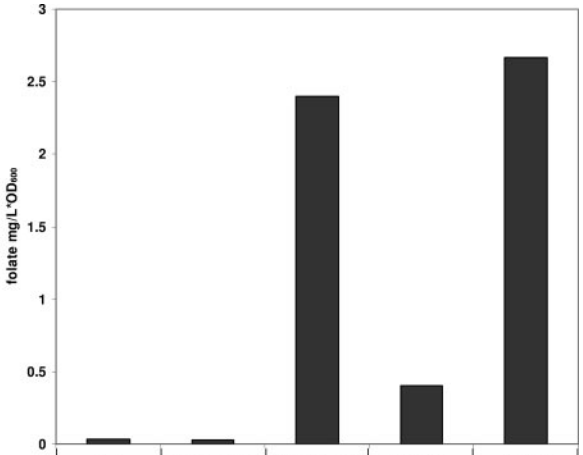

FIG. 4. Total folate production levels (mg/liter per OD_{600} unit) of three different strains: *L. lactis* NZ9000 (wild type [wt]), *L. lactis* NZ9000 harboring pNZ7019 (folate overexpression vector) cultivated in the presence and absence of *p*ABA, and *L. lactis* NZ7024 (NZ9000 strain harboring the folate and *p*ABA overexpression vector) cultivated without *p*ABA prior to and after the induction with nisin.

strain was able to produce 2.7 mg of folate/liter per OD_{600} unit, independently of *p*ABA supplementation of the growth medium (Fig. 4). Without induction, only 0.4 mg of folate/liter per OD_{600} unit was produced. In the *L. lactis* NZ7024 strain, the expression of the *p*ABA gene cluster results in the conversion of chorismate into high *p*ABA levels. Simultaneously, the overexpression of the folate gene cluster leads to the conversion of GTP into THF, which can be achieved only when high *p*ABA levels are present. Therefore, we conclude that the overexpression of the *p*ABA genes combined with the overexpression of the folate biosynthesis genes is essential for the overproduction of folate. The overexpression of only *p*ABA or the folate gene cluster separately cannot boost folate production.

DISCUSSION

Based on the observation in a previous study that *L. lactis* MG1363 is capable of producing folate (41), it was expected that all genes for the synthesis of *p*ABA are present in *L. lactis*. And indeed after bioinformatics analysis it was found that *L. lactis* IL-1403 (3), a strain closely related to *L. lactis* MG1363, possesses the three genes needed for *p*ABA production. *L. lactis* MG1363 contains *pabA* as a separate open reading frame and *pabBC* as a fused open reading frame. These merged *pabBC* genes were found not only in *L. lactis* but also in other gram-positive bacteria as well as in several gram-negative bacteria, suggesting that this gene fusion was the result of an ancient event. Another interesting feature of the *p*ABA genes is the homology they share with the genes for tryptophan biosynthesis. The *pabA* gene is homologous with the *trpG* gene, and *pabB* is homologous with *trpE* (23). However, no *pabC* homologue has been found, suggesting that *pabC* is very specific for converting 4-amino-4-deoxychorismate into *p*ABA. In the tryptophan biosynthesis pathway, chorismate is converted via TrpE and TrpG into 2-ABA (anthranilate), whereas in the *p*ABA biosynthesis pathway, chorismate is converted via PabA, PabB, and PabC into 4-ABA (*p*ABA). The facts that two enzymes are required to produce 2-ABA and that three enzymes are needed for the synthesis of *p*ABA highlight the important role of PabC and the crucial difference between the tryptophan and the *p*ABA biosynthesis pathway.

The *p*ABA biosynthetic enzymes (PabA, PabB, and PabC) play an important role in the biosynthesis of folate. The deletion of the *pabA-pabBC* gene cluster in *L. lactis* led to a strain that was unable to grow in the absence of *p*ABA, glycine, serine, and nucleobases/nucleosides. Adding all nucleobases/nucleosides to the medium restored the growth of strain NZ9000Δ*p*ABA completely but did not restore folate production. It was demonstrated that single additions of purines to the spent medium restored the growth of the NZ9000Δ*p*ABA strain. From this it can be concluded that the addition of purines circumvents the requirement for folate as a cofactor for the synthesis of IMP. Purines are synthesized from 5-phospho-D-ribosyl-1-pyrophosphate via several steps into IMP (14). In this biosynthetic pathway, 10-formyltetrahydrofolate is used twice as a cofactor, showing the key role of 10-formyltetrahydrofolate in the formation of purines. But the role of 5,10-methylenetetrahydrofolate is not so clear for both the conversion of dUMP to dTMP and the conversion of glycine to serine. Glycine, serine, and pyrimidines can be omitted from the medium, and still the NZ9000Δ*p*ABA strain is able to grow, providing the presence of purines in the medium. This demonstrates that the pyrimidine biosynthesis pathway seems to operate in the absence of 5,10-methylenetetrahydrofolate. This raises the question of which cofactor, other than folate, is used by thymidylate synthase (EC 2.1.1.45) for the conversion of dUMP to dTMP. However, the complex defined medium contains components that can circumvent the necessity of 5,10-methylenetetrahydrofolate as a cofactor for the glycine-serine interconversion. From the model of the metabolic network of *L. plantarum* WCFS1 (44), it could be deduced that threonine can be converted into glycine via threonine aldolase (EC 4.1.2.5.). In addition, the reconstructed metabolic network also predicts that cysteine can be converted into serine by cysteine synthetase (EC 4.2.99.8) and serine *O*-acetyltransferase (EC 2.3.1.30). In a similar experiment, the inhibitory effect of antifolates like methotrexate and trimethoprim can also be counteracted by the addition of the end products of folate biosynthesis (1, 12). These antifolates compete with folate in the cell for the enzyme dihydrofolate reductase (EC 1.5.1.3.), thereby depleting the folate pool which serves as the cofactor in the nucleotide biosynthesis pathway.

One other interesting feature of the medium depletion experiments is the fact that somehow protein translation is initiated in the absence of folate. This was previously investigated in *S. faecalis* R by Samuel et al. (33), who suggested that there might be an alternative C_1 donor for the initiation of protein synthesis or that no formylation is needed to initiate translation in *S. faecalis* R.

Overexpression of the *p*ABA gene cluster on three different vectors, namely, two nisin-inducible vectors (pNZ7020 and pNZ7023) and one constitutive vector (pNZ7022), led to the accumulation of *p*ABA. The overproduction of *p*ABA, however, did not lead to elevated folate pools. This confirms earlier results of Sybesma (38), who demonstrated that folate levels could not be increased solely by the overexpression of the folate biosynthesis genes of *L. lactis*. Hence, we constructed a strain (*L. lactis* NZ7024) overexpressing the *p*ABA and the folate biosynthesis gene clusters simultaneously. This strain was found to produce high folate levels independently of *p*ABA supplementation.

Although overproduction of *p*ABA alone did not result in elevated (total) folate pools, we observed a shift in the folate distribution across the cytoplasmic membrane (accumulated versus secreted folate). The overproduction of *p*ABA leads to relatively low intracellular folate pools and a relatively high secretion of folate. An explanation for this phenomenon is that elevated *p*ABA pools might inhibit the activity of the enzyme responsible for the elongation of the polyglutamate tail of the folate molecule folylpolyglutamate synthetase. It is known that monoglutamate THF molecules diffuse more readily out of the cell than the more highly charged polyglutamate THF derivates (19, 22, 34). In *L. lactis*, it was found that overexpression of the gene coding for folylpolyglutamate synthetase (*folC*) resulted in increased polyglutamate tails, which caused higher intracellular retention (42).

The observation that folate production was already restored in the *p*ABA KO strain without nisin induction suggests that the inducible nisin promoter in the vectors pNZ7020 and pNZ7023 exhibited a low but significant activity in the absence

of its inducer molecule (nisin). As a result, the wild-type phenotype is restored in the absence of the inducer nisin. The use of high- and intermediate-copy-number plasmids, such as pNZ7020 and pNZ7023, respectively, can amplify the effect of low promoter activity, resulting in the restoration of the wild-type phenotype.

This study shows that the activities of the pathways for folate and *p*ABA biosynthesis in *L. lactis* are tightly correlated. First, the deletion of the *p*ABA genes in *L. lactis* eliminated its ability to synthesize folate, causing a complete inability to grow in the absence of purine nucleobases/nucleosides. In the presence of purine nucleobases/nucleosides, folate is not required for growth. Furthermore, we have shown that folate is not strictly required for the pyrimidine biosynthesis pathway. The overproduction of folate or *p*ABA alone did not result in a strain with increased folate pools. However, the combined overexpression of folate and *p*ABA biosynthesis pathways led to a strain that produces a high folate concentration and that does not rely on the supplementation of precursors in the medium. This strongly suggests that in the wild-type cells, the production of *p*ABA and 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine (the intermediate of the folate biosynthesis pathway at the *p*ABA junction) are tightly correlated. In fact, both pathways need to be overexpressed simultaneously to increase total folate pools 80-fold in the absence of an external *p*ABA source.

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