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Increased Production of Folate by Metabolic Engineering of *Lactococcus lactis*

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The dairy starter bacterium *Lactococcus lactis* is able to synthesize folate and accumulates large amounts of folate, predominantly in the polyglutamyl form. Only small amounts of the produced folate are released in the extracellular medium. Five genes involved in folate biosynthesis were identified in a folate gene cluster in *L. lactis* MG1363: *folA*, *folB*, *folKE*, *folP*, and *folC*. The gene *folKE* encodes the biprotein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I. The overexpression of *folKE* in *L. lactis* was found to increase the extracellular folate production almost 10-fold, while the total folate production increased almost 3-fold. The controlled combined overexpression of *folKE* and *folC*, encoding polyglutamyl folate synthetase, increased the retention of folate in the cell. The cloning and overexpression of *folA*, encoding dihydrofolate reductase, decreased the folate production twofold, suggesting a feedback inhibition of reduced folates on folate biosynthesis.

Folate is an essential nutrient in the human diet. Folate deficiency leads to numerous physiological disorders, most notably anemia and neural tube defects in newborns (33) and mental disorders such as psychiatric syndromes among the elderly and decreased cognitive performance (7, 21). In addition, folate is assumed to have protective properties against cardiovascular diseases and several types of cancer (5, 6, 33). The daily recommended intake of dietary folate for an adult is 400 μg. For pregnant women, 600 μg is recommended. Recent studies done in The Netherlands and Ireland have indicated that folate deficiency is common even among various population groups in the developed countries, including women of childbearing age (24, 37).

Folate is a general term for a large number of folic acid derivatives that differ by their state of oxidation, one-carbon substitution of the pteridine ring, and the number of glutamate residues. These differences are associated with different physicochemical properties which may influence folate bioavailability, i.e., folate that can directly be absorbed in the gastrointestinal tract. The in vivo function of folate is that of a cofactor that donates one-carbon units in a variety of reactions involved in the de novo biosynthesis of amino acids, purines, and pyrimidines.

Many plants, fungi, and bacteria are able to synthesize folate and can serve as a folate source for the auxotrophic vertebrates. Due to the ability of lactic acid bacteria to produce folate (31), folate levels in fermented dairy products are higher than those in the corresponding nonfermented dairy products (1). The natural diversity among dairy starter cultures with respect to their capacity to produce folate can be exploited to design new complex starter cultures which yield fermented dairy products with elevated folate levels.

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Lactococcus lactis is by far the most extensively studied lactic acid bacterium, and over the last decades a number of elegant and efficient genetic tools have been developed for this starter bacterium. These tools are of critical importance in metabolic engineering strategies that aim at inactivation of undesired genes and/or (controlled) overexpression of existing or novel ones. In this respect, especially the nisin-controlled expression (NICE) system for controlled heterologous and homologous gene expression in *L. lactis* has proven to be very valuable (9). The design of rational approaches to metabolic engineering requires a proper understanding of the pathways that are manipulated and the genes involved, preferably combined with knowledge about fluxes and control factors. Most of the metabolic engineering strategies so far applied to lactic acid bacteria are related to primary metabolism and comprise efficient rerouting of the lactococcal pyruvate metabolism to end products other than lactic acid, including diacetyl (8, 20, 36, 44) and alanine (18), resulting in high-level production of both natural and novel end products. Metabolic engineering of more complicated pathways involved in secondary metabolism has only recently begun by the engineering of exopolysaccharide production in *L. lactis* (3, 30, 32, 43). Another complicated pathway is the biosynthesis of folate (13). This biosynthesis includes parts of glycolysis, the pentose phosphate pathway, and the shikimate pathway for the production of the folate building block *p*-aminobenzoate, while the biosynthesis of purines is required for the production of the building block GTP (Fig. 1, top). In addition, a number of specific enzymatic steps are involved in the final assembly of folate and for production of the various folate derivatives. The annotated genome sequence of *L. lactis* subsp. *lactis* IL1403 (4) reveals the existence of a folate gene cluster containing all genes encoding the folate biosynthesis pathway (Fig. 1, bottom).

In the present and previous studies we have used *L. lactis* subsp. *cremoris* MG1363 for metabolic engineering experiments (3, 18, 20). Although *L. lactis* IL1403 and MG1363 show a high degree of homology on the genome level, there are

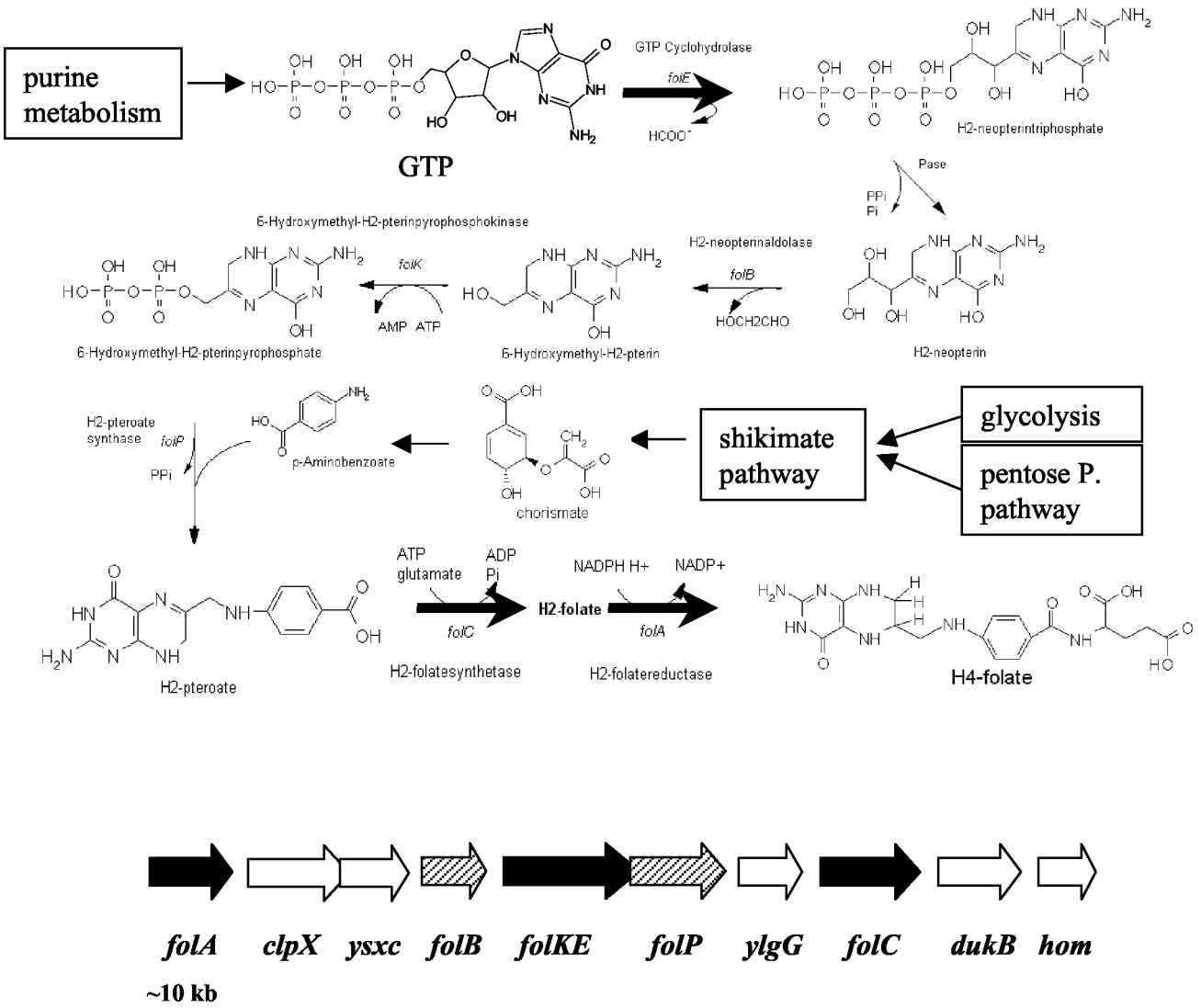

FIG. 1. (Top) Chemical structure of tetrahydrofolate and folate biosynthesis pathway. Thick arrows indicate enzymatic reactions that are controlled in metabolic engineering experiments (see text for details). (Bottom) Schematic representation of the *L. lactis* folate gene cluster as identified in *L. lactis* MG1363 and *L. lactis* IL1403. *folKE* encodes a bifunctional protein. Hatched arrows represent genes involved in folate biosynthesis, black arrows represent genes involved in folate biosynthesis that are overexpressed in metabolic engineering experiments, and white arrows represent genes that are not expected to be involved in folate biosynthesis.

considerable differences (28). For successful application of metabolic engineering in the final steps of the complicated biosynthesis pathway of folate in *L. lactis* MG1363, characterization of the folate gene cluster in this strain is necessary. The results presented here are an important step in the development of fermented foods with increased bioavailable and natural folate.

MATERIALS AND METHODS

Bacterial strains and plasmids, media and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in tryptone yeast medium (40). *L. lactis* was grown at 30°C in M17 medium (47) supplemented with 0.5% (wt/vol) glucose. When appropriate, the media contained chloramphenicol (10 μg/ml) or kanamycin (50 μg/ml).

DNA manipulations and transformations. Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook et al. (40). Large-scale isolation of *E. coli* plasmid for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed, Bad Oeynhausen, Germany). Isolation of chromosomal and plasmid DNAs from *L. lactis* and transformation of plasmid DNA to *L. lactis* was performed as previously described (11). Restriction enzymes and T4 DNA ligase were purchased from Life Technologies BV, Breda, The Netherlands.

PCR amplification of DNA and nucleotide sequence analysis. Several *L. lactis* genes were amplified from chromosomal DNA by PCR with 25 ng of DNA in a final volume of 50 μl containing deoxyribonucleoside triphosphates (0.25 to 0.5 mM each), oligonucleotides (50 pM) (Table 2), and 1.0 to 3.0 U of *Pfx* polymerase (Invitrogen, Paisley, Great Britain) or *Taq-Tth* polymerase mix (Clontech, Palo Alto, Calif.). Amplification was performed on a Mastercycler (Eppendorf, Hamburg, Germany) with 30 cycles of denaturation at 95°C for 30 s (3 min in the first cycle), annealing at 50°C for 30 s, and elongation at 68°C (*Pfx*) or 72°C (*Taq-Tth*) for 1 to 8 min. Sequence analysis of the genes involved in folate biosynthesis was done after amplification of a 9-kb DNA fragment flanked by the upstream regions of *folA* (29) and *hom* (34) by using primers Fol-F and Hom-R (Table 2) and cloning of the fragment in pCR-BLUNT (Invitrogen), generating pCR-BLUNT-FOL. The generated plasmid was transformed by electroporation

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
L. lactis strains		
MG1363	*L. lactis* subsp. *cremoris*, plasmid free	12
NZ9000	MG1363 *pepN*::*nisRK*	26
IL1403	*L. lactis* subsp. *lactis*, plasmid free	4
E. coli strain TOP10	Cloning host	Invitrogen
Plasmids		
pCR-blunt	Kan^r	Invitrogen
pCR-blunt-fol	Derivative of pCR-blunt carrying lactococcal folate gene cluster	This study
pNZ8048	Cm^r; inducible expression vector carrying *nisA* promoter	26
pNZ8160	Cm^r; derivative of pNZ8048 carrying terminator of *pepV*	This study
pNZ8161	Cm^r; derivative of pNZ8160 carrying constitutive promoter of *pepN*	This study
pNZ7010	Cm^r; pNZ8048 derivative containing a functional lactococcal *folKE* gene behind the *nisA* promoter	This study
pNZ7017	Cm^r; pNZ8161 derivative containing a functional lactococcal *folKE* behind the constitutive *pepN* promoter	This study
pNZ7011	Cm^r; pNZ8048 derivative containing a functional lactococcal *folKE* and *folC* gene behind the *nisA* promoter	This study
pNZ7012	Cm^r; pNZ8048 derivative containing a functional lactococcal *folP* gene behind the *nisA* promoter	This study
PNZ7013	Cm^r; pNZ8048 derivative containing a functional lactococcal *folA* gene behind the *nisA* promoter	This study
PNZ7014	Cm^r; pNZ8048 derivative containing antisense RNA of a lactococcal *folA* gene behind the *nisA* promoter	This study

to *E. coli* TOP10 (Invitrogen). The nucleotide sequence of the amplified folate gene cluster was determined by automatic double-stranded DNA sequence analysis with a MegaBACE DNA analysis system (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Primer sequences were obtained from published sequence data for *folA* (29) and *hom* (34) and by subsequent primer walking. Amplification, cloning, and sequencing were performed twice in independent experiments. Differences in both DNA sequences were reanalyzed after independent amplification and cloning of the regions flanking the ambiguous sequences.

Construction of plasmids and transformation of strains. Lactococcal plasmid pNZ8048 (25, 26) is a translational fusion vector used in nisin-controlled expression systems. The vector contains a *nisA* promoter and an *Nco*I cloning site. The gene *folKE*, encoding a bifunctional protein predicted to display both 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I activities, was amplified from chromosomal DNA by using primers *FolKE*-F and *FolKE*-R (Table 2). The forward primer *FolKE*-F was extended at the 5′ end, introducing an *Nco*I restriction site resulting in a slight modification of the mature gene (Table 2). The reverse primer *FolKE*-R was extended at the 5′ end, introducing a *Kpn*I restriction site. The amplification product was digested with *Nco*I and *Kpn*I and cloned in pNZ8048 (digested with *Nco*I and *Kpn*I), thereby placing the *folKE* gene under the control of *nisA*. The new plasmid is pNZ7010. The gene *folC*, encoding a bifunctional protein predicted to display both folate synthetase and polyglutamyl folate synthetase activities, was amplified from chromosomal DNA by using the primers FolC-F and FolC-R (Table 2). Both primers were extended at the 5′ end, introducing a *Kpn*I restriction site and an *Xba*I restriction site. The amplification product includes a ribosome binding site and was digested with *Kpn*I and *Xba*I. Next, the gene was cloned in pNZ7010 downstream of *folKE*, generating pNZ7011. The gene *folP*, encoding a protein predicted to display dihydropteroate synthase activity, was amplified from chromosomal DNA by using primers FolP-F and FolP-R (Table 2). Both primers were extended at the 5′ end, introducing a *Kpn*I restriction site and an *Xba*I restriction site. The amplification product includes a ribosome binding site and was digested with *Kpn*I and *Xba*I and cloned behind *folKE* in pNZ7010, generating pNZ7012. The gene *folA*, encoding dihydrofolate reductase, was amplified from chromosomal DNA by using primers FolA-F and FolA-R (Table 2). The forward primer folA-F was extended at the 5′ end, introducing an *Nco*I restriction site resulting in a slight modification of the mature gene (Table 2). The reverse primer FolA-R was extended at the 5′ end, introducing a *Hin*dIII restriction site. The amplification product was digested with *Nco*I and *Hin*dIII and cloned in pNZ8048 (digested with *Nco*I and *Hin*dIII), thereby placing the *folA* gene under the control of *nisA*. The new plasmid is pNZ7013. The cloning of the antisense RNA of the gene encoding dihydrofolate reductase was achieved in a way similar to that described for *folA*, except for the orientation, by using primers FolA-ASF and FolA-ASR (Table 2). The new plasmid is pNZ7014. The generation of a plasmid containing a constitutive promoter and a nisin-inducible promoter separated by a terminator (pNZ8161) was as follows. The terminator from *pepV* (17) was amplified from chromosomal DNA by using primers TpepV-F and TpepV-R (Table 2). The forward primer TpepV-F was extended at the 5′ end, introducing a *Bgl*II restriction site. The reverse primer TpepV-R was extended at the 5′ end, introducing a *Bam*HI restriction site. The amplification product was digested with *Bgl*II and *Bam*HI and cloned in pNZ8048-Δ*Sph*I (digested with *Bgl*II), generating pNZ8160. The constitutive promoter from *pepN* (48) was amplified from plasmid pNZ1120 (48) by using primers Pcon-F and Pcon-R (Table 2). The forward primer Pcon-F was extended at the 5′ end, introducing a multiple cloning site including a *Bgl*II restriction site. The reverse primer Pcon-R was extended at the 5′ end, introducing *Bam*HI and *Sph*I restriction sites. The amplification product was digested with *Bgl*II and *Bam*HI and cloned in pNZ8160 (digested with *Bam*HI), generating pNZ8161. Next, the gene *folKE* was amplified from chromosomal DNA by using primers *FolKE*2-F and *FolKE*2-R (Table 2). Both primers were extended at the 5′ end, introducing an *Sph*I restriction site. The amplification product was digested with *Sph*I and cloned in pNZ8161 (digested with *Sph*I), thereby placing the *folKE* gene under the control of the constitutive promoter of *pepN*. The new plasmid is pNZ7017.

L. lactis strain NZ9000 (26) was used as a host for the plasmids described in Table 1. In NZ9000 the genes for a nisin response regulator and a nisin sensor, *nisR* and *nisK*, respectively, are stably integrated at the *pepN* locus in the chromosome, and they are constitutively expressed under the control of the *nisR* promoter.

Nisin induction. An overnight culture of *L. lactis* NZ9000 harboring pNZ8048 or one of the plasmids described above was diluted (1:100) in GM17 supplemented with chloramphenicol and grown to an optical density at 600 nm (OD_{600}) of 0.5. The cells were induced with different concentrations of nisin A (referred to as nisin) ranging from 0.1 to 5 ng/ml, incubated for 2 h, and harvested for further analysis. The addition of nisin and the subsequent overexpression of genes did not affect the growth characteristics of the engineered strains. Folate was analyzed in cell extracts and fermentation broth, and overproduction of

TABLE 2. Oligonucleotides used for DNA amplification by PCR

Primer	Sequence[a]
Fol-F	CATACCACTTCTTTTTCGATTTGTAAAGG
Hom-R	CGATCCCGGGAAGCCCTGTGCCAACTGTCC
FolKE-F	CATGCCATGGGGCAAACAACTTATTTAAGCATGG
FolKE-R	GGGGTACCGATTCTTGATTAAGTTCTAAG
FolC-F	GAAGAGGTACCAGAAGAGTTTAAAAAGTATTATCG
FolC-R	TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG
FolP-F	GAATGGTACCTTTAGGAGGTCTTTTATGAAAATCTTAGAAC
FolP-R	GAGAAATCAAATCCTCATTCTAGATTAAAATTCC
FolA-F	GGAATTCCATGGTTATTGGAATATGGGCAGAAG
FolA-R	GCCTCAAGCTTCATGGTTGTTTCACTTTTTC
FolA-ASF	GAGGGGTACCTATGATAATTGGAATATGG
FolA-ASR	GCCCAAAAATTGATTTTGCCATGGTTG
Pcon-F	GAAGATCTGTCGACCTGCAGTAGACAGTTTTTTTAATAAG
Pcon-R	CGGGATCCGCATGCCTTCTCCTAAATATTCAGTATTAA
TpepV-F	CGGGATCCTTATGAACTTGCAAAATAAG
TpepV-R	GAAGATCTCACCTCTATTTCTAGAATAAAG
FolKE2-F	ATACATGCATGCAAACAACTTATTTAAGCATGGG
FolKE2-R	ATACATGCATGCGATTCTTGATTAAGTTCTAAG

[a] Underlined nucleotides represent modifications with regard to the mature gene.

proteins was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (3).

Analysis of intra- and extracellular folate concentrations. Folate was quantified by using a *Lactobacillus casei* microbiological assay (19). To measure intra- and extracellular folate concentrations, both cells and supernatant were recovered from a full-grown cell culture (5 ml) after centrifugation (12,000 × *g*, 10 min, 20°C). The supernatant was diluted 1:1 with 0.1 M sodium acetate buffer (pH 4.8)–1% ascorbic acid. The cells were washed with 0.1 M sodium acetate (pH 4.8)–1% ascorbic acid and resuspended in 5 ml of the same buffer. Folate was released from the cells and from folate binding proteins by incubating the samples at 100°C for 5 min, which was determined to be optimal for maximum folate release. Moreover, the heating inactivates the folate-producing bacteria and prevents their interference in the microbiological folate assay. The microbiological folate assay has nearly equal responses to monoglutamyl folate, diglutamyl folate, and triglutamyl folate, while the response to longer-chain polyglutamyl folate (more than three glutamyl residues) decreases markedly in proportion to chain length (46). Consequently, total folate concentrations can be measured only after deconjugation of the polyglutamyl tails in samples containing folate derivatives with more than three glutamyl residues. The analysis of total folate concentration, including polyglutamyl folate, was done after enzymatic deconjugation of the folate samples for 4 h at 37°C and pH 4.8 with human plasma (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) as a source for γ-glutamyl hydrolase activity. The deconjugation reaction mixture was prepared as follows: 1 g of human plasma was diluted in 5 ml of 0.1 M 2-mercaptoethanol–0.5% sodium ascorbate and cleared from precipitates by centrifugation (10,000 × *g*, 2 min), and a 2.5% (vol/vol) concentration of the clarified human plasma solution was added to the folate samples. The standard deviation of the microbiological assay varied between 0 and 15%. A 1% yeast extract medium solution (Difco, Becton Dickinson Microbiology Systems, Sparks, Md.), containing almost exclusively polyglutamyl folates, with a previously determined total folate content was used as a positive control for actual deconjugation.

Dihydrofolate reductase activity. Forty milliliters of a culture of *L. lactis* NZ9000 harboring pNZ8048, pNZ7013, or pNZ7014 was grown and induced with nisin as described previously. At an OD_{600} of 2.5, cells were harvested, washed, and resuspended in 1 ml of buffer (10 mM KPO_4, 0.1 mM dithiothreitol, 0.1 mM EDTA [pH 7.0]). A cell extract was made by addition of 1 g of silica beads to the cell suspension followed by disruption of the cells in an FP120 Fastprep cell disrupter (Savant Instruments Inc., Holbrook, N.Y.) and centrifugation (20,000 × *g*, 10 min, 0°C). Twenty to 100 μl of the cell extract was used to measure dihydrofolate reductase activity as described previously (38).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to the GenBank database under accession number AY156932.

RESULTS

Sequencing and annotation of a folate gene cluster. Based upon the genetic organization of a folate gene cluster in *L. lactis* IL1403 (4), a 9-kb DNA fragment flanked by *folA*, encoding dihydrofolate reductase, and *hom*, encoding homoserine dehydrogenase, was amplified from the genome of *L. lactis* MG1363. In the latter strain the sequence of the genes involved in folate biosynthesis was not yet known, except for *folA* (29). Its nucleotide sequence was determined and revealed the presence of nine open reading frames, all of which have the same orientation. Sequence comparison with the genome of *L. lactis* IL1403 showed that the two strains have an identical genetic organization. The nucleotide identity of the folate gene clusters in *L. lactis* MG1363 and IL1403 is 89%. Only five or six genes in the folate gene cluster appeared to be involved in folate biosynthesis: *folA*, encoding dihydrofolate reductase (EC 1.5.1.3); *folB*, predicted to encode neopterine aldolase (EC 4.1.2.25); *folK*, predicted to encode 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3); *folE*, predicted to encode GTP cyclohydrolase I (EC 3.5.4.16); *folP*, predicted to encode dihydropteroate synthase (EC 2.5.1.15); and *folC*, predicted to encode folate synthetase/folyl polyglutamate synthetase (EC 6.3.2.12/6.3.2.17).

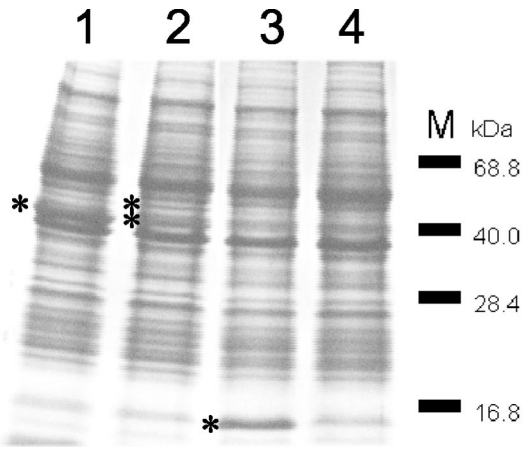

FIG. 2. Coomassie brilliant blue-stained gel after SDS-PAGE of cell extracts from cultures induced with nisin. Molecular markers are indicated on the right. Lanes: 1, overproduction of biprotein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I, encoded by *folKE*; 2, overproduction of the same biprotein and polyglutamyl folate synthetase, encoded by *folC*; 3, overproduction of dihydrofolate reductase, encoded by *folA*; 4, control strain. Increased band intensities are indicated by asterisks.

The remaining genes that were identified in the gene cluster are *clpX*, predicted to encode an ATP binding protein for ClpP; *dukB*, predicted to encode a deoxynucleoside kinase (EC 2.7.1.113); and *ysxC* and *ylgG*, both encoding unknown proteins. The genes *clpX* and *ysxC* may be involved in stress responses (22). The overall amino acid identity of these nine putative proteins between the two *L. lactis* strains is 90%, ranging from 73% identity for *ylgG* to 98% for both *clpX* and *dukB*. It has been reported previously that in *L. lactis folA* contains an identified promoter region (29) and that *folKE*, *folP*, *ylgG*, and *folC* are cotranscribed in a multicistronic operon (45).

Analysis of the nucleotide sequence of the putative *folK* and *folE* genes could identify neither a stop codon at the end of the putative *folK* gene nor a start codon at the beginning of the putative *folE* gene. To verify the nature of *folK* and *folE*, a DNA sequence comprising both genes was fused to the *nisA* promoter of pNZ8048, generating pNZ7010, and introduced in *L. lactis* strain NZ9000. Cells were induced with nisin, and cell extracts were prepared for SDS-PAGE. The Coomassie brilliant blue-stained gel revealed one intense protein band with an apparent molecular mass of 40 kDa, which corresponds to the combined molecular masses of the predicted enzymes encoded by *folK* and *folE*. The intense band was absent in a noninduced strain (Fig. 2). It appears that, in contrast to the case for many other microorganisms, in *L. lactis* the enzymes 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I are produced as one bifunctional protein and are encoded by one gene, here designated *folKE*.

Increased extracellular folate production by overexpression of *folKE*. GTP cyclohydrolase I, part of the biprotein encoded by *folKE*, is the first enzyme in the folate biosynthesis pathway (Fig. 1A). Compared to that in a noninduced strain, the overexpression of *folKE* in strain NZ9000 harboring pNZ7010 caused an increased concentration of extracellular folate from

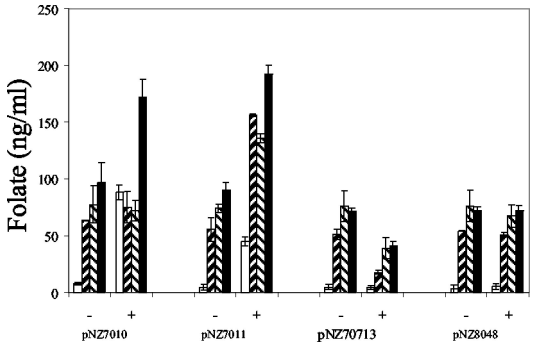

FIG. 3. Folate concentrations in different *L. lactis* strains harboring pNZ8048 (empty vector), pNZ7010 (overexpressing *folKE*), pNZ7011 (overexpressing *folKE* and *folC*), or pNZ7013 (overexpressing *folA*). Strains were induced with 0 (−) or 2 (+) ng of nisin per ml at an OD_{600} of 0.5. Folate concentrations were determined at the end of growth, at an OD_{600} of approximately 2.5. White bars, extracellular folate production; black hatched bars, intracellular folate production; white hatched bars, intracellular folate production after deconjugation; black bars, total folate production. Error bars indicate standard deviations.

approximately 10 to 80 ng/ml. Furthermore, the extracellular folate concentration measured for the control strain NZ9000 harboring pNZ8048 was not affected by induction with nisin (Fig. 3). The folate samples were enzymatically deconjugated with human plasma in order to determine whether part of the extracellular folate was present as polyglutamyl folate with more than three glutamate residues that could not be measured by the microbiological assay. However, no difference in folate concentration was measured with or without deconjugase treatment, indicating that the polyglutamyl folate was not excreted by the cells (Fig. 3). The intracellular folate concentration was measured by analyzing cell extracts for the presence of folate. Under inducing conditions, the *folKE*-overexpressing strain displayed a minor increase in intracellular folate production compared to a control strain or noninduced strain NZ9000 harboring pNZ7010. After deconjugation of the cell extracts, the intracellular folate concentrations were about 80 ng/ml in both strains (Fig. 3). The total folate production by *L. lactis* was determined by combining the extra- and intracellular folate concentrations. It can be concluded that by overexpression of *folKE*, the folate production is more than doubled compared to that of a control strain or noninduced NZ9000 harboring pNZ7010. The majority of the extra folate produced is present as extracellular mono-, di-, or triglutamyl folate. The constitutive expression of *folKE* behind the constitutive promoter of *pepN* that could be achieved in NZ9000 harboring pNZ7017 resulted in the same increase of folate production as observed by using the NICE system (results not shown).

Increased intracellular folate production by combined overexpression of folate genes. The extra- and intracellular folate distribution is assumed to be controlled by the ratio of mono- and polyglutamyl folates (35). The enzyme responsible for the synthesis of polyglutamyl folate is polyglutamyl folate synthetase encoded by *folC*. The simultaneous overexpression of *folKE* and *folC* (NZ9000 harboring pNZ7011) could be visualized by SDS-PAGE (Fig. 2). The overexpression of both genes resulted in a more than twofold increase in total folate production, similar to what was observed with overexpression of only *folKE*. However, differences were detected in the folate distribution. In contrast to the folate produced by the overexpression of *folKE* only, the majority of the extra folate produced was present as intracellular folate in the *folKE*- and *folC*-overexpressing strain. After deconjugation of the intracellular folate, no increased folate concentrations were detected, indicating that the overexpression of *folKE* and *folC* had no significant effect on the amount of polyglutamyl folates with more than three glutamate residues (Fig. 3). The overexpression of *folKE* and *folP*, encoding dihydropteroate synthase, was achieved by inducing strain NZ9000 harboring pNZ7012. However, no differences in folate concentration or folate distribution were observed compared to the overexpression of only *folKE* (results not shown).

Altered folate production by overexpression of *folA* or antisense *folA*. To gain further insight into folate biosynthesis control in *L. lactis*, the gene *folA*, encoding dihydrofolate reductase, was also overexpressed. In a similar way as described previously, the induction of strain NZ9000 harboring pNZ7013 resulted in production of the enzyme with a predicted molecular mass of 15 kDa at a level that could be visualized by SDS-PAGE (Fig. 2). The overexpression of *folA* caused a twofold decrease in folate production compared to that in a control strain or a noninduced strain (Fig. 3). The intracellular folate distribution and the relative amount of polyglutamyl folates remained unchanged. In a similar experiment we studied the effect of the production of antisense RNA encoding dihydrofolate reductase. The complementary sequence of the coding strand of *folA* was cloned under the control of the nisin promoter *nisA* in pNZ8048, starting at the 5′ end with the complementary sequence of the stop codon of *folA* and finishing at the 3′ end with the complementary start codon of the gene. The plasmid generated, pNZ7014, was transformed into *L. lactis* NZ9000. Under inducing conditions, a small but reproducible increase of approximately 20% in the total folate production was observed compared to that in a control strain (results not shown). To confirm the effect of the transcription of *folA* antisense RNA, the enzymatic activity of dihydrofolate reductase was determined. Cell extracts of strain NZ9000 harboring pNZ7014, transcribing antisense RNA of *folA*, showed a twofold decrease in dihydrofolate reductase activity (Fig. 4). In contrast, cell extracts of *L. lactis* strains overexpressing *folA* showed a more-than-1,000-fold increase in dihydrofolate reductase activity compared to a control strain (Fig. 4).

DISCUSSION

We have described successful metabolic engineering of the final part of the complicated biosynthetic pathway of folate biosynthesis and the cloning, sequencing, and analysis of the folate gene cluster in *L. lactis* MG1363. Homology studies with nonredundant databases show that the folate gene cluster contains *folA*, encoding dihydrofolate reductase; *folB*, predicted to encode dihydroneopterin aldolase; *folK* and *folE*, encoding the biprotein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I; *folP*, predicted to encode dihydropteroate synthase; and *folC*, encoding the bifunctional protein folate synthetase and polyglutamyl folate synthetase. The cloning and overexpression of the area

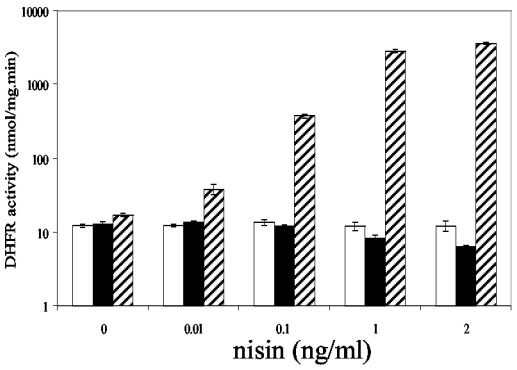

FIG. 4. Effect of nisin concentration on dihydrofolate reductase activity measured in cell extracts of *L. lactis* strains harboring pNZ8048 (empty vector) (white bars), pNZ7013 (overexpressing *folA*) (hatched bars), or pNZ7014 (overexpressing antisense RNA of *folA*) (black bars). Error bars indicate standard deviations.

comprising *folK* and *folE* showed the existence of a bifunctional protein encoded by only one gene, *folKE*. The other genes present in the folate gene cluster, *clpX*, *ysxC*, and *ylgG*, are not likely to be involved in folate biosynthesis. The gene *folE*, encoding GTP cyclohydrolase I, was always identified as an independent gene. Comparative genome analysis with nonredundant databases reveals that the gene *folK*, encoding 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, may exist as a single gene, but in several microorganisms, e.g., *Streptococcus pneumoniae*, *Clostridium perfringens*, *Chlamydia trachomatis*, *Chlamydia muridarum*, and *Rickettsia conorii*, *folK* forms a biprotein with either *folB* (neopterin aldolase) or *folP* (dihydropteroate synthase).

Folate gene clusters have previously been identified in some related microorganisms. In *S. pneumoniae* and *Lactobacillus plantarum*, *folP*, *folC*, *folE*, *folB*, and *folK* are clustered, but in a different order (23, 27). In *Lactobacillus plantarum*, a second *folC* gene was identified outside the folate gene cluster. In *Bacillus subtilis*, *folP*, *folB*, and *folK* are clustered together with genes involved in *p*-aminobenzoate synthesis, while *folE* and *folC* are far apart in the genome (42).

The NICE system was used to induce overexpression of genes involved in folate biosynthesis. At least three of the genes from the folate gene cluster appeared to be involved in controlling folate biosynthesis and folate distribution in *L. lactis*: controlled overexpression of *folKE* increases the extracellular folate production almost 10-fold and the total folate production almost 3-fold; in contrast, the overexpression of *folA* decreases the total folate production approximately 2-fold. The combined overexpression of *folKE* and *folC* favors the intracellular accumulation of folate. Overexpression of the first enzyme of a biosynthetic pathway (GTP cyclohydrolase I) can be a successful strategy to increase the flux through the pathway. Moreover, GTP cyclohydrolase I seems to be a good target for overexpression, since this enzyme in *B. subtilis* has a low turnover and is not regulated by feedback inhibition (10). The use of an inducible promoter system enables study of the effect of various expression levels of the folate biosynthesis enzymes. However, in food fermentations the use of constitutive promoters is preferred. The cloning of *folKE* behind the constitutive promoter of *pepN* resulted in the same increase of folate production as observed by using NICE, although the enzyme production levels were clearly lower. This demonstrates not only that functional expression of folate biosynthesis genes can also be achieved by using a constitutive promoter but also that a further increase in folate production can, presumably, be achieved only by combining *folKE* overexpression with altered expression of other folate biosynthesis genes.

Most of the folate produced by *L. lactis* is intracellularly accumulated, and only a minor part of the folate is secreted by the cells. More than 90% of the intracellular folate pool is present in the polyglutamyl form with four, five, and six glutamyl residues (unpublished results). One of the suggested functions of polyglutamylation is retention of folate within the cell (20, 35). Almost all of the extra folate produced by overexpression of *folKE* is excreted into the environment. We suggest that by the increased flux through the folate biosynthesis pathway, due to the overexpression of *folKE*, the enzymatic capacity of folate synthetase/polyglutamyl folate synthetase is not sufficient to transform all extra produced folate into the polyglutamyl form, which is necessary for retention of folate within the cell. As a consequence, the retention of folate in the cell is decreased. However, when *folKE* and *folC* are simultaneously overexpressed, the majority of the extra folate produced remains intracellular. This confirms that an increased capacity of folate synthetase leads to increased retention of folate in the cell due to an increased enzymatic capacity to elongate the glutamyl tail of the extra folate produced by the overexpression of *folKE*.

The decrease in folate production by overexpression of *folA*, encoding dihydrofolate reductase, may indicate a feedback inhibition of its reaction product, tetrahydrofolate, on one of the other enzymes involved in folate biosynthesis. Vinnicombe and Derrick (49) report an inhibiting effect of tetrahydrofolate on dihydropteroate synthase in *S. pneumoniae*. To further analyze the observed controlling effect of *folA*, we used the NICE system to produce the antisense RNA of *folA* and we measured the dihydrofolate reductase activity in vitro. Enzymatic activity was decreased approximately twofold in cells expressing *folA* antisense RNA. The same cells showed a 20% increase in folate production, confirming the presumable controlling effect of the *folA* gene product. To further improve our knowledge about the suggested effect of tetrahydrofolate on total folate production, we are working on the substitution of the *folA* promoter in the chromosome with the nisin-inducible promoter *nisA*.

It can be assumed that the increase of extracellular folate by overexpression of *folKE* is due to an increased production of folate with a short polyglutamyl tail, such as monoglutamyl folate. It has been established that the bioavailability of monoglutamyl folate is higher than that of polyglutamyl folate (for reviews, see references 14, 15, and 16). Polyglutamyl folates are available for absorption and metabolic utilization only after enzymatic deconjugation in the small intestine by a mammalian deconjugase enzyme. Only monoglutamyl folate derivatives can be directly absorbed in the human gut. The activity of these deconjugases is susceptible to inhibition by various constituents found in some foods (2, 39, 41). Furthermore, the intracellular polyglutamyl folate may not be available for absorption by the gastrointestinal tract of the consumer if the folate is not released by the (mostly dead) microorganisms. In

feeding trials, using rats as an animal model, we will investigate whether besides the increase in folate production, the folate bioavailability also will increase in cells overexpressing *folKE*.

Previous studies have shown that metabolic engineering can be well applied in rerouting of the lactococcal primary metabolism to end products other than lactic acid. This study has demonstrated that metabolic engineering can also be used for controlling secondary metabolism, such as the more complex folate biosynthesis pathway. Moreover, the results described here provide a basis for further development of functional foods with increased levels of folate. By using high-folate-producing starter bacteria, fermented dairy products with increased folate levels will become available, which will have a much higher contribution to the human daily folate intake than the 15 to 20% that, on average, is currently contributed by dairy products. Recent studies have shown that fermented foods are among the 15 most important food items contributing to the folate intake (25). In some countries other important sources of folate are synthetic folic acid supplements. The differences between bioavailabilities of synthetic forms of folate and natural forms of folate have not been unambiguously determined (15). However, folate-fortified foods are not widely available all over the world, because of either legislation or limited industrial development. In such cases the increase of folate bioavailability from natural sources may contribute significantly to the general health status of the population.

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