Modulation of Folate Production in Lactic Acid Bacteria

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Abstract

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Food fortification has proven to be very useful in reducing health problems associated with mal-intake of essential nutrients, such as the B-vitamin folate. Folate is used as one-carbon donor/acceptor in several biochemical processes like synthesis of DNA, RNA and some amino acids. Sufficient intake of folate is essential for neural tube development in early life but it has also been described to aid to brain power in the elderly. The daily recommended intake level for folate, however, are still not met by the whole human population. Fermentation fortification is a new concept which can help to increase the intake levels of nutrients and vitamins such as folate. By this method, the level of the nutrient of interest in the food product is increased as a result of microbial activity in the fermentation process. In this study we have focused on modulation of folate levels in food products using lactic acid bacteria. To be able to modulate folate levels it is essential to gain insight in the genes, which are involved in the biosynthesis of folate. Moreover, it is important to gain insight in pathways, which are involved in the production of folate. The missing gene in the folate biosynthesis pathway of *Lactococcus lactis* and *Arabidopsis thaliana* has been identified. Moreover, the role of *p*ABA biosynthesis in the production of folate in *Lactococcus lactis* was addressed. It was observed that disruption of *p*ABA biosynthesis abolished the production of folate. In addition we have shown that a folate-consuming *Lactobacillus gasseri* can be converted into a folate-producing strain by heterologous overexpression of the folate gene cluster of *Lactococcus lactis*. Moreover, a folate-overproducing *Lactobacillus plantarum* strain has been constructed and the impact of folate overproduction on the gene expression and metabolite formation was monitored. The observed discrepancy between the limited metabolic response and the reduced growth rate was further investigated. Propagation of the folate-overproducing strain of *L. plantarum* results in an instable phenotype. We have shown a clear correlation between growth rate, plasmid copy numbers and folate production levels. A minimal medium was developed for *L. plantarum,* which was used to study the impact of extremely low folate pools on growth. In addition, we found that folate overproduction results in resistance towards the folate antagonist methotrexate (MTX). Remarkably, mutants that showed resistance towards MTX were isolated and one of these was found to produce 70% more folate in comparison to the wild-type. Finally we demonstrated that two lactobacilli (*L. reuteri* and *L. plantarum*) can be used to increase the folate content of melon juice by fermentation. This example illustrates the significance of fermentation fortification for increasing the nutritional value of a fermented beverage

In this study we have shown that folate production can be modulated from very low levels (1 ng/L per OD_{600} unit) to very high levels (3 mg/L per OD_{600} unit). Essential for the modulation of folate levels is the presence and absence of *p*ABA in the growth medium. Another critical factor that influences the folate production pools is the expression of the folate gene cluster.

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Introduction, Aim and
Outline of the Thesis

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Chapter 1

Introduction

Tetrahydrofolate is a one-carbon carrier that plays a key role in the synthesis of purines, pyrimidines, amino acids and formylation of tRNA^{Met}. The property of folate biosynthesis can be found in two of the three domains of the phylogenetic tree. The exception is the domain of the Archaea. Species in this domain use tetrahydromethanopterin as one-carbon carrier (25, 27). However, exceptions of the ability to synthesize folate can also be found within the group of the Bacteria and Eukarya. In particular, the position of Animalia is remarkable since members of this Kingdom require folate but are not capable of synthesis (26). Species that can not synthesize folate are therefore dependent on the consumption of folate rich food sources like: meat, plants, fruits and fermented food products. Dietary intake of folate is not the only source of biological available folate. Studies with folate-depleted rats have shown that the folate supply for the animals could be increased through folate coming from cecal-bacteria (20, 23).

Folate deficiency in humans is associated with health problems, such as cancer, cardiovascular diseases as well as neural tube defects in newborns (13, 18, 31, 36). The daily recommended intake (DRI) in European Union (EU) is set at 200 and 400 µg/day for adults and women in the periconceptional period, respectively (5). This increase in folate intake for women in the periconceptional period is important since, only 10% of women at childbearing age have a folate level in red blood cells that is sufficiently high to decrease the risk of neural tube defects (11). There are several ways to increase the folate levels of food products: i) fortification of food products, ii) selection of special plant cultivars, or fruits with increased folate pools, iii) fermentation fortification.

Methods such as fortification have proven to be very useful in reducing health problems associated with folate mal-intake. The addition of folate to food products in the USA and Canada has reduced the prevalence of neutral tube defects with 25% and 46%, respectively (3, 10). However, it was recently shown that high-level intake of chemically synthesized folate might have some adverse health effects (29). It was proposed that these problems could be circumvented by consumption of a mixed diet containing folate-rich foods (29, 38). Bacterial strain selection can be an important contributor to increased folate intake levels, e.g. in fermented food products. The ability to produce folate can differ remarkably between different lactic acid bacteria. The levels may vary from 2 to 214 µg/L folate (34).

Another way to increase folate content in food products is by fermentation fortification. The natural folate content of rye sourdoughs is 6.5 µg/100g and after fermentation by yeasts the folate content was shown to have increased approximately three-fold (19). This increased folate content is a direct result of folate that is produced by the yeast present in the rye sourdough. Fermentation fortification is a new concept which can be improved in several ways: i) bacterial strain selection, ii) delivery engineering and iii) metabolic engineering. There is a growing interest in using lactic acid bacteria for improvement of the nutritional value of fermented food products and/or adding health benefits to these products. Several known lactic acid bacteria are marketed as probiotics, defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (WHO (2)). Also these probiotic lactic acid bacteria can be used for fermentation fortification to increase the nutritional value of the food products.

Lactic acid bacteria

Lactic acid bacteria are a remarkable group of bacteria. Mankind has used lactic acid bacteria without any scientific knowledge for thousands of years, principally for preventing spoilage of food products like meat, vegetables, fruits, and dairy products. In these fermentation processes, the carbohydrates are rapidly converted into lactate and other organic acids. The production of lactate results in acidification of the food products, which helps to preserve these food products. During the last century, these food production processes have been industrialized in large parts of the world. Currently, a wide range of fermented food products are part of the daily diet of billons of people: e.g. cheeses, yoghurts, sauerkraut, sausages, sourdough, beer, wine. The market of fermented food products and beverages is a multi-billion Euro business, showing the importance of gaining insight in controlling and optimizing the processes of food fermentation (4, 8). Besides the production of organic acids some lactic acid bacteria are also able to produce antimicrobial peptides (35). It is believed that the rapid acidification either in combination with this antimicrobial peptide production can prevent growth of unwanted bacteria (1). Two important species of lactic acid bacteria are *Lactobacillus plantarum* and *Lactococcus lactis*. The best studied lactic acid bacterium is *Lactococcus lactis*. This bacterium is used for as starter culture for the manufacturing of cheese, yielding in 10^7 tons of cheese annually (12). *L. plantarum* is a versatile and flexible organism that can be found in several ecological niches, such as dairy, meat and plant material fermentations. However, this bacterium can also be found as a natural inhabitant of the human gastrointestinal tract, and is known to survive passage through the stomach in an active form (9). The genome of *L. plantarum* has one of the largest genomes of all Lactobacilli which are currently known down to the level of the full genome sequence (22, 28).

Folate biosynthesis pathway and folate related reactions

Folate is produced by plants, fungi, certain protozoa and some bacteria (7, 14, 17, 37). In this pathway GTP is converted via several steps into the biological active cofactor tetrahydrofolate (Fig. 1). Inspection of the molecular structure of the folate molecule shows that folate is a tripartite molecule, composed of a pterin residue, *p*ABA and a γ-linked glutamate moiety. These three structural elements reflect the three pathways (pterin, *p*ABA and glutamate pathway) that are involved in the synthesis of the building blocks of folate (Fig. 1). The pterin pathway reflects the conversion of GTP by GTP cyclohydrolases (I or II). Metabolites that arise from this conversion step are called pterins.

The tetrahydrofolate that is produced is converted in the one-carbon pathway into one-carbon carriers such as 5-methyl tetrahydrofolate, 10-formyl tetrahydrofolate, 5,10-methenyl tetrahydrofolate, and 5,10-methylene tetrahydrofolate. These latter components are involved in several reactions in the synthesis of purines, pyrimidines, amino acids and initiation of protein synthesis by formylation of tRNA[Met]. Another remarkable feature of the folate biosynthesis pathway is that folate is involved in the synthesis of its own precursor, GTP (see Fig. 1).
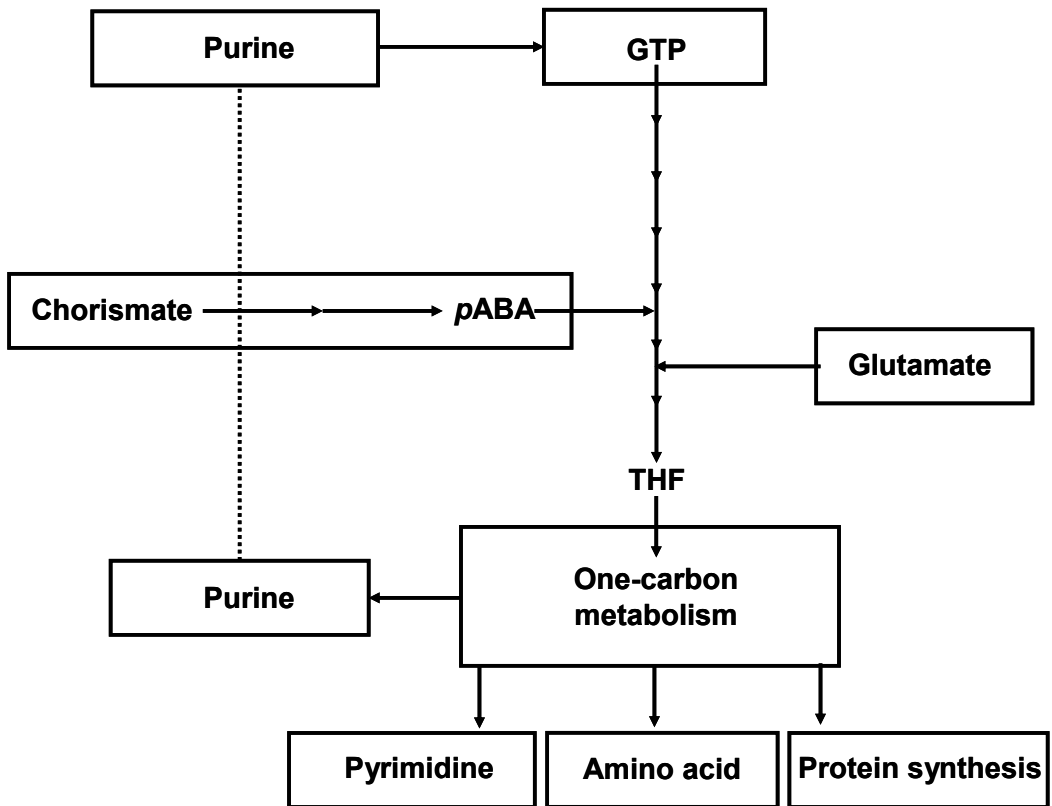
FIG. 1: Schematic representation of the folate and one-carbon pathway. Whereby, GTP is guanosine triphosphate and *p*ABA is *para*-aminobenzoic acid.

Folate biosynthesis genes in lactic acid bacteria

In order to modulate the folate content in fermented products it is crucial to have knowledge of the genes involved in folate biosynthesis. The presence and absence of enzymes involved in folate, one-carbon and *p*ABA metabolism were predicted in 17 species of lactic acid bacteria and two of the best studied bacteria; *Bacillus subtilis* and *Escherichia coli* (Table 1).

TABLE 1: The presence of the folate -, one-carbon - and *p*ABA biosynthesis enzymes for 19 bacterial species predicted from their genomes. The presence of the enzymes is indicated with grey shading that also depicts the number of enzymes predicted to be present. The following enzymes are involved in folate production; FolE, FolQ, FolB, FolK, FolP, FolC1,2 and FolA. One-carbon reactions are catalyzed by the following enzymes; PurN, PurH, ThyA, ThyX, FolD, MetF, FthC, Fmt, GlyA, FolD, MetF2 and Fhs. Finally, *p*ABA biosynthesis is catalyzed by the following enzymes; PabA, PabB and PabC. See Appendix 1 for a list of corresponding enzyme names along with the E.C. numbers and corresponding reactions.

Bacterial species and type-strain	FolE	FolQ	FolB	FolK	FolP	FolC	FolA	PurN	PurH	ThyA	FolD	MetF	FthC	Fmt	GlyA	MetF2	Fhs	PabA	PabB	PabC
B. subtilis 168	1		1	1	1	1	1	2	1	2	1	1	1	1	1			1	1	1
E. coli K12 MG1655	1		1	1	1	1	1	2	1	1	1	1	1	1	1	1		2	1	1
L. acidophilus NCFM						1	1	1	1	1	1		1	1	1	1	2			
L. brevis ATCC367						1	1			1	1		1	1	1	1	1			
L. casei ATCC334						1	1	1	1	1	1	1	1	1	1					
L. delbrueckii bulgaricus	1		1	1	1	2	1	1	1	1	1	1	1				1			
L. delbrueckii subsp. bulgaric	1		1	1	1	2	1	1	1	1		2	1				1			
L. gasseri ATCC-33323						1	1			1	1		1	1	1	1				
L. johnsonii NCC 533						1	1			1	1		1	1	1	1				
L. plantarum WCFS1	1		1	1	1	2	1	1	1	1	1	1	1	1	1	1				
L. reuteri 100-23	1		1	1	1	2	1	1	1	1	1			1	1	1				
L. reuteri F275	1		1	1	1	2	1	1	1	1	1		1	1	1	1				
L. reuteri JCM 1112	1		1	1	1	2	1	1	1	1	1		1	1	1	1				
L. sakei subsp. sakei 23K	2		1	2	1	2	1	1	1	1	1		1	1	1					
L. salivarius subsp. Salivarius						1	1	1	1	1	1		1	1	1	1				
L. lactis IL 1403	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
L. lactis MG1363	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L. lactis SK11	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	2	1	1
S. thermophilus CNRZ1066	1		1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1

It is evident that the three *Lactococcus lactis* strains are the only ones that have complete pathways for folate and *p*ABA biosynthesis as well as for one-carbon metabolism (Table 1). Up to know, *Lactococcus lactis* is the only species for which all folate biosynthesis genes are annotated (21). Three species, *Bacillus subtilis*, *Escherichia coli* and *Streptococcus thermophilus*, appear to lack FolQ (dihydroneopterin triphosphate pyrophosphohydrolase), however, these species are capable of folate production (30, 34, 40), suggesting that an enzyme with dihydroneopterin triphosphate pyrophosphohydrolase activity is waiting to be discovered. Knowing this, we assume that all species which lack FolQ, possess an unannotated gene coding for an enzyme with dihydroneopterin triphosphate pyrophosphohydrolase activity. Moreover, our genome comparison also shows that 7 of the 13 lactobacilli have a complete folate biosynthesis pathway, showing that folate production within this genus is strain dependent. Remarkably, none of the lactobacilli seems to be capable of *p*ABA production, suggesting that within the *Lactobacillus* genus folate production is only possible when free *p*ABA is present in the environment.

The location of the folate genes on the chromosome can be a relevant factor for metabolic engineering purposes, since organization of the genes in a single gene cluster facilitates metabolic engineering strategies (Fig. 2). The sequence alignment of *folP* in *B. subtilis*, *E. coli* and *S. thermophilus*, shows that the genes for folate biosynthesis in these species are not located in a single gene cluster. Instead, the genes appear to be distributed at different locations on the chromosome (data not shown). However, the genes *folQ*, *xtp2* and *mutT* appear to be part of the folate gene cluster. The involvement of *folQ* in folate production is analyzed and discussed in Chapter 3. In the genus *Lactobacillus* and *Lactococcus* all of the folate biosynthesis genes, except for *folA*, are located in a single gene cluster. The gene

cluster of *L. lactis* is well described and the impact of overexpression of some of these genes on the folate production levels have been well established (32-34). Metabolic engineering is used to alter metabolite or protein levels in the cell. This can be done by overexpression or disruption of metabolic or regulatory genes. Metabolic engineering is often used to redirect fluxes through metabolic pathways (16).
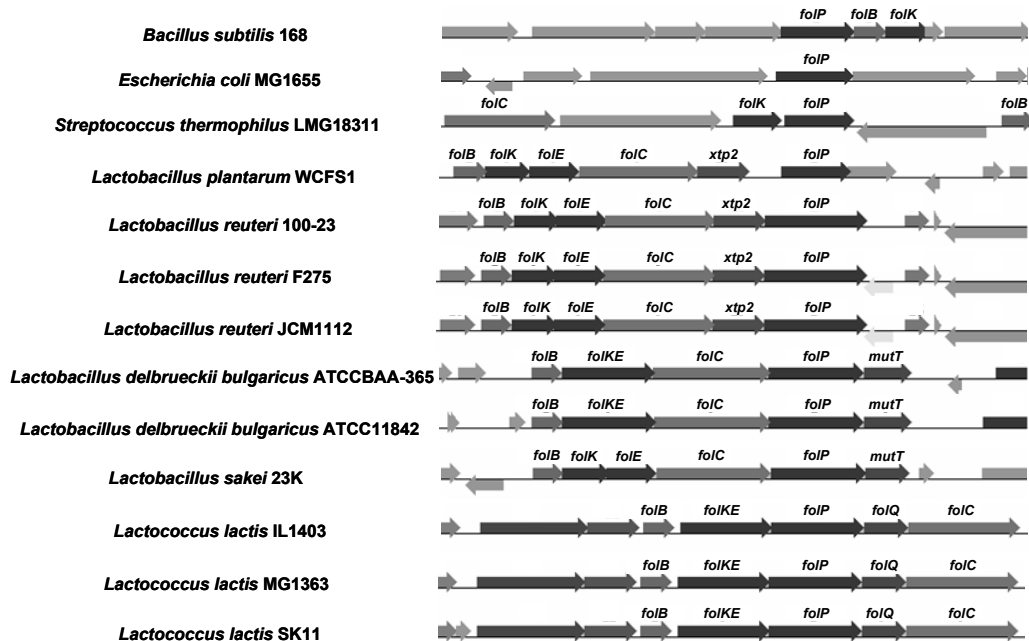

FIG. 2: Alignment of *folP* and adjacent genes in several bacteria (analysis using the ERGO™ database).

In lactic acid bacteria several metabolic pathways have been rerouted or overexpressed; e.g. those for alanine, riboflavin, sorbitol, mannitol and folate production (6, 15, 24, 37, 39). Traditionally, a single pathway is engineered and many aspects of this pathway are investigated. However, the development of metabolic models and functional genomic tools like genomics, transcriptomics, metabolomics and proteomics can help to investigate impact of metabolic engineering on the overall performance of the host organism. Here we describe how folate production levels in lactic acid bacteria can be modulated by gaining insight in the genes, pathways and metabolites that are involved.

Aim
In this thesis we have focused our research on fermentation fortification using lactic acid bacteria to modulate folate production pools on several media. A wide range of methods has been employed to modulate folate production capabilities: (i) gene identification, (ii) metabolic engineering, (iii) impact of medium composition and (iv) identification of novel

food matrices. Crucial for modulation of the folate content in lactic acid bacteria was the understanding of the folate biosynthesis pathway and the genes involved in folate production.

Outline of the thesis

Chapter 2 provides a general introduction on the role of folate and related metabolites in *Bacteria* and *Archaea*. In all biology and biochemistry textbooks, folate is considered to be an essential cofactor in one-carbon (C1) metabolism. However, in many studies on a number of bacterial species, this strict dependency on folate could not be demonstrated experimentally.

Until recently, the gene coding for the enzyme that catalyses the conversion of dihydroneopterin triphosphate into dihydroneopterin monophosphate had not been identified in folate-producing organisms. In **Chapter 3**, we describe the identification of such a gene in the folate gene cluster of *Lactococcus lactis* as the missing gene in the biosynthesis pathway of folate. The biochemical features of this purified enzyme were determined. In addition, a homologue of this gene with conserved NUDIX-motif was identified and characterized in *Arabidopsis thaliana*.

Chapter 4 describes the characterization of the role of *para*-aminobenzoic acid on folate production in *Lactococcus lactis*. Using a bioinformatics approach, all genes for the production of *p*ABA were identified in the genome of *L. lactis*. Moreover, the impact of disruption and overexpression of the *p*ABA gene cluster on the production on folate was determined.

We also converted a folate-consuming *Lactobacillus gasseri* strain into a folate-producing strain. Description of this metabolic engineering strategy and the phenotype of the engineered strain can be found in **Chapter 5**.

In **Chapter 6**, we describe the construction of a folate-overproducing strain which displays a decreased growth rate. The growth rate reduction was further characterized by competitive growth experiments. The maintenance of the folate overproduction plasmid was monitored in propagation experiments in the presence and absence of selectable antibiotic markers.

Chapter 7 describes the physiological response of *Lactobacillus plantarum* WCFS1 towards folate overproduction. Using a functional genomics approach (transcriptomics, metabolomics and metabolic models), the global response of the organism towards elevated folate pools was described in detail. This study delivered understanding of the factors that contribute to the reduced growth performance of the folate-overproducing strain.

Chapter 8 deals with the development of a minimal growth medium for *L. plantarum* WCFS1. This minimal medium allowed us to determine the minimal folate production levels required for growth of *L. plantarum* WCSF1.

Chapter 9 describes folate overproduction as a mechanism of resistance towards the folate antagonist methotrexate in *L. plantarum*. Many modes of resistance of against methotrexate have been reported and we identified a novel modus. This specific mechanism can be used for selecting natural folate overproducing microorganisms.

Chapter 10. Fortification of food products with folate is not mandated in EU since the sole addition of folate can mask the deficiency of vitamin B12. In this chapter it was demonstrated that the lactic acid bacterium *L. reuteri* is capable of folate and cobalamin production on synthetic medium and liquid food products.

Chapter 11 Summarizes results of this study and highlights the opportunities for vitamin fortification through fermentation.

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Chapter 2

The Role of Tetrahydrofolates and
Tetrahydromethanopterins in
Bacteria and Archaea

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Abstract

The biosynthesis of tetrahydrofolate (THF) and the one-carbon metabolism has been well studied. In this review we give a comprehensive insight in the THF biosynthesis- and one-carbon pathways of bacteria. Moreover, we describe the role and importance of THF in bacterial metabolism by summarizing one-carbon donation on molecules that participate in folate dependent reactions. Finally, we give some examples in Bacteria and Archaea where specific one-carbon carriers of THF and tetrahydomethopterin, respectively, are not essential for growth.

Introduction

One-carbon tetrahydrofolate (THF) carriers are involved as one-carbon donor in several metabolic pathways for the the synthesis of purines, pyrimidines, some amino acids and for initiation of protein synthesis in all organisms. Exceptions of the ability to synthesize folate can be found within the group of the Bacteria and Eukarya. The position of Animalia is remarkable since members of this Kingdom require folate but are not capable of synthesis. Species that can not synthesize folate are therefore dependent on the consumption of folate rich food sources like meat, plants, fruits and fermented food products. Folate transporters are involved in Eukarya and Bacteria for the uptake of folates. In Animalia these transporters are well described and identified on the chromosome of some organisms. However, in folate-auxotrophic bacteria these transporters have been characterized (18, 31), but the genes have not been found. The ability to produce THF is not only found in bacteria but also in green plants, fungi and certain protozoa (5, 15, 23, 58). In Archaea, however, folate production has not been established. In Archaea, several of the one-carbon reactions take place with tetrahydromethanopterin (THMPT) as co-factor; a molecule that is structurally related to THF (Fig. 1, panel A and B).
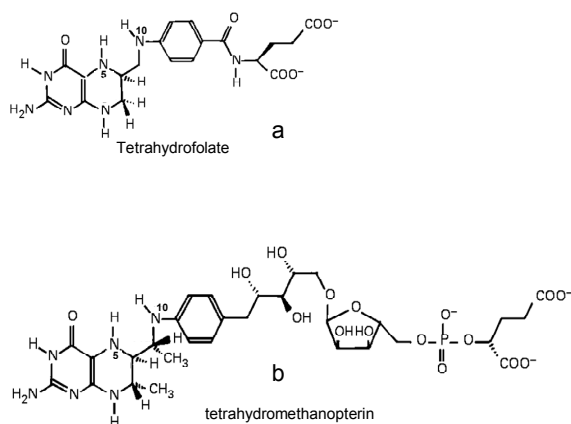

FIG 1: Structure of THF (panel A) and THMPT (panel B).

In many lower organisms the absolute requirement for THF or THMPT as cofactor in the purine, amino acid and protein synthesis could not be established as is described below. In Archaea, for example, an alternative one-carbon donor is involved in the one-carbon metabolism. In bacteria the addition of specific medium components (folate-dependent metabolites, such as purines, pyrimidines and some amino acids) can circumvent the need for folate. In some organisms protein synthesis can be initiated without the need for folates. Furthermore, comparative genomics studies have shown that the genomes of several bacterial species are lacking one or more genes encoding for folate biosynthesis and one-carbon metabolism

Folate biosynthesis and one-carbon reactions

The biosynthesis pathway of THF is complex and involves the purine, *p*ABA and glutamate pathway. THF is used in one-carbon metabolism, for the synthesis of purine, pyrimidine and amino acid but also for the initiation of protein synthesis (Fig. 2).

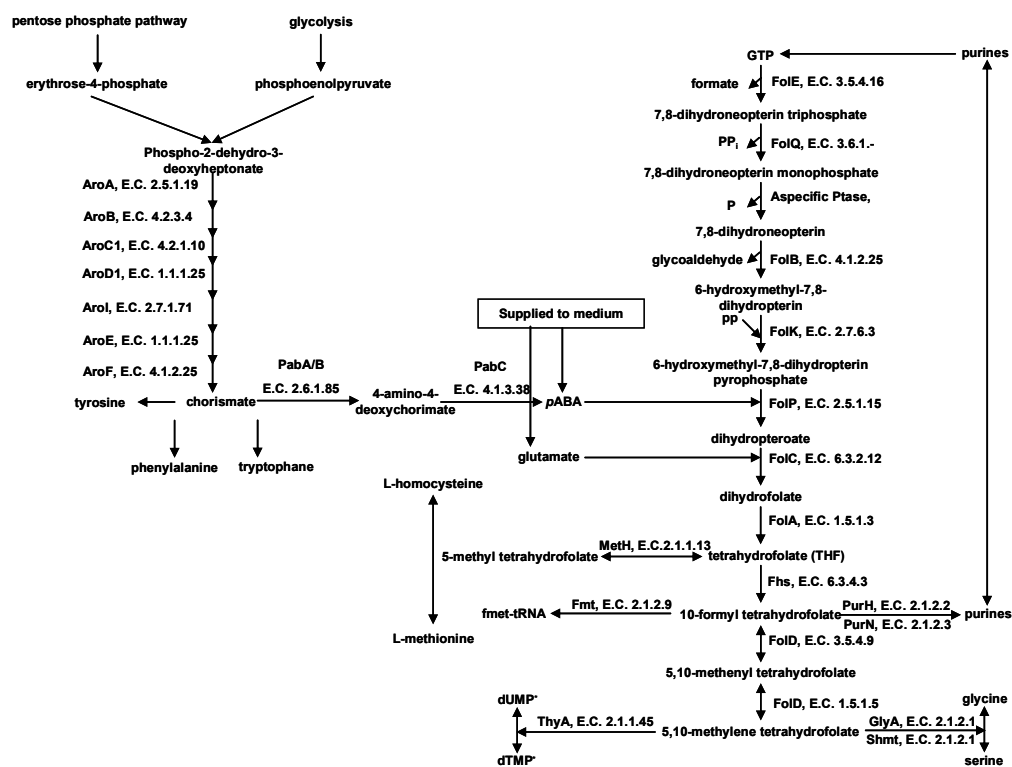FIG 2: The biosynthesis pathway of THF and *p*ABA. Along with the one-carbon metabolism with all reactions were THF carriers are involved as one-carbon donor or acceptor. In all one-carbon donation reactions, the THF one-carbon carrier is converted into THF after the donation reaction, the only exception is the conversion of dUMP into dTMP, where 5,10-methylene THF is converted into dihydrofolate[*].

In the biosynthesis pathway of THF (Fig. 2) the nucleotide GTP is converted via GTP cyclohydrolase I (GCHYI, E.C. 3.5.4.16, FolE) into 7,8-dihydroneopterin triphosphate. Then the three phosphate groups are removed by two consecutive reactions. First a pyrophosphate is removed by dihydroneopterin triphosphate pyrophosphohydrolase (E.C. 3.6.1.-, FolQ). The other phosphate group is removed by an a-specific phosphatase. Next, 7,8-dihydroneopterin is converted by dihydroneopterin aldolase (DHNA, E.C. 4.1.2.25, FolB) into 6-hydroxymethyl-7,8-dihydropterin. The latter component is pyrophosphorylated by hydroxymethyldihydropterin pyrophosphokinase (HPPK, E.C. 2.7.6.3, FolK), resulting in 6-hydroxymethyl-7,8-dihydropterin pyrophosphate. This component is condensed with *p*ABA, mediated by dihydropteroate synthase (DHPS, E.C. 2.5.1.15., FolP), into dihydropteroate. Subsequently, dihydrofolate is synthesized by coupling of glutamate with dihydropteroate by dihydrofolate synthase (DHFS, E.C. 6.3.2.12, FolC). Dihydrofolate is converted through dihydrofolate reductase (DHFR, E.C. 1.5.1.3, FolA) into THF. Finally, folylpolyglutamate synthase (FPGS, E.C. 6.3.2.17, FolC) adds a γ-glutamyl tail, to allow intracellular retention (53). *p*ABA is synthesized from chorismate. The latter intermediate is synthesized in the shikimate pathway were 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. Chorismate is converted via chorismate synthetase component I and II (E.C. 6.3.5.8, PabA and PabB) into 4-amino-4-deoxychorismate. Subsequently, a pyruvate-group is cleaved of from the latter intermediate by 4-amino-4-deoxychorismate lyase (E.C. 4.1.3.38, PabC), to result in *p*ABA. Glutamate is an important moiety of the folate molecule, but most lactic acid bacteria are auxotrophic for glutamate (54).

THF and its one-carbon carriers (5-methyl THF, 10-formyl THF, 5,10-methenyl THF, and 5,10-methylene THF) are involved in a number of single-carbon-transfer reactions (Fig. 2). The one-carbon carrier 10-formyl THF is synthesized from formate and THF via formate-tetrahydrofolate ligase (E.C. 6.3.4.3, Fhs). 10-Formyl THF is used twice for the synthesis of inosine monophosphate (IMP). Firstly, phosphoribosylglycinamide formyltransferase (E.C. 2.1.2.2, PurN) converts 5-phospho-ribosyl-glycineamine into 5-phospho-ribosyl-N-formylglycineamine. Secondly, aminoimidazolecarboxamide transformylase (E.C. 2.1.2.3, PurH) catalyzes the reaction from aminoimidazolecarboxamide ribotide into phosphoribosyl-formamido-carboxamide, which is subsequently converted by an additional enzymatic reaction (IMP cyclohydrolase E.C. 3.4.5.10) into IMP. The cofactor 10-formyl THF is also required for the initiation of protein synthesis. In this reaction, methionyl-tRNA is formylated into formyl-methionyl-tRNA by methionyl-tRNA formyltransferase (E.C. 2.1.2.9, Fmt). The one-carbon carrier 5,10-methylene THF is synthesized from the conversion of serine into glycine by hydroxymethyl transferase (E.C. 2.1.2.1, Shmt). The latter component is used for the conversion of dUMP into dTMP via thymidylate synthase (E.C. 2.1.1.45, ThyA), The cofactor 5,10-methylene THF is converted via methylene tetrahydrofolate dehydrogenase (E.C. 1.5.1.5, FolD) into 5,10-methenyl THF, which is sequentially converted via methenyl tetrahydrofolate cyclohydrolase (E.C. 3.5.4.9, FolD) into 10-formyl THF. From these reactions it can be seen that specific one-carbon THF carriers can

be interconverted. Moreover, THF plays a key-role in the production of purines, pyrimidines, amino acids and for the initiation of protein synthesis. However, there are some exceptions to this rule, as described below.

One-carbon metabolism in Archaea.

One-carbon metabolism in Archaea is quite distinct from that found in Bacteria. In methanogenic and sulfate-reducing Archaea a molecule structurally related to THF is produced (Fig. 2) (1). This molecule, THMPT, is involved in one-carbon metabolism in Archaea. Remarkably, cell extracts of Archaea containing THMPTs could not be used to evoke growth of a folate auxotrophic bacteria (32), showing the molecular mechanisms of THMPT is distinct from THF. In the bacterium *Methylobacterium extorquens* AM1, the THF and the THMPT biosynthetic pathways coexists; this micro-organism is considered to be an evolutionary progenitor for both pathways (56). In this bacterium one-carbon molecules such as methanol are converted via THMPT one-carbon metabolism into CO_2, the resulting CO_2 and methanol is used by the THF one-carbon metabolism for the assimilation of all cellular components (38).

Biochemical evidence indicates that formyl donation by 5-formyl THMPT or 10-formyl THMPT in Archaea is biochemically unfavorable, which reflects to the fact that protein synthesis in Archaea is initiated with unformylated methionyl-tRNA (35, 43). In addition, in several Archaea it has been established that formyl-THMPT is not required or essential for the synthesis of purines (43, 49). Instead, in these species alternative one-carbon molecule such as formate are used directly for the synthesis of purines (60). Additional biochemical evidence has shown that one-carbon donation of methyl- and methylene-THMPT is possible. However, it is believed that one-carbon donation with THMPTs carriers is not as efficient as with THF carriers (35). It has been suggested from genomic analyses and enzymatic assays that a small number of highly homologous enzymes of folate biosynthesis among Archaea are expected to use THMPTs (4, 21, 35, 50). Remarkably, by checking for the presence of the folate biosynthesis and one-carbon genes we identified a substantial number of archaeal species to carry several genes, which are annotated as folate or one-carbon genes (ERGO database) (Table 1). In fact we observed that 12 of the 13 genomes possess four or more annotated folate biosynthesis or one-carbon metabolism genes, however, it remains unclear whether these genes are expressed. Moreover, each of these 12 genomes contains *folP* and *glyA*, coding for dihydropteroate synthetase, and glycine hydroxymethyltransferase, respectively. In *Nanoarchaeum equitans* Kin4-M 1, none of the genes associated with folate- or one-carbon metabolism were predicted to be present, which is compatible with its symbiotic lifestyle. In summary, although the genes encoding enzymes for the biosynthesis of THF and THMPT are distinct from each other, both groups of molecules are involved in many identical reactions in one-carbon metabolism.

Growth in the absence of folates.

A recent study of de Crecy-Lagard et al (6) has shown that nearly 40% of all sequenced bacteria (150 out of 400) are predicted to be auxotroph for folate production. The *a priori* assumption is that these auxotrophic bacteria are therefore dependent on the uptake of

folate. The folate transporters of two folate auxotrophic bacteria, *Lactobacillus casei* and *Lactobacillus salivarius*, have been well described (18, 31).

TABLE 1: The presence of 6 folate biosynthesis genes (*folE*, *folB*, *folK*, *folP*, *folC* and *folA*) and 6 folate-dependent one-carbon metabolism genes (*purN*, *purH*, *thyA*, *thyX*, *fmt* and *glyA*) is shown for 15 genomes. The top 13 organisms are randomly selected Archaea and the two lower are the Bacteria; *B. subtilis* and *E. coli*. A grey cell indicates the presence of that particular gene and the digit indicates the number of copies present on the genome.

	folE	folB	folK	folP	folC	folA	purN	purH	thyA	thyX	fmt	glyA
Ferroplasma acidarmanus fer1 (JGI)	1			1	1		1	1				2
Haloarcula marismortui ATCC 43049	1			3	1	2		1		1		1
Haloquadratum walsbyi				2	1	2	1	1	1			1
Methanobrevibacter smithii ATCC 35061	1			1					1			1
Methanococcus jannaschii DSM 2661				1			1		1			1
Methanocorpusculum labreanum Z				2			1	1	1		1	1
Methanospirillum hungatei JF-1				2			1	1	1		1	1
Nanoarchaeum equitans Kin4-M												
Natronomonas pharaonis DSM 2160				2	1	1		1	1			1
Pyrobaculum calidifontis JCM 11548	1			1			1			1		1
Pyrococcus abyssi				1			1			1		1
Sulfolobus acidocaldarius DSM 639	1		1	1			1			1		1
Sulfolobus solfataricus P2	1			1			1			1		1
Bacillus subtilis 168	1	1	1	1	1	1	2	1	2		1	1
Escherichia coli K12 MG1655	1	1	1	1	1	1	2	1	1		1	1

Remarkably, it was found that some folate-auxotrophic lactic acid bacteria were able to grow in the absence of folate. The bacterium *Lactobacillus acidophilus* (51) is unable to produce folate, however, the sole addition of folate-dependent metabolites (the end products of folate dependent biosynthesis pathways; e.g. serine, methionine, purines and thymine) was found restore growth completely. Remarkably, also *Lactobacillus casei* ATCC 7469 a strain that is well established as indicator organism in the folate microbiological assay (19, 20), was found to grow in the absence of folate when high levels of thymine and guanine were supplied to the medium (39). These examples show that the need for folate as essential cofactor for one-carbon metabolism can be circumvented when folate-dependent metabolites are supplied to the growth medium (purines, pyrimidines and some amino acids). Folate-dependent metabolites can also circumvent the need for folate when the folate biosynthesis pathway is blocked by gene deletion. A *p*ABA deletion-strain of *E. coli* was unable to produce folate, which prevented growth. However, the supplementation of folate, *p*ABA or the folate-dependent metabolites restored growth of this strain (12, 29). Deletion of the complete *p*ABA gene cluster in *Lactococcus lactis* abolished folate production completely (Chapters 4, and references 5,8). Growth of this deletion-strain was dependent on 10-formyl THF, but this requirement could be circumvented by the addition of purines to the growth medium. The latter study indicates that alternative cofactors must exist for the synthesis of amino acids and thymidylate. The lactic acid bacterium *Lactobacillus plantarum* WCFS1 is unable to produce folate in the absence of *para*-aminobenzoic acid. However, this strain was able to grow on a minimal medium that lacks *p*ABA, folate and all folate-dependent metabolites (Chapter 8). When comparing folate production of this strain in the presence and absence of *p*ABA a 12,000-fold reduction in folate pool size still did not affect the growth rate of this strain

(Chapter 8). This experiment strongly suggests that in *L. plantarum*, alternative one-carbon donors participate in the synthesis of metabolites, which were thought to be folate-dependent. This overview shows that many bacteria do not depend on the presence of folate in the growth medium. Instead, the supplementation of folate-dependent metabolites was found to restore growth completely.

In natural folate producers, the biosynthesis pathway of folate can be blocked by chemical agents which are mostly structural analogues of folate or folate intermediates. Folate antagonists like methotrexate (MTX) are chemically produced compounds with a broad use in therapeutics and medicine. MTX, for example, can be used as medicine against malaria to prevent growth of the parasite *Plasmodium falciparum* (24). A number of these antagonists compete with dihydrofolate for the binding site of the enzyme dihydrofolate reductase (DHFR) (E.C. 1.5.1.3) (59). Blocking DHFR will deplete THF pools. Reduction of THF pools can have a dramatic effect on the growth rate. In *E. coli*, for example, the addition of the folate antagonist trimethoprim to minimal medium severely reduced the growth rate (16, 57). The addition of purines and pyrimidines to the growth medium restored growth rate to almost normal levels. Interestingly, it was suggested that the supplementation of the purines and pyrimidines can not bypass the need for formylated methionyl-tRNA, which explains the slight reduction in growth rate. Similar experiments were performed with *L. plantarum* WCFS1 (Chapter 9). It was found that growth of this strain is severely reduced on a growth medium lacking folate-dependent metabolites in the presence of the methotrexate. In our laboratory we have shown that there are two ways to cope with the growth inhibitory effect of methotrexate: i) the addition of the folate-dependent metabolites to the growth medium, ii) overproduction of folate. In the first way, the need for folate is bypassed through the presence of the folate-dependent metabolites. In the engineered folate overproducer, the high intracellular folate pools outcompete intracellular methotrexate (MTX) on the level of dihydrofolate reductase (DHFR). These experiments clearly show that components such as trimethoprim and methotrexate, will have limited antimicrobial effect when applied in niches that are rich in folate or folate-dependent metabolites, such as the intestine or food products.

In conclusion, one-carbon THF carriers are used in several reactions. Often the uptake of folate is considered to be essential for growth, especially, for folate auxotrophic bacteria; however, many documented examples exist of bacteria that have a conditional dependency on folate as a cofactor. Blocking of the folate production by gene disruption or by applying folate biosynthesis inhibitors does not lead to growth impairment when the folate-dependent metabolites are supplied to the growth medium. All these examples show that folate production is only essential in a medium that lacks the folate-dependent metabolites.

10-Formyl THF for initiation of protein synthesis.

In addition to the production of the folate-dependent metabolites, folate has a unique position in the formylation of methionyl-tRNAs for protein synthesis. For protein synthesis, two types of methionyl—tRNA's exist: (i) a formylated (fMet-tRNA) and (ii) an unformylated molecule (Met-tRNA). The formylated methionyl-tRNA is essential for initiation of protein synthesis, whereas the methionyl-tRNA is needed for elongation of the polypeptide chain (Fig. 3).
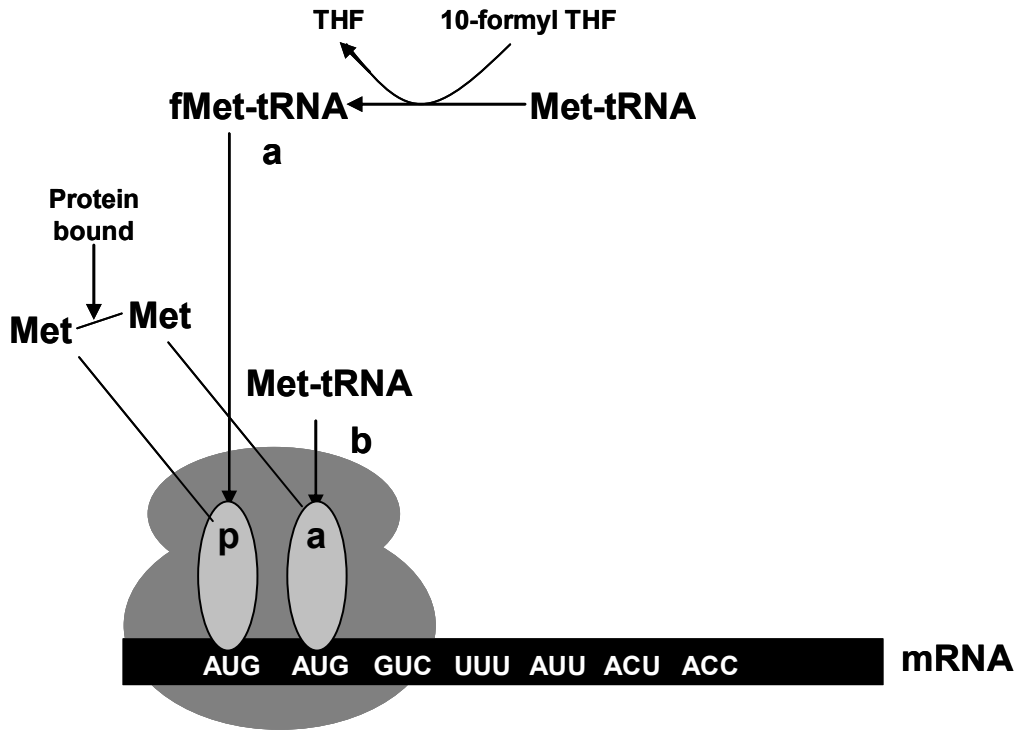
FIG 3: Involvement of 10-formyl THF in the initiation of protein synthesis in Bacteria. The cofactor 10-formyl THF is converted into THF and simultaneously, the formyl-group is donated to methionyl tRNA. The formylated methionyl-tRNA (panel A) can only bind to the p-site of the ribosome, whereas the unformylated methionyl-tRNA (panel B), can only bind to the a-site of the ribosome.

Initiation of protein synthesis with formylated methionyl-tRNA takes place in most prokaryotic cells, chloroplasts, and mitochondria all possessing 70S type ribosomes (37, 48). The initiation of protein synthesis with formylated methionyl-tRNA is catalyzed by methionyl-tRNA formyltransferase (E.C. 2.1.2.9). In this reaction, the formyl group is donated by 10-formyl THF to tRNA^{Met} (7). Remarkably, it has been reported that some bacteria do not require formyl-THF for initiation of protein synthesis. In *Enterococcus faecalis* R, for example, protein initiation could be initiated in the absence of 10-formyl THF (44). The authors have suggested the presence of an alternative formyl-molecule, such as formate, that can substitute the function of 10-formyl THF (8, 36, 45). In addition, similar experiments were performed using *E. coli*. At high magnesium concentrations (>10 mM Mg^{2+}) initiation of protein takes place with methionyl-tRNA (28, 42). At magnesium concentration below 6 mM, 10-formyl THF is needed for formylation of tRNA^{Met} (28, 42). Deletion of the gene encoding methionyl-tRNA formyltransferase (*fmt*) in *E. coli* resulted in viable cells (13), demonstrating that protein initiation takes place without *fmt*. Recently, it was

found that the genomes of *M. hyopneumoniae* and *Aster yellows phytoplasma* OY-M lack methionyl-tRNA formyltransferase (*fmt*). From this it was concluded that these species are able to initiate protein synthesis without formyl THF (6). Within the domain of Archaea and Eukarya it is well established that protein synthesis takes place without need for formylated methionyl-tRNA (43, 49). The evolutionary purpose of formylated methionyl-tRNA^Met for protein synthesis in bacteria is well established. it has been shown that formylated methionyl-tRNA has an increased sensitivity to select the start codon in frame (52). The presented overview of literature illustrates that a wide range of organisms do not depend on 10-formyl THF for the initiation of protein synthesis, in fact, in some species protein synthesis can be initiated in the absence of an alternative formyl donor.

Disruption of the folate biosynthesis pathways in bacteria

Gene disruption is a suitable way to obtain more insight in functional aspects of the metabolic pathway of interest. Some genes will be essential, whereas, the deletion of other genes might help to reveal alternative pathways. Many viable deletions can be made in the genes for the folate biosynthesis- and carbon-one pathways. Some of these deletion-strains require folate for growth; others can be salvaged by the addition of *p*ABA or folate-dependent metabolites. Finally some deletion-genotypes force the organism to express new pathways. In an attempt to systematically delete all genes of *E. coli* (the Keio collection) it was found that *folE*, *folK*, *folC*, and *folA* were essential on rich medium (2). In contrast to this, *folP* and *folB* were the only genes marked as essential on minimal medium (2, 26). In this experiment no-growth was defined when the optical density was lower than one third of the average optical density. As a consequence, slow growth of these putative viable deletion-strains can be easily missed. A closer look at these deletions-strains showed that some strains were able to grow after all. In *E. coli*: *folA*, *folP*, *folX, folK* and *thyA* were successfully deleted from the genome as single deletion (9, 17, 22, 25, 40). Deletion of *folA* in *E. coli* resulted in viable cells when plated on minimal medium plates supplemented with thymidine (30). Also a double deletion, Δ*thyA* and Δ*folA*, was able to grow on minimal medium, supplemented with thymidine (11). Growth of this deletion-strain has been attributed to the expression of an alternative dihydrofolate reductase. This enzyme was called pteridine reductase, and was assigned as FolM. This enzyme reduces dihydrobiopterin into tetrahydrobiopterin. The rate of dihydrobiopterin reduction was only 10% in comparison to the reduction of dihydrofolate into THF (11). Based on the rate of THF synthesis by this alternative dihydrofolate reductases (*folM*) it has been calculated that these THF pools are still not high enough to support growth of *E. coli* (14, 40). Based on the argument presented above, we assume that alternative genes coding for enzymes that reduce folates still wait to be discovered (14, 40). The combined disruption of *folM*, *folA* and *thyA* in *E. coli* did not result in viable cells, except when *folP* of *Helicobacter pylori* was transferred to this deletion-strain (33). From this experiment it was concluded that the *H. pylori* dihydropteroate synthase (*folP*) can complement an *E. coli* mutant which lacks dihydrofolate reductase activity. Recently, it was shown that FolP of *H. pylori* can convert dihydropteroate into tetrahydropteoate, this reaction is dependent on flavins (such as FMN and reduced flavin adenine dinucleotide) for reduction (34), it is expected that tetrahydropteroate is directly converted into THF. Pteridine reductases (PTR1)

were also found in the protozoan parasite *Leishmania major*. Deletion of PTR1 in *L. major* exhibited an absolute requirement for dihydrobiopterins and tetrahydrobiopterins (3). Moreover, it was found that some PTR1 enzymes show dihydrofolate reductase activity, and therefore it was suggested that PTR1 could provide an alternative source of dihydrofolate (3, 47). Another successful gene disruption did not lead to growth impairment. Disruption of *folX* from *E. coli*, a *folB*-analogue, did not show any sign of growth reduction on a minimal medium (17). It was found that FolX, dihydroneopterin-triphosphate epimerase, catalyzes the formation of dihydro-L-monapterin triphosphate. However, the function of the pterin-molecule remains unknown. Multiple and single deletion analysis of some of the folate genes have thus shown new features of the folate biosynthesis pathway and one-carbon metabolism.

Comparative genomics on folate biosynthesis genes.
        Comparative genomics is a valuable approach to explore sequenced genomes and to obtain increased insight in metabolic pathways. As described above it was shown that nearly 40% of all sequenced bacteria lack one or more genes involved in the production of THF (6). In biology textbooks it is stated that the functional coupling between *folA* and *thyA* is essential for all bacteria (40). However, in several bacterial species (*Rickettsia rickettsii* and *Borrelia burgdorferi*) *folA* and *thyA* were not identified on the chromosome, instead a flavin-dependent thymidylate synthase, *thyX*, was found (40). It was shown that dihydrofolate accepts the carbon-one group from 5,10-methylene THF, but the electrons needed for the formation of the methyl-group are supplied by the reduced flavin-nucleotide. From these findings it was concluded that two major pathways exist for dTMP formation in bacteria: (i) *thyA* dependent, (ii) *thyX* dependent (40, 41). In this context, the folate-auxotrophic microorganism, *M. hyopneumoniae*, is of particular interest. The genome of *M. hyopneumoniae* possesses only one gene, coding for a folate-dependent bioconversion step (55). The enzyme encoded by this gene (*glyA*), serine hydroxymethyltransferase (E.C. 2.1.2.1), is believed to require folate for the conversion of serine into glycine. However, in rabbit liver cells it was found that threonine could be converted into glycine in the absence of reduced folate carriers (46). Therefore, it was suggested that in the presence of threonine, growth of *M. hyopneumoniae* is completely independent from folate production or folate uptake. A closer look at the folate biosynthesis pathway reveals that the lactic acid bacterium *L. lactis* is the only organism known to possess a complete biosynthesis route (6, 27). In this organism the missing step in the folate biosynthesis pathway has been characterized and an enzyme named FolQ was found to catalyze the reaction from dihydroneopterin triphosphate into dihydroneopterin monophosphate (Chapter 3 and (27). The folate biosynthesis genes (*folE*, *folB*, *folK*, *folQ*, *folP*, *folC*, *folA*) are very well described and their sequence is known. However, several genomes lack some of these genes, whereas other genomes hold several copies of particular genes (6). Gene deletion studies and comparative genomics on the folate genes have shed new light on many putative genes involved in the production of folate or in the one-carbon THF carriers. The staggering rate at which new genome sequence data becomes available holds the promise that in the near future new genes associated with folate pathways will be discovered.

Conclusions

This review described many of the exceptions to THF and THMPT biosynthesis and one-carbon metabolism in Bacteria and Archaea. Species in the latter taxonomic group possess alternative one-carbon donors, which are structurally related to THF, but not capable of one-carbon donation in bacteria. A large number of bacteria are unable to grow produce folate, whereby several of these micro-organisms are able to grow in the absence of folate when folate-dependent metabolites are supplied to the medium. Protein synthesis can proceed in the absence of 10-formyl THF and 10-formyl THMPT in several members of the domain of Bacteria and all Archaea. Many of the known folate - and one-carbon related genes could be disrupted; in these strains alternative one-carbon pathways were discovered. It was found that a *L. plantarum* strain producing 4 orders of magnitude less folate compared to the production levels under standard growth conditions, still showed normal growth behavior (Chapter 8). This motivates the search for new one-carbon donors in these organisms.

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Chapter 3

A Nudix Enzyme Removes
Pyrophosphate from Dihydroneopterin
Triphosphate in the Folate Synthesis
Pathway of Bacteria and Plants

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Abstract

Removal of pyrophosphate from dihydroneopterin triphosphate (DHNTP) is the second step in the pterin branch of the folate synthesis pathway. There has been controversy over whether this reaction requires a specific pyrophosphohydrolase or is a metal ion-dependent chemical process. The genome of *Lactococcus lactis* has a multicistronic folate synthesis operon that includes an open reading frame (*ylgG*) specifying a putative Nudix hydrolase. Because many Nudix enzymes are pyrophosphohydrolases, YlgG was expressed in *Escherichia coli* and characterized. The recombinant protein showed high DHNTP pyrophosphohydrolase activity with a K_m value of 2 μM, had no detectable activity against deoxynucleoside triphosphates or other typical Nudix hydrolase substrates, required a physiological level (~1 mM) of Mg^{2+}, and was active as a monomer. Essentially no reaction occurred without enzyme at 1 mM Mg^{2+}. Inactivation of *ylgG* in *L. lactis* resulted in DHNTP accumulation and folate depletion, confirming that YlgG functions in folate biosynthesis. We therefore propose that *ylgG* be redesignated as *folQ*. The closest *Arabidopsis* homolog of YlgG (encoded by Nudix gene At1g68760) was expressed in *E. coli* and shown to have Mg^{2+}-dependent DHNTP pyrophosphohydrolase activity. This protein (AtNUDT1) was reported previously to have NADH pyrophosphatase activity in the presence of 5 mM Mn^{2+} (Dobrzanska, M., Szurmak, B., Wyslouch-Cieszynska, A., and Kraszewska, E. (2002) *J. Biol. Chem.* 277, 50482–50486). However, we found that this activity is negligible at physiological levels of Mn^{2+} and that, with 1 mM Mg^{2+}, AtNUDT1 prefers DHNTP and (deoxy) nucleoside triphosphates.

Introduction

Enzymes and genes for all except one of the specific steps of the folate biosynthesis pathway have been identified in bacteria (1) and plants (2–7). The exception is the second step of the pterin branch of the pathway, in which pyrophosphate is cleaved from dihydroneopterin triphosphate (DHNTP),[1] yielding dihydroneopterin monophosphate (DHNP) (Fig. 1*A*). Furthermore, there has been controversy over whether this step is enzymatic (8–10).

In favor of an enzymatic mechanism, Suzuki and Brown (9) partially purified a small (17 kDa) *Escherichia coli* protein able to catalyze the release of pyrophosphate from DHNTP but not nucleoside triphosphates. The enzyme was accordingly named DHNTP pyrophosphohydrolase. The K_m for DHNTP was 11 μM, and activity was Mg^{2+}-dependent. No evidence was presented showing this activity to be necessary for folate synthesis, however. More recent work in *Bacillus subtilis* has questioned the requirement for an enzyme, because chemical conversion of DHNTP to DHNP was observed at pH 8 and 37 °C in the presence of Mg^{2+} or Ca^{2+} (10). Unfortunately, the Mg^{2+} and Ca^{2+} concentrations studied were non-physiologically high (5–12 mM), with typical cytosolic levels of free Mg^{2+} and Ca^{2+} being ~1 mM and ~0.1 μM, respectively, in both bacteria and plants (11–14).

The mechanism of the DHNTP →DHNP step is of considerable interest. First, if there is a specific DHNTP pyrophosphohydrolase, it could be a novel target for antibacterial drug discovery in the folate synthesis pathway (8). Second, modestly higher fluxes to folate can be engineered in bacteria (15) and plants (16, 17) by greatly increasing the production of DHNTP, but it is not yet clear whether this DHNTP is efficiently converted to DHNP. If it is

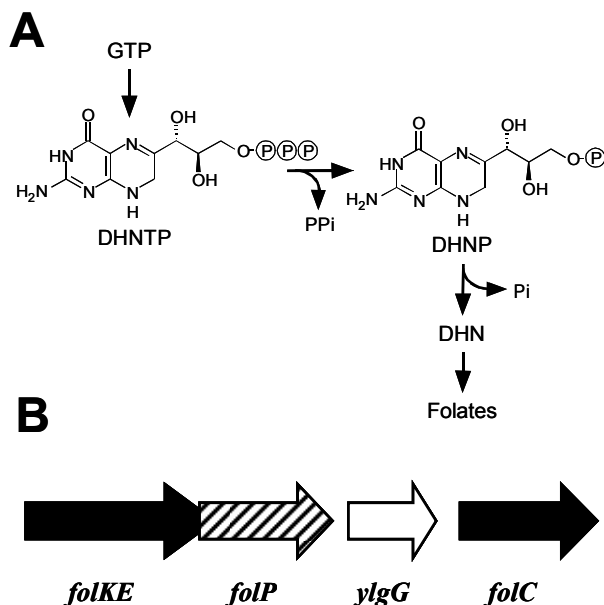not, engineering the overexpression of a DHNTP pyrophosphohydrolase could be used to correct the problem.

FIG. 1: Schemes of the reaction that removes pyrophosphate from DHNTP (*A*) and of the multicistronic folate biosynthesis operon of *L. lactis* (*B*). The *folKE* gene encodes a bifunctional GTP cyclohydrolase I/hydroxymethyldihydropterin pyrophosphokinase; *folP* encodes dihydropteroate synthase; *folC* encodes dihydrofolate synthase, and *ylgG* encodes a Nudix protein.

The annotated genome sequence of the lactic acid bacterium *Lactococcus lactis* (18) offers a clue to the existence of a specific DHNTP pyrophosphohydrolase, for it reveals a folate biosynthesis gene cluster in which known folate genes flank a gene (*ylgG*) encoding an unknown protein from the Nudix hydrolase family. Nudix (nucleoside diphosphate linked to x) hydrolases are typically small proteins (15–25 kDa), contain a characteristic motif ($GX_5EX_7REUXEEXGU$, where U is a hydrophobic residue), and many have nucleoside triphosphate pyrophosphohydrolase activity (19). Furthermore, *ylgG* was shown to be cotranscribed with three of the flanking folate genes in a multicistronic operon (15, 20) (Fig. 1*B*). Genomic evidence thus identifies YlgG as a candidate for the elusive DHNTP pyrophosphohydrolase.

In this study, we set out to find whether recombinant *L. lactis* YlgG has specific DHNTP pyrophosphohydrolase activity, and whether *ylgG* deletants are defective in pteridine and folate synthesis. When this proved to be the case, we sought homologs of YlgG among the Nudix proteins of *Arabidopsis* (21), and we showed that the closest homolog has DHNTP pyrophosphohydrolase activity.

Materials and methods

Chemicals and Reagents

Pteridines were obtained from Schircks Laboratories (Jona, Switzerland). The Biomol Green[TM] phosphate assay reagent was from Biomol (Plymouth Meeting, PA). Ni^{2+}-nitriloacetic acid superflow resin was from Qiagen (Valencia, CA). PD-10 columns were from Amersham Biosciences. Oligonucleotides were from MWG Biotech (High Point, NC). Reduced nicotinamide mononucleotide (NMNH) was a gift of G. Magni (University of Ancona, Italy). Other chemicals were from Sigma or Fisher.

Inactivation of ylgG by Double Crossover Recombination

The bacterial strains and plasmids used are listed in Table 1. *E. coli* was grown at 37 °C in TY medium (22), and *L. lactis* was grown at 30 °C in M17 medium (23) containing 0.5% glucose (GM17 medium) or in chemically defined medium (CDM), prepared as described (24) except that folic acid was omitted.

TABLE 1: Bacterial strains and plasmids used in *ylgG* inactivation

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
L. lactis NZ9000	*MG1363 pepN*:nisRK	27
E. coli EC1000	Cloning host: RepA+ MC1000, Km[r], carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	26
Plasmids		
pORI280	EM[r], LacZ+, ori+ of pWV01, replicates only in strains providing *repA* in trans	26
pORI280-folP-ylgG-folC	pORI280 with *ylgG* and flanking regions of *folP* and *folC* inserted in multiple cloning site	This study
pORI280-folP-folC	pORI280-*folP-ylgG-folC* containing a 477 bp in-frame deletion of *ylgG*	This study

E. coli was transformed by heat shock and *L. lactis* by electroporation (25). For *E. coli*, kanamycin and erythromycin were used at final concentrations of 50 and 200 μg/ml, respectively. PCRs were performed with *Pfx* polymerase (Invitrogen). The pre-integration vector for inactivation of *ylgG* was constructed by using pORI280 (26) and an amplified linear DNA fragment (2287 nucleotides) consisting of 450 bp of the 3'-end of *folP*, the entire *ylgG* gene, and the entire *folC* gene, by using primers folPNcoI-F and folCXbaI-R, which contained an NcoI site and an XbaI site, respectively (Table 2). The amplicon was digested with NcoI and XbaI and cloned between the matching sites in pORI280, giving plasmid pORI280-folP-ylgG-folC. This plasmid was transformed to *E. coli* and harvested. The final integration vector, containing an in-frame deletion of almost the entire *ylgG* gene, was obtained by PCR amplification using pORI280-folP-ylgG-folC as a template and primers ylgGPstI-F and ylgGPstI-R (Table 2). Both primers contained a terminal PstI site. The amplicon was digested with PstI and ligated, generating plasmid pORI280-folP-folC, which was transformed to *E. coli* and isolated for subsequent introduction into *L. lactis* strain NZ9000 (27). *L. lactis* transformants in which plasmids had integrated via single crossover were grown overnight in

GM17 with 2 µg/ml erythromycin and plated on GM17 agar containing 2 µg/ml erythromycin and 80 µg/ml 5-bromo-4-chloro-3-indoyl-galactopyranoside (X-gal). The orientation of the single crossover event was tested by PCR amplifications directly on cell material from a blue colony using the primers pORIc-ntr-F (sequence present downstream of multiple cloning site of pORI280) and folP-F (sequence present in *folP* but not the integration vector) (Table 2). Subsequently, cells that contained the plasmid integrated over the short *folP*-flanking region were grown in medium without antibiotics and re-inoculated in the same medium to a density of ~1–10 cells/ml. After growth for ~30–40 generations, dilutions of the culture were plated on agar containing X-gal. After 48 h white colonies were selected, and the presence or absence of *ylgG* was determined by PCR amplification from cell material by using primers folP-F and folCXbaI-R (Table 2).

TABLE 2: PCR primers used in the inactivation of *ylgG*

Primer	Sequence (5' → 3')
folP*Nco*I-F	GATATTATTAATGATATccATGGTTTTGACACAGCG
folC*Xba*I-R	TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG
ylgG*Pst*I-F	GAAGAAGctgCAGAAGAGTTTAAAAAGTATTATCG
ylgG*Pst*I-R	CCACTGCAGTTATTTGAGAAATCAAATCC
folP-F	ATGAAAATCTTAGAACTTAATC
pORIcntr-F	CGACCCGTGCTATAATTATACTAA

Nucleotides in lowercase letters represent modifications with regard to the native gene. Restriction sites are underlined.

Gene Cloning and Protein Expression in *E. coli*.

The *ylgG* gene was amplified from a pNZ8048 derivative (28) using KOD polymerase (Novagen, Madison, WI) and the primers 5'-CATGCACCATGGATGAGGATTTGATTTCT-3' (forward) and 5'-CCCGCTCGAGTATGTTCTTATAACGATA-3' (reverse), containing NcoI and XhoI sites, respectively. Using the NcoI site for cloning changed the second amino acid of YlgG from Asn to Asp. A full-length cDNA (*Arabidopsis* Biological Resource Center U50379 [GenBank]) served as template to amplify the At1g68760 coding sequence, using the primers 5'-ACCCACATGTCGACAGGAGAAGCG-3' (forward) and 5'-CCCGCTCGAGGTCTCCACCACCATGAGT-3' (reverse), containing AflIII and XhoI sites, respectively. The *ylgG* and At1g68760 amplicons were digested with appropriate enzymes and cloned between the NcoI and XhoI sites of pET-28b (Novagen), which added a hexahistidine tag to the C terminus. The sequence-verified constructs were electroporated into *E. coli* BL21-CodonPlus®-RIL (DE3) cells (Stratagene, La Jolla, CA). For enzyme production, cells were grown at 37 °C in 1 liter of LB medium containing 100 µg/ml ampicillin and 20 µg/ml chloramphenicol until the A_{600} reached 0.5. Isopropyl-D-thiogalactopyranoside was then added (final concentration 1 mM) and incubation continued for 16 h at 25 °C.

Protein Isolation and Molecular Mass Determination

E. coli cells from a 1-liter culture were harvested by centrifugation, resuspended in 20 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M KCl (buffer A), and broken in a Mini-BeadBeater (Biospec Products, Bartlesville, OK) using 0.1-mm zirconia/silica beads. Protein purification by Ni^{2+}-affinity chromatography under native conditions followed the manufacturer's protocol (Qiagen). All steps were carried out at 0–4 °C. Purified proteins were desalted on a PD-10 column equilibrated in buffer A. For YlgG, a second purification step was carried out using a Waters 626 high pressure liquid chromatography system. Proteins were loaded on a MonoQ HR 5/5 column (Amersham Biosciences) equilibrated in buffer A containing 0.1 M NaCl. Proteins were eluted with a linear gradient of 0.1–0.5 M NaCl (0.2 ml/min), and fractions with DHNTP pyrophosphohydrolase activity were pooled. Purified proteins were stored at 4 °C for up to 6 months, which did not cause significant activity loss. Protein concentration was determined by Bradford's method (29) using bovine serum albumin as standard. The native molecular mass of YlgG was estimated using a calibrated Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM HEPES-KOH, pH 8.0, containing 150 mM NaCl, using a flow rate of 0.3 ml/min. The standards were carbonic anhydrase, bovine serum albumin, β-amylase, apoferritin, and thyroglobin.

Preparation of DHNTP

Recombinant *E. coli* GTP cyclohydrolase I (30) was used to prepare DHNTP. The reaction was run in 1.5 ml of 50 mM HEPES-KOH containing 0.1 M KCl (buffer B) plus 1 mM GTP, 10 mM β-mercaptoethanol, and 30 µg of purified GTP cyclohydrolase I for 2 h at 37 °C. DHNTP formation was followed by absorption at 330 nm until all GTP was converted; the molar extinction coefficient of DHNTP is 6300 $M^{-1} cm^{-1}$ at 330 nm (31). The solution was then deproteinized using a Centricon YM-10 unit (Millipore, Billerica, MA). Aliquots containing 30–80 nmol of DHNTP were frozen in liquid N_2 and stored at –80 °C. After thawing, aliquots were used at once and not re-frozen.

Enzyme Assays

Assays were routinely made in 50–200-µl reaction mixtures at 30 °C for 5–180 min in buffer B containing the specified concentrations of substrates and divalent metal ions (as chloride salts). The 0.1 M KCl in this buffer did not significantly affect enzymatic reactions. DHNTP pyrophosphohydrolase activity was assayed by fluorometric HPLC. Reactions were stopped by adding oxidizing solution (0.5% I_2, 1% KI, w/v, in 1 M HCl, 10 µl per 100-µl reaction mix) and incubating for 1 h at 4 °C. Then 10 µl of 2% sodium ascorbate and 4.1 µl of 1 M Na_2HPO_4, 1 M NaOH were added to destroy excess I_2 and adjust the pH to 6.0, and denatured protein was pelleted. Separation of neopterin and its phosphates was basically as described (4), using a Waters 2695 instrument (Waters, Milford, MA). Briefly, samples (20–50 µl) were injected on a C_{18} Synergi 4µ Fusion-RP 80 column (4 µm, 4.6 x 250 mm, Phenomenex, Torrance, CA) and eluted isocratically with 10 mM sodium phosphate buffer, pH 6.0, at 1 ml/min. Peaks were detected using a Waters 2475 Multi λFluorescence Detector (excitation 350 nm, emission 450 nm) and quantified relative to neopterin monophosphate (Schircks). A neopterin diphosphate standard was prepared from DHNTP by acid phosphatase

treatment and oxidation. Pyrophosphate was quantified using the Biomol Green[TM] phosphate detection reagent after addition of 1–2 units of inorganic pyrophosphatase (Sigma) to enzymatic assays (32). To measure NADH hydrolysis, samples (20 µl) were injected on a C_{18} symmetry column (3.5 µm, 4.6 x 250 mm, Waters) and eluted in 50 mM sodium phosphate, pH 6.0, containing 8 mM tetrabutylammonium bisulfate using a linear gradient from 12 to 30% methanol. The NMNH peak was quantified fluorometrically (excitation 340 nm, emission 460 nm) relative to authentic NMNH. Hydrolysis of nucleoside triphosphates and their 2'-deoxy derivatives was assayed by pyrophosphate release as described above. Reactions were stopped by adding either excess EDTA (2–3 times the quantity of M^{2+} ions) or the Biomol Green[TM] reagent. Hydrolysis of other substrates (Ap4A, Ap3A, NAD, ADP-ribose, UDP-glucose, and coenzyme A) was assayed by treating the reaction products with shrimp alkaline phosphatase (Roche Applied Science) as described (32) and determining phosphate with the Biomol Green[TM] reagent. Kinetic data were analyzed by direct fitting to the Michaelis-Menten equation using nonlinear regression.

Folate and Pteridine Analysis

Total folate was quantified using the *Lactobacillus casei* microbiological assay (33) after enzymatic deconjugation, as described previously (15). Pteridines were extracted from *L. lactis* cells in buffer C (0.2 M sodium citrate, pH 4.5, containing 5 mM β-mercaptoethanol) and analyzed by HPLC. Briefly, cells of a 50-ml overnight culture (30 °C) were harvested by centrifugation, resuspended in buffer C, and broken in a Mini-BeadBeater. Samples were centrifuged, and the supernatant was incubated for 10 min at 37 °C plus or minus 3 units of wheat germ acid phosphatase (Sigma). Acid hydrolysis conditions were used to avoid formation of cyclic DHNP, which is not attacked by phosphatase (4). Reactions were stopped by adding 15 µl of oxidizing solution per 100-µl sample and then incubated for 1 h at 4 °C. Then 15 µl of 2% sodium ascorbate and 6.1 µl of 1 M Na_2HPO_4, 1 M NaOH were added, and the samples were centrifuged and analyzed by HPLC as described above except that the flow rate was 1.5 ml/min.

Results
Inactivation of *ylgG*

The *ylgG* gene was inactivated in *L. lactis* strain NZ9000ΔylgG by double crossover recombination using modifications of a published method (26). The NZ9000ΔylgG strain, which lacks 95% of the *ylgG* gene, had the same growth rate as the wild type on minimal (CDM) medium (Fig. 2*A*, *inset*). However, the mutant had intracellular folate levels 3-fold lower than the wild type and, unlike the wild type, secreted almost no folate to the medium (Fig. 2*A*). The complementation of *ylgG* into the NZ9000ΔylgG strain restored folate level to that of the wild type (not shown). These data indicate that YlgG mediates a step in folate biosynthesis. HPLC analysis showed this step to be dephosphorylation of DHNTP, for mutant cells accumulated a pteridine with the retention time of DHNTP (Fig. 2*B*) that gave dihydroneopterin (DHN) upon phosphatase treatment (Fig. 2*B* and Table 3). (Pteridines were oxidized before HPLC to convert them to their fluorescent aromatic forms, so that DHN was analyzed as neopterin and DHNTP as neopterin triphosphate.) Two other features of Fig. 2*B*

are noteworthy. First, the mutant cells accumulated dihydroneopterin di- and monophosphates as well as DHNTP, showing that sequential removal of single phosphate residues was occurring. Second, wild type cells contained 10-fold more DHNTP than dihydroneopterin, showing that the dephosphorylation process is far from equilibrium *in vivo*.
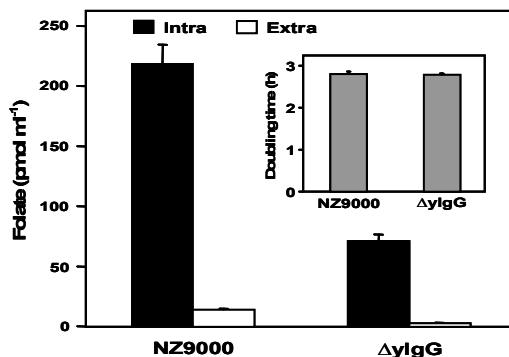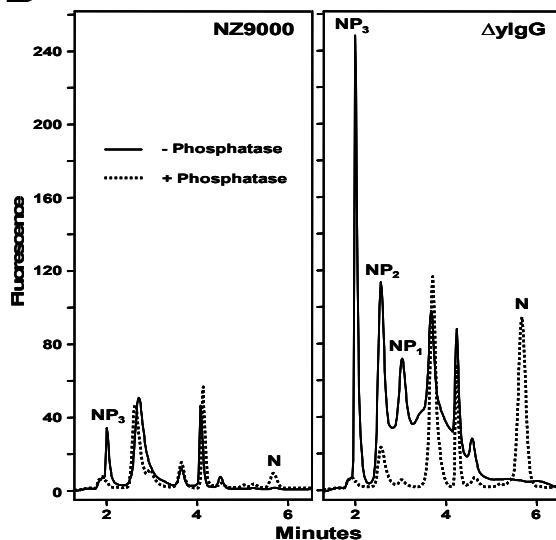
FIG. 2: Effect of *ylgG* inactivation on *L. lactis* folate content, growth, and pteridine profile. *A*, intracellular levels (*intra*) and extracellular secretion (*extra*) of folate in wild type strain NZ9000 and mutant strain NZ9000ΔylgG (Δ*ylgG*) grown in 10 ml of CDM for 18 h to an A_{600} value of 2.1 ± 0.1. Total folate was assayed microbiologically. Data are means of 6–10 determinations and S.E. *Inset* shows the doubling time of each strain and S.E. *B*, HPLC analysis of oxidized dihydroneopterin and its phosphates from strains NZ9000 and NZ9000ΔylgG, without (*solid lines*) or with (*broken lines*) phosphatase treatment. *N*, neopterin; *NP₃*, neopterin triphosphate; *NP₁*, neopterin diphosphate; *NP₂*, neopterin phosphate. The phosphatase-insensitive peak at 3.7 min that is higher in the mutant strain is an unidentified pteridine.

TABLE 3. Effect of *ylgG* inactivation on intracellular pteridine levels in *L. lactis*

Pteridine	Intracellular pteridine concentration			
	Wild type NZ9000		NZ9000ΔylgG	
	Untreated	+ Phosphatase	*Untreated*	+ Phosphatase
	μM			
Neopterin triphosphate	0.33 ± 0.08	< 0.01	3.28 ± 0.65	< 0.01
Neopterin	0.03 ± 0.002	0.43 ± 0.02	< 0.01	5.69 ± 0.22

Cultures (50 ml) were grown in CDM medium to an A_{600} value of 3. One-half of the pteridine extract of the cell pellet was treated with acid phosphatase (see "Experimental Procedures"); the other half was not. Pteridines were then oxidized to their aromatic forms and quantified by fluorometric HPLC. Values are means of three determinations ± S.E. and are expressed as intracellular concentrations. The internal cell volume of a 50-ml culture at $A_{600} = 3$ was estimated as 93 µl (taking an A_{600} value of 1 as equivalent to 0.2 mg of protein ml^{-1} and *L. lactis* internal cell volume as 3.1 µl mg^{-1} protein).

Characterization of Recombinant YlgG

The *ylgG* open reading frame (Fig. 3*A*) was expressed in *E. coli* with a C-terminal hexahistidine tag. The expression level was insufficient to detect the recombinant YlgG in the total soluble protein fraction, but a polypeptide close to the predicted size (20.4 kDa, including the His tag) was readily isolated from this fraction by Ni^{2+} affinity and anion exchange chromatography (Fig. 4*A*). The purified YlgG constituted ~0.2% of total soluble protein. Enzyme assays showed that purified YlgG had high DHNTP pyrophosphohydrolase activity, *i.e.* that it cleaved DHNTP to give DHNP and pyrophosphate in a 1:1 ratio, with negligible production of free phosphate (Fig. 4*B*). This activity was dependent on Mg^{2+} and reached ~70% of its maximal value at 1 mM (Fig. 4*C*). Because 1 mM is approximately the physiological level of free Mg^{2+} (11, 12), this concentration was adopted for subsequent work. Ca^{2+} at 1 mM did not activate YlgG; Zn^{2+} and Mn^{2+} could not be tested as they caused rapid chemical breakdown of DHNTP (9). At 1 mM Mg^{2+}, chemical conversion of DHNTP to DHNP was almost undetectable (<0.5% of the maximal rate of the enzymatic reaction in Fig. 4*C*). This chemical conversion increased when Mg^{2+} or Ca^{2+} concentration was raised to 10–12 mM as reported (10), but at these concentrations other major breakdown products were also formed (not shown).

Kinetic characterization of YlgG (Table 4) gave a K_m value for DHNTP (2.1 µM) that is among the lowest reported for Nudix enzymes and gave a fairly typical k_{cat} value (0.21 s^{-1} at 30 °C) (34). YlgG had no detectable activity with GTP, ATP, TTP, CTP, the corresponding deoxynucleoside triphosphates, 8-oxo-dGTP, NADH, NAD$^+$, diadenosine tri- or tetraphosphate, ADP-ribose, UDP-glucose, or coenzyme A (the detection limit was ≦1% of the activity with DHNTP). Consistent with this lack of activity, the YlgG amino acid sequence (Fig. 3*A*) does not have the motifs associated with specificity for NADH, diadenosine polyphosphates, ADP-ribose, or coenzyme A (35–38). The native molecular mass of recombinant YlgG was estimated to be 24.2 kDa on the basis of its elution from a calibrated size exclusion column. This value is close to the calculated mass of the His-tagged polypeptide (20.4 kDa), indicating that the native enzyme is a monomer.

TABLE 4. Kinetic constants of YlgG with DHNTP as substrate

K_m	V_{max}	K_{cat}	K_{cat}/K_m
μM	$\mu mol\ min^{-1}\ mg^{-1}$	s^{-1}	$s^{-1}\ M^{-1}$
2.1 ± 0.5	0.65 ± 0.11	0.21 ± 0.04	1.0×10^5

Values were determined at 30 °C in HEPES-KOH buffer, pH 8.0, containing 100 mM KCl and 1 mM Mg^{2+}. Data are means of three independent determinations ± S.E.

A

```
YlgG       MNEDLISQIKEVVTAENQEKLIKIIQLLESSNYELRGKINEDLQLSASAL
At1g68760                                 MSTGEAIPRVAVVVFIL

YlgG       VFKEDKLFFIEHPYQK-ELLLPAGHVELKESPLDTAIREFHEETGFFAKK
At1g68760  NGNSILLGRRRSSIGNSTFALPGGHLEFGESFEECAAREVMEETGLKIEK

YlgG       MGKLVDVNLIDIPENETKNEKKHQHIDFRYLLELEEQEAELAELPFFLLE
At1g68760  MKLLTVTNNV---EKEAPTPSHYVSVSIRAVLVDPSQEPKNME-------

YlgG       LEFAPEEFKKYYRYKNI
At1g68760  -PEKCEGWDWYDWENLPKPLFWPLEKLFGSGFNPFTHGGGD
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B
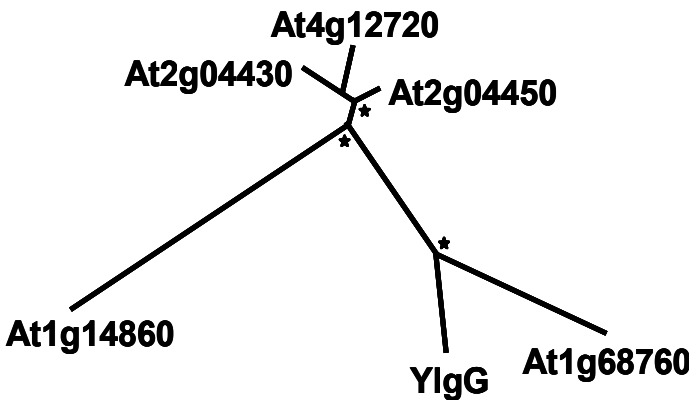
FIG. 3: Alignment and phylogenetic analysis of the deduced amino acid sequences of YlgG and selected *Arabidopsis* Nudix proteins. *A*, alignment of YlgG (GenBank[TM] AAN64308 [GenBank] with its closest *Arabidopsis* homolog, the At1g68760 gene product. Identical residues are shaded in *black*, and similar residues are shaded in *gray*. *Dashes* are gaps introduced to maximize alignment. The Nudix motif is *overlined*. *B*, unrooted phylogram, generated using the neighbor-joining method and 1,000 bootstrap iterations of YlgG, the At1g68760 protein, and other *Arabidopsis* proteins sharing homology with YlgG outside the Nudix box. *Asterisks* show branch points with bootstrap values >70%. Phylogenetic analysis was carried out using PHYLIP at the Institute Pasteur server (bioweb.pasteur.fr). Sequences were aligned using Multalin (prodes.toulouse.inra.fr/multalin/multalin.html).

FIG. 4: Isolation, reaction products, and Mg^{2+} response of recombinant YlgG. *A*, SDS-PAGE of soluble proteins from *E. coli* BL21-CodonPlus®-RIL cells induced by isopropyl-D-thiogalactopyranoside (*lane 1*) and YlgG protein purified by Ni^{2+}-affinity and anion exchange chromatography (*lane 2*). The gel was stained with Coomassie Blue. Positions of molecular mass standards (kDa) are marked. *B*, analysis of the products of the acti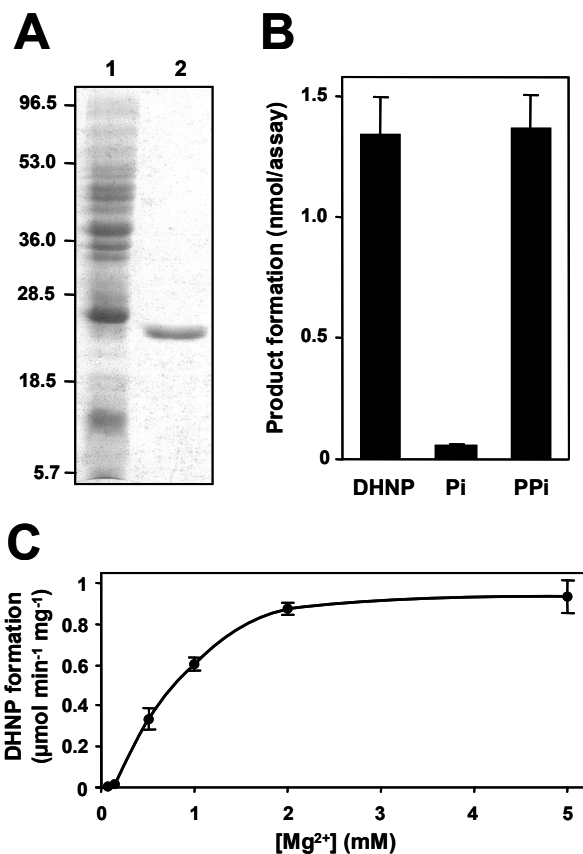on of purified YlgG on DHNTP. Reactions were run for 10 min in the standard assay conditions in the presence of 1 mM Mg^{2+}, 100 mM KCl, and 0.1 mM DHNTP. DHNP was quantified by fluorometric HPLC. Pyrophosphate was determined as phosphate after treating with pyrophosphatase. Data are means of three determinations and S.E. *C*, response of DHNTP pyrophosphohydrolase activity to Mg^{2+}. Assays contained 0.2 mM DHNTP and 100 mM KCl. Data are means of three determinations ± S.E.

Arabidopsis Homologs of YlgG

BLAST searches of the *Arabidopsis* genome revealed 20 proteins with a canonical Nudix motif (see supplemental Table 1). Most of these share homology with YlgG only in the area of the Nudix box, but five (shown in Fig. 3*B*) have some degree of overall homology. The overall homology is greatest for the At1g68760 gene product, which is 34% identical and 44% similar to YlgG in a 73-residue region spanning the Nudix box (Fig. 3*A*). Phylogenetic analysis confirmed that this protein is more closely related to YlgG than the other four (Fig.

3*B*). Moreover, the At1g68760 protein is of similar size (16.4 kDa) to YlgG and, like other early enzymes in plant folate biosynthesis (4, 6), has no obvious organellar targeting sequence. Like YlgG, the At1g68760 protein lacks the motifs associated with preference for NADH, diadenosine polyphosphates, ADP-ribose, or coenzyme A (35–38). Finally, the At1g68760 protein is by far the closest *Arabidopsis* homolog (33% identity, 51% similarity) of the Nudix moiety of a predicted *Parachlamydia* fusion protein (GenBank[TM] YP008156) whose other domain is the folate synthesis enzyme dihydroneopterin aldolase (FolB). (This fusion arrangement strongly implies that the Nudix domain is the *Parachlamydia* DHNTP pyrophosphohydrolase.) Several lines of bioinformatic evidence thus point to the At1g68760 gene product as the best candidate for DHNTP pyrophosphohydrolase in *Arabidopsis*.
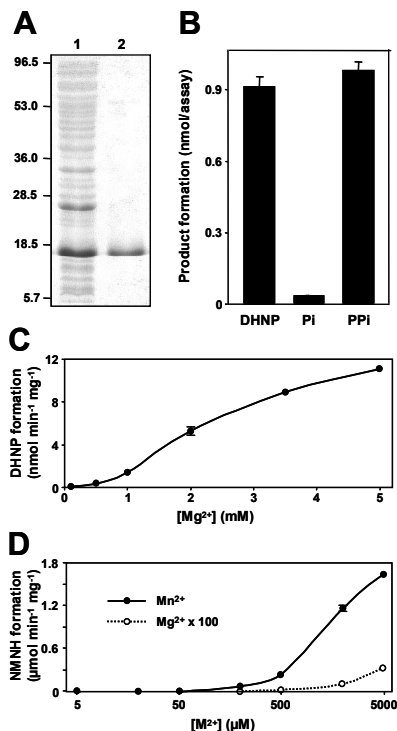
FIG. 5. Isolation, reaction products, and metal responses of recombinant At1g68760 protein. *A*, SDS-PAGE of soluble proteins from *E. coli* BL21-CodonPlus[®]-RIL cells induced by isopropyl-D-thiogalactopyranoside (*lane 1*) and the At1g68760 protein purified by Ni^{2+}-affinity chromatography (*lane 2*). The gel was stained with Coomassie Blue. Positions of molecular mass standards (kDa) are marked. *B*, analysis of the products of the action of the purified protein on DHNTP. Reaction conditions are as shown in Fig. 4 except that incubation time was 30 min and DHNTP concentration was 0.3 mM. Data are means of three determinations and S.E. *C*, response of DHNTP pyrophosphohydrolase activity to Mg^{2+} concentration. Assays contained 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, and 300 μM DHNTP. Data are means of three determinations ± S.E. *D*, response of NADH pyrophosphatase activity to Mn^{2+} or Mg^{2+} concentration. Assays were carried out in 50 mM Tris-HCl, pH 8.0, in the presence of 1 mM dithiothreitol and 2 mM NADH (21). Data are means of three determinations ± S.E. Note that values for Mg^{2+} are multiplied x100. Where no error bars are shown in *C* and *D*, they were smaller than the symbols.

Characterization of the At1g68760 Gene Product

The At1g68760 gene product with or without a C-terminal His tag was abundantly expressed in *E. coli* (Fig. 5*A*). Pilot assays with desalted extracts indicated that the tag made no difference to the enzymatic characteristics of the protein, so all further work was done on the tagged protein after purification to homogeneity by Ni^{2+} affinity chromatography (Fig. 5*A*). Like YlgG, the At1g68760 protein had DHNTP pyrophosphohydrolase activity, producing DHNP and pyrophosphate in a 1:1 ratio but essentially no free phosphate (Fig. 5*B*). Also like YlgG, activity required Mg^{2+} (Fig. 5*C*), and this could not be replaced by Ca^{2+} (1 mM). Unlike YlgG, however, in the presence of 1 mM Mg^{2+} the At1g68760 protein was far more active against dGTP, 8-oxo-GTP, dTTP, and other nucleoside and deoxynucleoside triphosphates than against DHNTP (Table 5). Activities with other Nudix substrates were negligible (Table 5).

TABLE 5. Substrate preference of the At1g68760 protein

Substrate	Specific activity
	nmol min^{-1} mg^{-1}
NTPs[a]	7.3 –446
dNTPs[b]	69.4 – 695
DHNTP	1.70
Ap3A	< 0.01
Ap4A	0.85
ADP-ribose	< 0.01
UDP-glucose	0.019
NADH	0.021
NAD$^+$	< 0.01
Coenzyme A	< 0.37[c]

[a]Individual NTP specific activities (nmol min^{-1} mg^{-1}) were: ATP, 7.3; CTP, 12.9; GTP, 223; TTP, 446.
[b]Individual dNTP specific activities (nmol min^{-1} mg^{-1}) were: dCTP, 69.4; dATP, 300; dTTP, 499; 8-oxo-dGTP, 590; dGTP, 695.
[c]The detection limit was high due to liberation of the 3′-phosphate group.
Substrate concentrations were 0.5 mM. Activities were determined at 30 °C in HEPES-KOH, pH 8.0, containing 100 mM KCl and 1 mM Mg^{2+}.

To further explore this striking contrast with the substrate specificity of YlgG, we evaluated the kinetic properties of the At1g68760 protein with DHNTP or dGTP as substrate in the presence of 1 mM Mg^{2+} (Table 6). The K_m value for DHNTP was fairly similar to that for dGTP, but the k_{cat} value was 500-fold lower, so that the catalytic efficiency was far higher for dGTP.

TABLE 6. Kinetic constants of the At1g68760 protein (AtNUDT1)

Substrate	K_m	V_{max}	K_{cat}	K_{cat}/K_m
	μM	*μmol min^{-1} mg^{-1}*	*s^{-1}*	*s^{-1} M^{-1}*
DHNTP	147 ± 6	2.0 ± 0.1 × 10^{-3}	6.0 × 10^{-4}	4.1
dGTP	81 ± 3	1.02 ± 0.02	0.30	3.6 × 10^3

Values were determined at 30 °C in HEPES-KOH buffer, pH 8.0, containing 100 mM KCl and 1 mM Mg^{2+}. Data are means of three independent determinations ± S.E.

The At1g68760 gene product was reported by Dobrzanska *et al.* (21) to have high NADH pyrophosphatase activity and was designated AtNUDT1 (we will use this name henceforth). This activity against NADH was, however, characterized using an Mn^{2+} concentration of 5 mM, which is ~10^3-fold greater than typical levels of Mn^{2+} in growing cells (39). Low activity was also found in the presence of 5 mM Mg^{2+} (21). We therefore measured NADH pyrophosphatase activity at various Mn^{2+} and Mg^{2+} concentrations (Fig. 5*D*). This activity fell drastically as Mn^{2+} concentration approached the normal physiological range, and at 5 μM Mn^{2+} was only 0.001% that at 5 mM (Fig. 5*D*). With Mg^{2+}, activity was insignificant except at the highest concentrations tested (Fig. 5*D*).

Discussion

Our genetic and biochemical results with *L. lactis* identify the YlgG protein as a specific DHNTP pyrophosphohydrolase that participates in the folate pathway. We therefore propose that the gene encoding this protein be redesignated *folQ*. The Mg^{2+} requirement, substrate specificity, K_m value, and native molecular mass of YlgG are very like those reported for the partially purified DHNTP pyrophosphohydrolase of *E. coli* (9). As *E. coli* and *L. lactis* are not closely related, the occurrence of similar DHNTP pyrophosphohydrolases in both species implies that specific DHNTP-cleaving Nudix enzymes are common to a wide range of bacteria.

Although deleting *ylgG* reduced folate production markedly in *L. lactis*, it did not eliminate it and did not affect growth. The residual folate synthesis in the deletion mutant is not unexpected, because broad spectrum phosphatases can attack DHNTP (40), and the massive accumulation of DHNTP seen in the mutant could potentially drive significant flux through non-specific, normally minor, enzymatic dephosphorylation reactions. The accumulation of dihydroneopterin diphosphate found in the YlgG-deficient cells supports this possibility, for it indicates that iterative removal of single phosphate residues has inefficiently replaced removal of pyrophosphate as the main route from DHNTP to DHNP.

Two lines of evidence show that metal ion-catalyzed chemical dephosphorylation of DHNTP (10) is certainly not a major process under normal *in vivo* conditions and is probably not even a significant minor one. First, the large build up of DHNTP in deletant cells indicates that most of the DHNTP →DHNP flux is normally mediated by YlgG. Second, the rate of chemical dephosphorylation was negligible in *in vitro* assays made at physiological pH and Mg^{2+} levels. The proposal that chemical dephosphorylation is important (10) was based solely on biochemical experiments involving non-physiological Mg^{2+} or Ca^{2+} levels and was not supported by genetic or other *in vivo* evidence.

Cleavage of pyrophosphate from DHNTP is a new addition to the list of reactions shown to be mediated by Nudix enzymes (19, 36, 41, 42). The Mg^{2+} response observed for YlgG is very similar to those of Nudix nucleoside triphosphate pyrophosphohydolases such as *E. coli* MutT (the archetype of this enzyme class), which requires two divalent cations, one coordinated by the nucleoside triphosphate and the other by conserved residues in the Nudix motif and elsewhere (43). The low K_m value observed (2.1 μM) for YlgG is consistent with the estimated *in vivo* concentration of DHNTP in wild type *L. lactis* cells (0.33 μM).

It is intriguing that the DHNTP concentration in wild type *L. lactis* is 10-fold higher than that of DHN, because this suggests that the rate of DHNTP dephosphorylation limits the pool size of DHN, and hence flux from DHN to folates. If this is the case, increasing YlgG expression could potentially enhance folate production in *L. lactis* strains engineered to overproduce DHNTP (15).

DHNTP is an intermediate in the biosynthetic pathway of tetrahydrobiopterin as well as of folate, and organisms such as pseudomonads, cyanobacteria, and fungi have both pathways (44–47). If these organisms use a DHNTP pyrophosphohydrolase, then this enzyme would be the one that commits pteridine moieties to folate synthesis. This differs from the usual situation in which the committing role is played by GTP cyclohydrolase I, the enzyme that produces DHNTP (1, 10). Regardless of whether DHNTP pyrophosphohydrolase has a committing role, it would seem to be a poor target for antibacterial drug discovery because inactivating it, in *L. lactis* at least, did not totally suppress folate synthesis and had no impact on growth.

The previously known bacterial folate synthesis enzymes all have homologs in plants (2–7), and the same is true for YlgG, although the homology in this case is weaker than usual. More generally, bacterial Nudix enzymes often share sequence features outside the Nudix box with eukaryotic enzymes having the same activity (35, 38, 47, 48). This pattern of cross-kingdom structural similarity between functionally equivalent Nudix hydrolases in itself makes the original identification of At-NUDT1 as an NADH pyrophosphatase (21) seem suspect, for this protein lacks the SQPWPFPXS motif found downstream of the Nudix box in other NADH pyrophosphatases (35, 38). (This motif is, however, present in another *Arabidopsis* protein, the At5g20070 gene product that has yet to be characterized, see Ref. 38.) This suspicion about the function of AtNUDT1 was corroborated by biochemical evidence, for we found that the activity against NADH requires non-physiological levels of Mn^{2+} and that DHNTP and nucleoside and deoxynucleoside triphosphates are far better substrates than NADH in the presence of physiological Mg^{2+} levels. A similar switch in substrate preference when Mg^{2+} replaces Mn^{2+} has been reported for another Nudix hydrolase (49).

The catalytic efficiency (k_{cat}/K_m) of AtNUDT1 for DHNTP is ~10^4-fold lower than that of YlgG and, unlike YlgG, AtNUDT1 is not at all specific for DHNTP, deoxynucleoside and nucleoside triphosphates being much better substrates. These findings, added to the probability that cytosolic deoxynucleoside and nucleoside triphosphate levels greatly exceed that of DHNTP (17, 50), raise the question of whether AtNUDT1 could account for DHNTP hydrolysis *in vivo* or whether another, more efficient, DHNTP pyrophosphohydrolase remains to be discovered. We cannot at this point exclude the existence of another enzyme, but two arguments suggest there is no need to invoke one. First, the DHNTP →DHNP flux is so small that it might well be sustained even by an inefficient enzyme operating in the presence of a large excess of better substrates. (This flux can be estimated from data on folate turnover in plants as ≦15 pmol h^{-1} g^{-1} fresh weight (51, 52), which is ≈10^6-fold smaller than the respiratory ADP →ATP flux (53), for example.) Second, there are precedents for Nudix hydrolases mediating key minor reactions while awash in alternative substrates that they are concurrently cleaving. MutT-type Nudix enzymes are thought to sanitize nucleotide pools by

removing oxidized, mutagenic derivatives such as 8-oxo-dGTP. These enzymes usually prefer the oxidized derivatives but, like At-NUDT1, also attack normal deoxynucleoside and nucleoside triphosphates, which are far more abundant *in vivo* (54–57). Because the substrate range of AtNUDT1 is like that of MutT-type hydrolases (54), it could be a bifunctional DHNTP pyrophosphohydrolase/sanitizing enzyme. If so, the metabolic inefficiency involved in hydrolyzing large amounts of normal nucleotides as well as DHNTP would add no extra energy cost because such wastage would already be part of the vital sanitizing function of the enzyme.

Footnotes
The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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Chapter 4

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

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Abstract

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 at its 5' end to 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 flux through the folate and *p*ABA biosynthesis pathways in the wild-type strain.

Introduction

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 certain 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 eight 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).
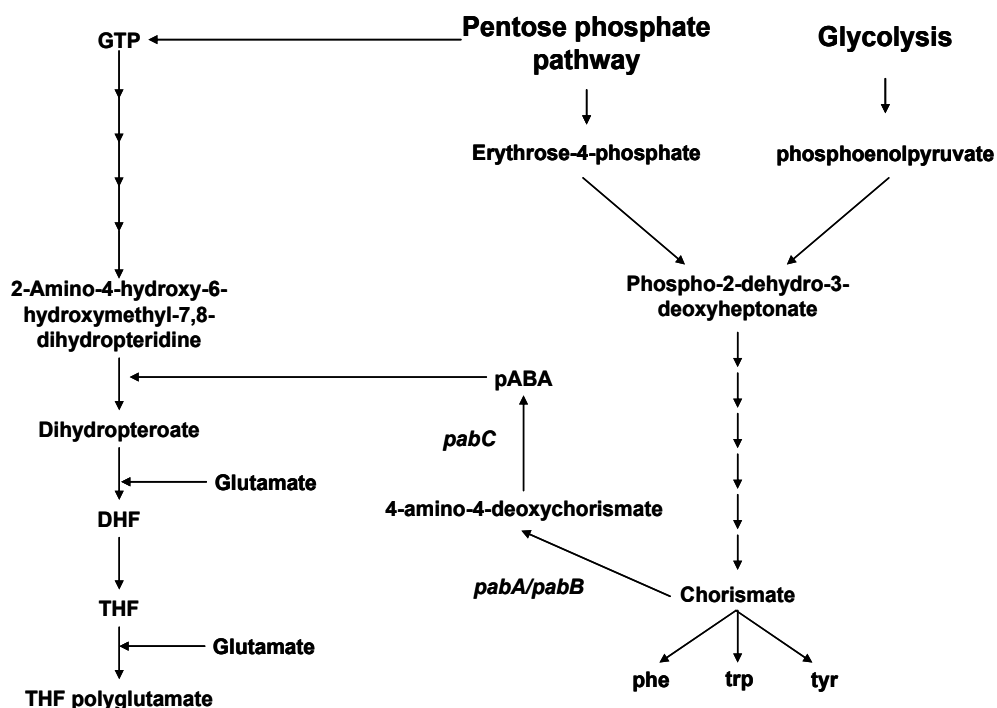
FIG. 1: Biosynthesis pathway of *p*ABA and folate, showing the conversion of GTP into THF, the route for *p*ABA biosynthesis from chorismate, and the incorporation of *p*ABA into THF. (DHF is dihydrofolate).

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 pABA, glycine, serine, and nucleobases/nucleosides (guanine, adenine, uracil, xanthine, inosine, thymidine, and orotic acid). Finally, we analyzed the effect of overexpression of the folate biosynthesis genes combined with overexpression of the pABA 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 B12, 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 $MnCl_2·4H_2O$, 0.003 g/liter $FeCl_3·6H_2O$, 0.005 g/liter $FeCl_2·4H_2O$, 0.005 g/liter $ZnSO_4·7H_2O$, 0.0025 g/liter $CoSO_4·7H_2O$, 0.0025 g/liter $CuSO_4·5H_2O$, 0.0025 g/liter $(NH_4)_6Mo_7O_{24}·4H_2O$, 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 pABA 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 pABA. When these components were added separately, the concentrations as described above were used. Spent medium was made by growth of the NZ9000ΔpABA strain on CDM lacking nucleobases, nucleosides, glycine, serine, and pABA, 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* NZ9000ΔpABA. The *E. coli* DH5α strain (47), used for the construction of the pABA 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*.

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

Material	Relevant feature(s)[a]	Source of reference
Strain		
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]	When indicated all pNZ plasmids contain the pSH71 or PWV01 origin of replication	
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 cross over 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; the final 15 bases pairs of *pabB/C* till 346 bp in *yneH*	this study
pIL253	Ery^R	(35)
pNZ7023	Ery^R, pIL253 derivative, containing the nisin promoter along with the *pabA, pabB/C* gene cluster of *L. lactis*	this study
pNZ5327	Tet^R pGHOST derivative, containing the resolvase gene for recombining of *LOX* sites	this study
pNZ5348	Tet^R NZ7110 derivative, containing the resolvase gene for recombining of *LOX* sites	(17)
pGHOST8	TetR, 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	
LclpabAsphI2F	AGTCGCATGCGAGGAGGATAAA*ATG*CGATTAC	
LclpabB/CXbaIR	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.

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 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).
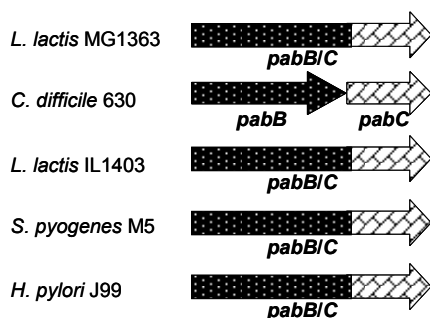
FIG. 2: Organization of the *pabBC* fusion gene in *Lactococcus lactis* MG1363 compared to those of the *pabB* (*p*ABA synthetase component I) and *pabC* (4-amino-4-deoxychorismate lyase) genes of *Clostridium difficile* 630, *Lactococcus lactis* IL-1403, *Streptococcus pyogenes* M5, and *Helicobacter 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 NZ9000ΔpABA, grown on CDM in the presence or absence of pABA and nucleobases/nucleosides (Table 2).

TABLE 2. Growth rates, final OD_{600} values, and total folate production levels of the wild-type and NZ9000ΔpABA strains grown on CDM with and without pABA and nucleobases/nucleosides.

		Wild-type		NZ9000ΔpABA		Mean conc (µg/liter) of folate (SD)	
Presence of pABA	Presence nucleobases/ nucleosides	Mean growth rate h^{-1} (SD)	OD_{600}	Mean growth rate h^{-1} (SD)	OD_{600}	Wild-type	NZ9000ΔpABA
-	-	0.31 (0.03)	3.2	0.02 (0.001)	0.5	91.7 (10.5)	0.05 (0.01)
+	-	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)
+	+	0.32 (0.03)	3.5	0.35 (0.06)	3.3	90.3 (16.0)	109.7 (10.3)

The growth rate and final OD_{600} of strain NZ9000ΔpABA on medium lacking pABA 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 NZ9000ΔpABA strain was able to grow poorly but consistently on medium lacking pABA and nucleobases/nucleosides is somewhat unexpected. Therefore, we checked whether the CDM contained trace amounts pABA, folate, glycine, serine, or nucleobases/nucleosides. To test this, the following experiment was conducted. Strain NZ9000ΔpABA was grown on CDM lacking pABA glycine, serine, and nucleobases/nucleosides until no further increase in optical density was observed (OD_{600} of 0.5). The spent medium of strain NZ9000ΔpABA was filter sterilized and adjusted to the initial pH, and finally, 0.5% additional glucose was added. The freshly inoculated NZ9000ΔpABA strain was unable to grow on the spent medium, even though glucose and other nutrients were still present in sufficient amounts. The addition of pABA 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 pABA. Interestingly, the growth of the pABA 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).

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 +.

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.

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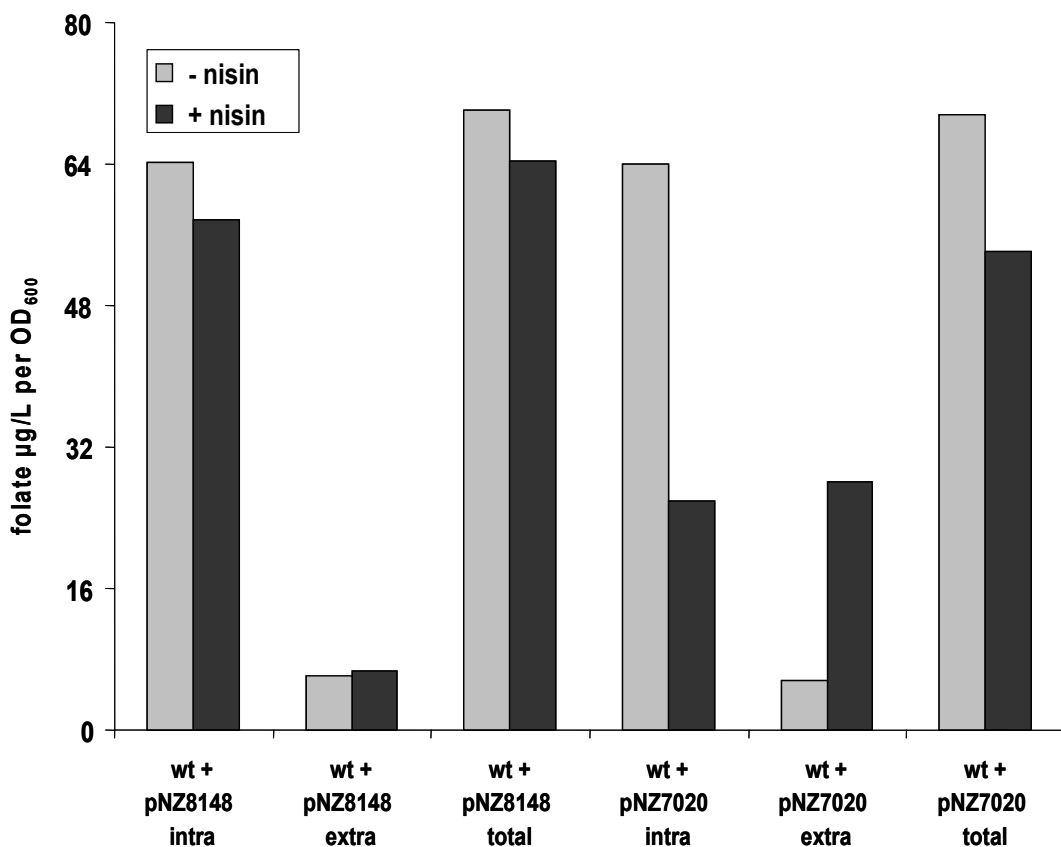
FIG. 3: Folate production (µg/ml per OD$_{600}$ unit) of the *L. lactis* wild-type strain (wt) harboring pNZ8148 (empty vector) and pNZ7020 (nisin-inducible *p*ABA overexpression vector). The folate pool quantities of intracellular, extracellular, and total fractions are shown. The uninduced folate levels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

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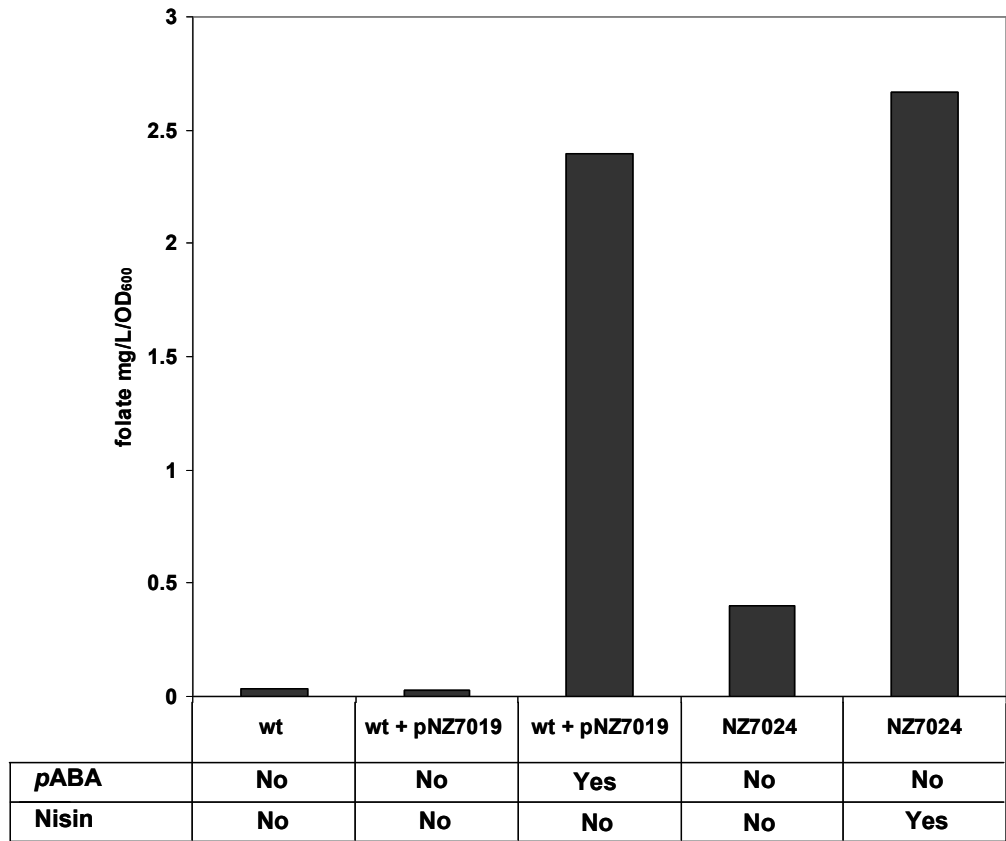
FIG. 4: Total folate production levels (mg/liter per OD_{600} unit) of three different strains: *L. lactis* NZ9000 (wild-type [wt]), *L. lactis* NZ9000 harboring pNZ7019 (folate overexpression vector) cultivated in the presence and absence of *p*ABA, and *L. lactis* NZ7024 (NZ9000 strain harboring the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

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*

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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 highlights 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, provided that purines are present 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.

Acknowledgments

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Chapter 5

Transformation of Folate-Consuming *Lactobacillus gasseri* into a Folate Producer

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Chapter 5

Abstract

      Five genes essential for folate biosynthesis in *Lactococcus lactis* were cloned on a broad-host-range lactococcal vector and were transferred to the folate auxotroph *Lactobacillus gasseri*. As a result *L. gasseri* changed from a folate consumer to a folate producer. This principle can be used to increase folate levels in many fermented food products.

Introduction

      Folate is an essential component in the human diet, and adequate intake of folate may prevent the occurrence of diseases and syndromes like neural tube defect, coronary heart disease, anemia, and certain types of cancer (9). Food products like green vegetables, meat, and fermented dairy products contain significant folate levels. Despite this, folate deficiency occurs throughout the world, including several well-developed countries. Recently, it has been shown that metabolic engineering can be used to increase folate levels in fermented foods (12). Lactic acid bacteria such as *Lactococcus lactis* and *Lactobacillus plantarum* have the ability to synthesize folate, which is a biological cofactor involved in their amino acid and nucleotide metabolism (7, 11). The genes for folate biosynthesis have been identified (6, 12). The biosynthetic pathway includes eight consecutive steps, in which the precursor guanosine triphosphate is converted into tetrahydrofolate (10). However, some lactic acid bacteria, such as *Lactobacillus gasseri* strain ATCC 33323, cannot synthesize folate, because the genes involved in folate biosynthesis are lacking in the genome except for the two genes, *folA* and *folC*, involved in regeneration and retention of reduced folates taken up from the medium (http://genome.jgi-psf.org/draft_microbes/lacga/lacga.draft.html).

      The folate biosynthetic genes of *L. lactis* MG1363 are organized in a folate gene cluster, consisting of six genes (*folA*, *folB*, *folKE*, *folP*, *ylgG*, and *folC*) (Fig. 1) (12). In the present work we describe the transformation of the folate-consuming *L. gasseri* into a folate producer by the transfer of a broad-host-range plasmid containing the folate gene cluster from *L. lactis*.

Cloning and transformation of the folate gene cluster of *L. lactis* into *L. gasseri*.

      The plasmid pNZ7017 (12) was digested by using XbaI and SphI (both Invitrogen, Paisley, United Kingdom) as restriction enzymes. The 3.1-kb DNA fragment that was obtained from the digestion consisted of the constitutive *pepN* promoter (14), a part of the multiple cloning site, chloramphenicol resistance marker, and replication genes that originated from pNZ12 (1). The folate gene cluster (*folB*, *folKE*, *folP*, *ylgG*, and *folC*) of *L. lactis* was amplified by PCR by using high-fidelity *Pwo* polymerase (Invitrogen). The forward primer SphfolB-F (5'-AGGAAGCATGCCTTACAAAATAAAACTTAATAATATG-3') was extended at the 5' end, creating a SphI restriction site overlapping the start codon of *folB*. The reverse primer folCXba-R (5'-TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG-3') was extended at the 5' end, creating an XbaI restriction site that overlapped the stop codon of *folC* (Fig. 1). The amplified PCR fragment was restricted with XbaI and SphI. Subsequently, the two fragments were ligated by using T4 ligase (Invitrogen), generating a translational fusion between the constitutive promoter of the *pepN* gene (14) and the folate gene cluster (Fig. 1). The resulting plasmid was designated pNZ7019.
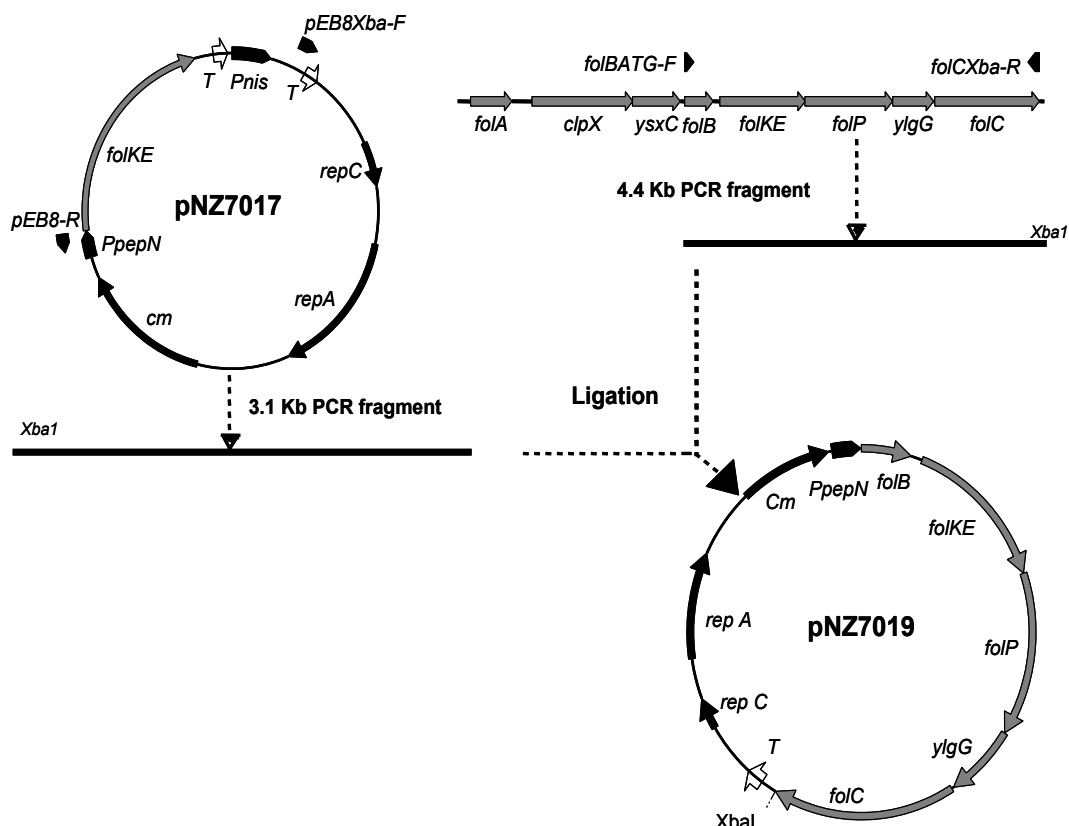
FIG. 1: Construction of pNZ7019 by restriction of pNZ7017 and the amplification of the folate gene cluster from chromosomal DNA of *L. lactis* MG1363.

After transformation to *L. lactis* NZ9000 and subsequent cultivation of the strain, the plasmid was harvested as described previously (2). *L. gasseri* (ATCC 33323) was transformed with purified pNZ7019 by using an established procedure (8) and was plated on MRS medium (Merck, Darmstadt, Germany) containing 10 μg of chloramphenicol/ml. After incubation for 40 h at 37°C, chloramphenicol-resistant colonies were examined for the presence of pNZ7019 by using restriction analyses. An *L. gasseri* colony harboring pNZ7019 was used for renewed cultivation by using the same growth conditions as previously described. Random amplified polymorphic DNA fingerprint analysis was used to confirm the identity of the transformant harboring pNZ7019 as *L. gasseri* ATCC 33323 (results not shown).

Conversion of folate consumer into folate producer.

A modified Folic Acid Casei Medium (FACM) (Difco, Becton Dickinson and Co., Sparks, Md.) was developed for growth and subsequent folate analysis of the *L. gasseri* wild-type strain and the *L. gasseri* strain harboring pNZ7019. The FACM was enriched with 1 mg

75

of vitamin B_{12} (Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany)/liter and 1 ml of Tween 80 (Merck, Darmstadt, Germany)/liter. The wild-type strain could not grow at 37°C unless folate was added (1.0 mg/liter), whereas the strain harboring pNZ7019 showed folate-independent growth.

Folate levels were quantified by using the *Lactobacillus casei* microbiological assay, including enzymatic deconjugation of the polyglutamate tail (5, 11, 12). The *L. gasseri* strain harboring pNZ7019 produced significant intracellular and extracellular folate levels (Fig. 2). As expected, the wild-type strain consumed folate from the medium and intracellular folate concentrations remained below the detection limit (Fig. 2).

Stability of the folate production in the *L. gasseri* strain.

L. gasseri strain ATCC 33323 harboring pNZ7019 was cultivated for approximately 30 generations on MRS medium supplemented with 10 µg of chloramphenicol/ml at 37°C. The culture was plated on MRS agar plates supplemented with 10 µg of chloramphenicol/ml. Subsequently, 100 colonies were transferred to folate-free FACM plates containing 10 µg of chloramphenicol/ml. Since all colonies grew on these plates, it appears that the folate biosynthesis is stably maintained in the pNZ7019 vector for more than 30 generations of growth in the presence of folate. Sequential cultivation in folate-rich medium resulted in decreased folate production by the transformant (data not shown). This is presumably a result of instability of the folate gene cluster.
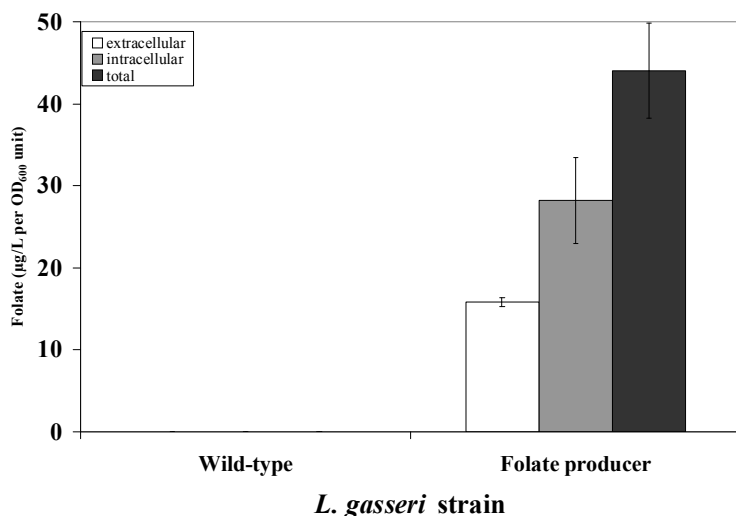
FIG. 2: Folate production of the *L. gasseri* wild-type strain and the *L. gasseri* strain harboring pNZ7019 (marked folate producer), grown on modified FACM. White bar, extracellular folate levels; gray bar, intracellular folate levels; and black bar, total folate levels. The *L. gasseri* wild-type strain could not grow without supplementation of folate. Therefore, folate levels depicted in the figure are corrected for folate added to the medium. Error bars indicate the standard deviation of the folate microbiological assay over two independent measurements.

Conclusion.

The five genes, i.e., *folB*, *folKE*, *folP*, *ylgG*, and *folC*, directing folate biosynthesis in *L. lactis* were transferred to *L. gasseri* by using a derivative of the broad-host-range vector pNZ12 (1). These genes are sufficient to introduce a folate biosynthesis pathway in this folate auxotroph lactic acid bacterium, thereby transforming a folate consumer into a folate producer. *L. gasseri* is currently marketed as a probiotic (4), and when the described strategy is used, this lactic acid bacterium can be used to enrich (fermented) foods with the essential B vitamin, folate, in addition to conferring its health-promoting effect on the consumer (3, 13).

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Chapter 6

Plasmid Maintenance in *Lactobacillus plantarum* WCFS1 Engineered for Folate Overproduction

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Abstract

Aim: This paper describes the impact of folate overproduction on plasmid maintenance in *Lactobacillus plantarum*.

Methods and results: Overexpression of the folate gene cluster in *L. plantarum* resulted in a 1000-fold higher folate production when compared to a control strain. Propagation of the overproducer in the absence of antibiotic selection pressure results in a 23- and 40-fold drop in plasmid copy number and folate pools, respectively. The growth rate of this strain was found to be 20-25% reduced when compared to the control strain. Single colony isolates with low folate pools, have a low plasmid copy number and display a higher growth rate.

Conclusion: Homologous overexpression of the folate gene cluster in *L. plantarum* resulted in a strain that produced high folate pools, but the growth rate of the strain was reduced. This correlated with high plasmid copy numbers. In competition experiments the folate-overproduction phenotype is rapidly lost, as cells with a reduced copy number will out-compete cells with a higher copy number.

Significance and impact of study: Improving phenotypic stability of strains engineered for high folate production is relevant for their application in industrial fermentation processes and can only be achieved if the instability phenomenon is well understood.

Introduction

Folate serves as an essential cofactor for all animals including humans. Humans do not have all the enzymes for folate biosynthesis. Of the seven enzymes needed for folate production, only two out of seven were found to be encoded in the human genome (1). One enzyme is dihydrofolate reductase (DHFR), which is needed for the conversion of dihydrofolate into tetrahydrofolate (24). The other enzyme is folylpoly(γ-glutamate) synthetase (FGPS) which plays a role intracellular retention of folate (13). To meet the daily requirement for folate, the vitamin has to be supplied in the diet. Dairy products, green-vegetables and fruits contain significant amounts of folate. Nevertheless, the daily recommended folate intake levels are not met in a large part of the western world. In the USA the daily recommended intake for adults has been set at 400 μg/day, for women in the periconceptional period this level is set at 600 μg/day (3, 22).

Folate intake at the population level can be elevated by food fortification programs such as the one implemented in the United States of America for folate fortified wheat. This program has led to a dramatic drop in neural tube prevalence (28). Alternatively, the vitamin content of food products can be increased via biofortification by improving either the raw materials or the fermentation process through the use of selected or metabolically engineered plants or microbes, respectively. In transgenic tomatoes for example, the combined overexpression of both GTP cyclohydrolase I (*GCHI*, synthetic mammalian gene) and aminodeoxychorismate synthase, (*AtADCS*, *Arabidopsis thaliana* gene) resulted in 25-fold increased folate pools when compared to control-tomatoes (9). In *Bacillus subtilis* 8-fold elevated folate pools were obtained by partial inhibition of pyruvate kinase activity and simultaneous overexpression of the GTP-cyclohydrolase gene and the *aroH*, coding for a

enzyme in the chorismate biosynthesis pathway (31). In the lactic acid bacterium *Lactococcus lactis*, the complete folate gene cluster and the *p*ABA gene cluster were simultaneously overexpressed on two different high copy number plasmids. This combined overexpression resulted in a strain that produced 80-fold increased folate levels (29). The use of such a strain in the fermentation fortification of a dairy drink could result in the supplementation of the daily folate dose by a single serving. High copy number plasmids are often used to increase production levels of metabolites or proteins, plasmids like pNZ8148 and pNZ7021 are commonly used in lactic acid bacteria to boost gene expression (20, 29).

In this paper we describe an analysis of the phenotypic stability (i.e. folate production and growth performance) of a high folate-producing *L. plantarum* WCFS1 strain. Furthermore, in serial propagation experiments the stability of the folate-overproduction phenotype was monitored on basis of copy number and folate production pools. Finally, low and high folate producing strains were selected to determine the relation between folate production, copy number and growth rate.

Materials and methods

Bacterial strains, media and culture conditions

The bacterial strains, plasmids and primers used in this study are listed in Table 1. All *Lactobacillus plantarum* WCFS1 strains were cultivated at 37ºC in a medium based on Chemically Defined Medium (CDM), as described before (21, 27). Unless stated otherwise, CDM is complete, however during some batch experiments *p*ABA was omitted from the medium. In case *p*ABA was added, it was applied at a concentration of 10 mg/L. Batch cultivation of the control strain (*L. plantarum* WCFS1 containing pNZ7021) and folate overproducing strain (*L. plantarum* WCFS1 containing pNZ7026) were performed with 56 mM glucose as main carbon and energy source. In continuous culture, 100 mM glucose was used. The chloramphenicol (CM) concentration both in the batch and continuous culture varied between 0 and 80 mg/L. For the serial propagation experiments, 40µl of full-grown culture was used for inoculation of 10 ml of fresh CDM, thereby assuming that 8 doubling events are needed to result in full-grown culture. MRS broth and agar was used (Difco, Surrey, U.K.) for selection of genetic engineered strain, using 10 mg/L CM. *Lactococcus lactis* was grown at 30ºC. Transformed *L. lactis* strains were cultivated and selected on M17 broth and agar using 10 mg/L CM. Growth rates for the *L. plantarum* WCFS1 strains were determined in batch culture and in 96 wells microtiter plates by turbidity measurements at 600nm using the Spectra Max384 spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, USA).

Continuous culture

Continuous cultivation was performed in a 1 liter reactor (Applikon Dependable Instruments, Schiedam, The Netherlands), containing 0.5 L, CDM. Temperature was controlled at 37ºC. The control strain and the folate overproducing *L. plantarum* strains were inoculated in the reactor; first exponential growth of the culture was allowed until the maximal turbidity at 600nm was reached. Hereafter the growth rate of both strains was set at

0.25 h^{-1}. Steady state was assumed after 5 volume changes. A stable pH of 5.5 was maintained by the titration of 5M NaOH, the pH was monitored by an ADI 1020 fermentation control unit (Applikon Dependable Instruments, Schiedam, The Netherlands). Anaerobic conditions were obtained by flushing the headspace of the reactor with nitrogen.

Construction of the folate overproducing strains

The genomic DNA of *L. plantarum* WCFS1 was isolated using established procedures (11). PCR was performed as follows; 30 sec. denaturation at 94ºC, 30 sec. primer annealing at 45ºC, and elongation at 68ºC for 1 min per kilo-base, this specific sequence was repeated for 30 cycles. Pfx polymerase (Invitrogen, Breda, The Netherlands) was used for amplification. DNA ligation was performed using T4 DNA ligase (Invitrogen) by overnight incubation at 16ºC, DNA fragments were mixed with a 1:5 ratio of plasmid: insert:. The folate gene cluster was amplified by PCR using folBKpnF (forward primer), and folPXbaR (reverse primer). The folBKpnF and folPXbaR primers were modified in their sequence to introduce a KpnI, and XbaI restriction site, respectively (modified bases underlined). The folate gene cluster was amplified from 18 base pairs upstream of *folB* to 52 bases downstream of *folP*. The linear stretch of amplified DNA was partially digested, by KpnI (Invitrogen) and XbaI (Invitrogen). Partial digestion is essential because *folP* contains an additional XbaI restriction site. The digested folate gene-cluster, contains the full length of the folate gene cluster. Plasmid pNZ7021 (containing constitutive promoter; *pepN* of *Lactococcus lactis*) (29) was also digested with XbaI and KpnI. Both digested pieces of DNA were mixed in a 1: 5 ratio (plasmid: insert), and were ligated by T4 DNA ligase. The ligated DNA was transferred to competent cells of NZ9000ΔylgG *L. lactis* (17) strains using electroporation by established procedures (8). Subsequently, the transformed *L. lactis* strain was cultivated for 40 hours on M17 plates with 10 mg/L CM. Chloramphenicol resistant colonies were checked for the presence of the plasmid by PCR using folPF (forward primer); and nisRKR (reverse primer). Positive transformants were picked and inoculated on M17 broth with 10 mg/L CM. Total plasmid content was isolated from full grown overnight cultures and plasmids were isolated using Jetstar columns (Genomed GmbH, Bad Oeynhausen, Germany). The plasmid was checked by restriction analysis and subsequently sequenced using standard procedures as described below. The resulting plasmid was designated as pNZ7026. Next, plasmids pNZ7021 and pNZ7026 were transferred to competent cells of *L. plantarum* WCFS1 (18) by electroporation using established procedures (16). Transformants were grown for 40 hours on MRS plates with 10 mg/L CM and CM resistant cells were analyzed for the presence of the appropriate plasmids by PCR. For *L. plantarum* containing the pNZ7026, primers folpF and nisRKR were used as probes. The primers CmdownF (forward primer) and nisRKR were used for monitoring the presence of pNZ7021 in the host strain. Both strains were cultivated on CDM with 10 mg/L CM and stored in glycerol stocks at -80ºC. From now on *L. plantarum* containing pNZ7021 was named the control strain, and *L. plantarum* containing pNZ7026 was named the folate overproducing strain.

Determining the relative copy number

The relative copy number was determined by quantitative PCR (qPCR). One primer-set was designed for the CM resistance gene on the plasmids pNZ7021 and pNZ7026, the other primer set was designed for the tryptophan gene, *trpE*, on the chromosome of *L. plantarum* WCFS1. The primers for the CM gene on the plasmid contain the following sequences, CTTAGTGACAAGGGTGATAAACTCAAA and CAATAACCTAACTCTCCGTCGCTAT, for the forward and reverse primers, respectively. The primer sequences of the tryptophan gene, *trpE*, on the chromosome of *L. plantarum* WCFS1 were as follows: GCTGGCGCGCCTAAGA (forward primer) and GCGGCACCTGCTCATAATG (reverse primer). The primers for the chromosome are used as marker for the chromosomal copy number to which all plasmid copy numbers were compared; this determines the relative copy number. Total DNA fraction was isolated from *L. plantarum* in the stationary phase. Total DNA was isolated from 5 ml of cell pellet using established procedures (4). For qPCR, 0.2 µg of total DNA was used. The amplification efficiency was determined for: genomic DNA of *L. plantarum* WCFS1, pNZ7021 plasmid DNA and pNZ7026 plasmid DNA, amplification factors ranging from 1.9 to 2.0 were considered to be reliable. Sybr Green (ABI, Cheshire, UK) was used as fluorescent dye for determining the level of amplification. The Critical threshold number (C_t) was determined using ABI Prism 7500 Fast Real-Time PCR system and software. The C_t value was used to calculate the relative gene copy number ($N_{relative}$) for the plasmid copy number in relation to the chromosomal copy number with the formula $N_{relative} = 2^{(C_{t,plasmid} - C_{t\,chromosome})}$. $C_{t,plasmid}$ is C_t value for plasmid and $C_{t,chromosome}$ is C_t value for the chromosome. All relative copy number determinations were performed in triplicate.

Folate, *p*ABA and pterin analysis

Folate was quantified using a microbiological assay, including enzymatic deconjugation of polyglutamate tails (15, 25). The folate levels were determined in the intracellular, extracellular and total fraction. The total fraction represents the folate pools in a sample in which 0.5 ml full grown culture was mixed with 0.5 ml acetate buffer (100mM Na-acetate, containing 1% vitamin C). Extracellular fraction was composed out of 0.5 ml of supernatant mixed with 0.5 ml acetate buffer. The folate content of the intracellular fraction was determined by; centrifuging 1ml of full grown culture, two times pellet washing (with 1ml of acetate buffer) and resuspending the pellet in 1ml acetate buffer. Intracellular, extracellular and total folate fractions were boiled for 5 minutes at 100ºC. To determine folate pools in the *p*ABA deprived strains, a folate concentration-procedure was applied (29). Samples of the folate overproducing strain were diluted in an acetate buffer for analysis in the microbiological folate assay.

Sequence analysis on the overproduction plasmid

The folate overproducing strain was cultivated for 56 generations in CDM containing CM. Of the full-grown culture an aliquot was plated on CDM plates containing CM. From this plate a single colony isolate was selected; cultivated and total plasmid content was isolated by a Jetstar-column (Genomed). The isolated folate overexpression plasmid was

amplified by PCR using the following primer combinations: AntibioticF and RepAR; RepAF and FolPR; FolPF and XTP2R; XTP2F and FolC2R; FolC2F and FolER; FolEF and AntibioticR, respectively. All amplified DNA fragments were put on agarose gel. The size of the amplified PCR fragment was compared to the predicted size. Deviations in the predicted size were used as marker for possible plasmid rearrangements. Sequence analysis was used to localize these arrangements. The first part of the folate gene cluster was sequenced (Baseclear, Leiden, The Netherlands) bidirectional using the following primers FolEF and AntibioticR.

Results

Genetic organization of the folate genes in Lactobacillus plantarum WCFS1.

The ability to produce folate is widespread within the *Lactobacillus* genus. In a recent study in our laboratory, 7 *Lactobacillus* species were analyzed for folate pools and three of the tested strains were able to produce folate (26). One of the natural folate producers is *Lactobacillus plantarum* WCFS1 of which the full genome sequence in publicly available (18). Genes for folate production were found as part of a 4559 base pair long gene cluster on the genome. The gene cluster of *L. plantarum* WCFS1 contains six genes: *folB*, *folK*, *folE*, *folC2*, *xtp2*, and *folP* (see Fig. 1).
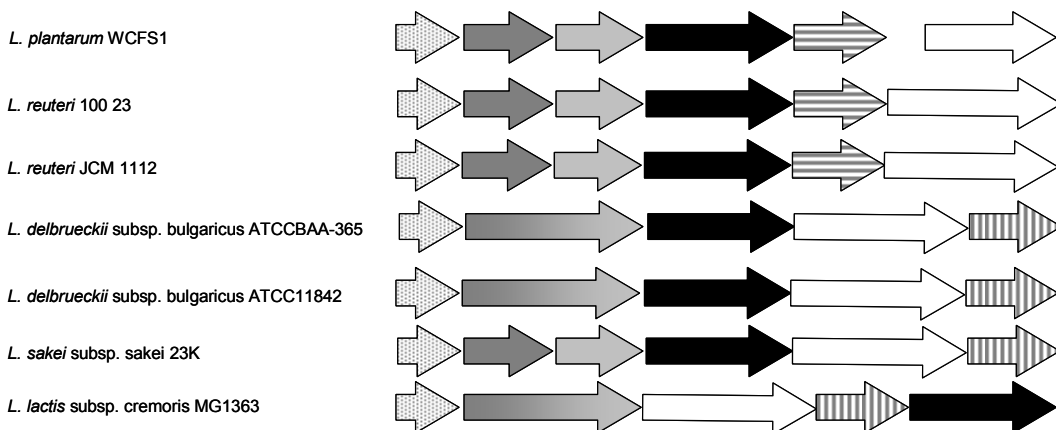
FIG. 1: The genetic organization of the folate gene cluster in *L. plantarum* WCFS1 was compared with the folate gene clusters of: *L. reuteri* 100-23, *L. reuteri* JCM-1112, *L. delbrueckii* subsp. *bulgaricus* ATCCBAA 365, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. sake* subsp. *sakei* 23 K and *L. lactis* subsp cremoris MG1363. The *L. plantarum* folate gene cluster contains the following genes: *folB*, *folK*, *folE*, *folC2*, *xtp2*, and *folP* coding for 7,8-dihydroneopterin aldolase (*folB*; spackled arrow), 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase (*folK*; dark grey arrow), GTP cyclohydrolase I (*folE*; light grey arrow), dihydrofolate synthetase (*folC2*; black arrow), xanthosine triphosphate pyrophosphatase (*xtp2*; horizontally striped arrow) and dihydropteroate synthetase (*folP*; white arrow). Finally, *mutT* is the vertically striped arrow. In both *L. delbrueckii* species and the *L. lactis* strain *folK* is fused with *folE* (dark gray to light gray gradient).

Currently, a total of 15 *Lactobacillus* species are completely or partially sequenced. We have determined the presence of the six folate genes in the 15 sequenced lactobacilli by BLAST-P (23) analysis using the ERGO™ database. Six species, including *L. plantarum* WCFS1, have the complete folate gene cluster (see Fig. 1). The presence of a gene was assumed for target sequences with E-scores higher than 10^{-20}. The six lactobacilli possess the complete folate gene cluster are: *L. plantarum* WCFS1, *L. reuteri* 100-23, *L. reuteri* JCM-1112, *L. delbrueckii* subsp. *bulgaricus* ATCCBAA 365, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, and *L. sake* subsp. *sakei* 23 K. The genetic organization of the folate gene cluster in the six *Lactobacillus* species, including the well described folate gene cluster of *L. lactis* MG1363 (25, 29, 30) have been compared (see Fig. 1). This analysis shows that the gene cluster of *L. plantarum* WCFS1 is very similar to that of *L. reuteri* 100-23 and *L. reuteri* JCM 1112. The organization of the gene cluster within the group consisting of *L. delbrueckii* subsp. *bulgaricus* ATCCBAA 365, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, and *L. sake* subsp. *sakei* 23 K shows a high similarity and is clearly different from the organization found in *L. plantarum* and *L. reuteri*. In addition, *folC* was found encoded in the genomes of all 15 *Lactobacillus* species. However, 6 species that possess the folate gene cluster have an additional *folC* copy (as part of the gene cluster). Based on this study, unpublished data and literature data, it can be concluded that folate is produced by *L. plantarum* WCFS1 (this paper), *L. reuteri* JCM 1112, *L. reuteri* 100-23 (A. Wegkamp, unpublished data) and *L. delbrueckii* subsp. *bulgaricus* (26). This is in full agreement with the annotated genome sequence information which predicts the presence of a complete folate gene-cluster for all strains.

Folate production in batch culture.

The engineered *L. plantarum* WCFS1 strains, containing the control plasmid (pNZ7021) and folate overexpression plasmid (pNZ7026), were cultivated on Chemically Defined Medium (CDM) with and without the supplementation of *p*ABA in the presence of 80 mg/L chloramphenicol (CM). In the absence of *p*ABA, total folate pools of both engineered *L. plantarum* strains were found to be below the detection limit (2 µg/L), indicating that in the absence of *p*ABA, folate could not be synthesized. This fits with the observation that the *p*ABA biosynthetic genes (*pabA*, *pabB* and *pabC*) were not found on the genome of *L. plantarum* WCFS1. To determine whether trace amounts of folate could be made on CDM without *p*ABA, the folate analysis was performed on a 500-fold concentrated intracellular cell fraction of the *L. plantarum* control strain. In the concentrated cell extract, 4.7 µg/L folate was detected. This pool is equivalent to a folate production level of 9.4 ng/L folate in the non-concentrated cells. Folate analyses on the concentrated cell extract shows that either low folate pools are made by *L. plantarum*, or that a highly concentrated intracellular component affects growth of the indicator strain in the folate assay. Still, these folate pools are more than three orders of magnitude lower in comparison to those found in normal wild type levels. In CDM with *p*ABA, *L. plantarum* containing the control plasmid was able to produce folate pools of 29 µg/L per OD_{600} unit (stdev of 2.7 µg/L), and the strain with the folate overproduction plasmid produced 3.28 mg/L per OD_{600} (stdev of 0.430 mg/L).

In the folate overproducing strain 90 % of the folate is secreted into the medium. In the *L. plantarum* strain harboring the control plasmid only 30% of the total folate can be found outside the cell (data not shown).

The growth rates of the engineered *L. plantarum* WCFS1 strains were determined in pH controlled batch culture and 96 well plate cultures. In both experiments it was observed that the *L. plantarum* WCFS1 strain harboring the folate overproduction plasmid grew 20-25% slower than the control strain. This suggests that overexpression of the folate genes imposes stress on the folate overproducing strain, resulting in a lower growth rate.

In summary, a high folate producing *L. plantarum* WCFS1 strain was constructed; this was done by overexpression of the complete folate gene cluster. High *p*ABA levels in the medium are required for elevated folate production and the overexpression of the folate gene-cluster resulted in 20-25% drop in growth rate.

Folate production in continuous culture.

The folate overproducing *L. plantarum* strain as well as the control strain, were cultivated in continuous culture ($D=0.25$ $h^{-1)}$ on various CM concentrations (10, 40 and 80 mg/L). When steady state was reached the folate pools and the colony forming units (CFU's) were determined on MRS plates with and without CM. Folate pools did not differ much between the three different chemostat experiments. Average folate pools (with a deviation lower than 10%) of 0.05 and 0.65 mg/L per OD_{600} unit were produced by the control and folate overproducing strain, respectively. Interestingly, a large difference in the ability to grow on MRS plates with CM was observed for the folate overproducing strain in comparison with the control strain. Only 10 % of the cells in the folate overproducing chemostat culture supplemented with 10 mg/L CM was able to grow on MRS plates with CM. This was unexpected since the chemostat culture contains the same CM concentration as the plates used for viable count analysis. Increasing the CM concentration in the chemostat culture to 40 mg/L assured that 45% of the folate overproducing cells was able to grow on MRS plates with CM. At CM concentrations of 80 mg/L nearly all folate overproducing cells were able to grow on MRS plates with CM. The discrepancy in growth performance in the chemostat culture supplemented with 10 mg/L CM and MRS plates might be caused by a drop in plasmid copy number in the folate overproducing strain when grown in chemostat. The relatively low specific growth rate in the chemostat culture could be related with the assumed lowering of the plasmid copy number. Increasing the CM concentration in chemostat prevents the copy number to drop.

Folate production upon prolonged propagation in batch culture.

Total folate pools and the plasmid copy number were used as marker to determine the stability of the folate overproduction phenotype in propagation experiments. First, different CM concentrations were used to determine the CM concentration that leads to the most stable folate production phenotype. The folate overproducer was propagated for 48 generations in batch culture on CDM with varying concentrations up to 80 mg/L CM; total folate content was analyzed in overnight cultures. It was found that 80 mg/L CM resulted in the most stable folate production phenotype (data not shown). Moreover, these experiments also revealed that

10 mg/L CM, which is commonly used for plasmid maintenance, is not sufficient for maintaining a stable folate-overproduction phenotype in *L. plantarum* WCFS1.

The *L. plantarum* control and folate-overproducing strains were propagated in 6 (control) and 7 (folate overproducer) sequential batch cultures, thereby reaching a total of 48 and 56 generations, respectively. Several analyses were performed on the full grown cultures: total folate pools, turbidity at 600 nm (OD_{600}), and relative copy number were determined (Fig. 2 and Fig. 3).
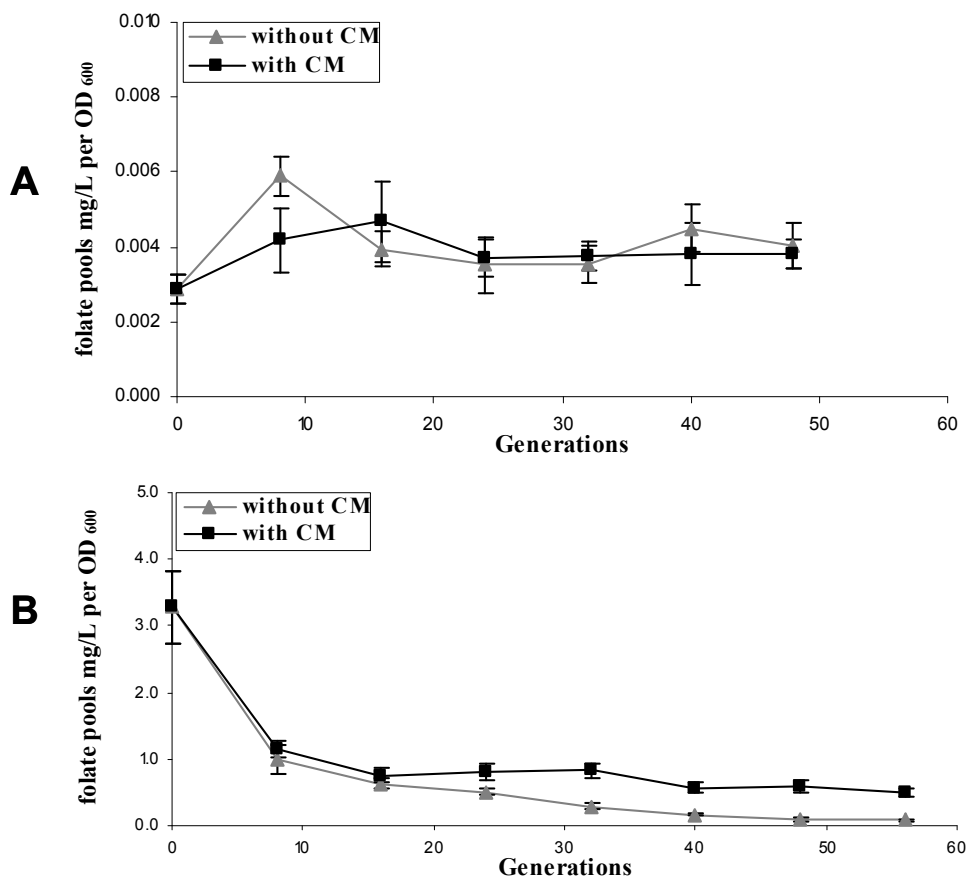
FIG 2: Folate production pools in mg/L per OD_{600} unit of the control (A) and folate overproducing strain (B) in the absence (black line) and presence (grey line) of CM as a function of the number of generations. The standard deviation of each measuring point is indicated in the graph.

Folate production pools in the control strain did not change remarkably when propagated for 48 generations in the presence and absence of CM, folate levels remained stable between 3 and 6 µg/L per OD_{600} unit (Fig 2A). In contrast, the folate overproducing strain shows a rapid decrease in the capacity to produce folate when propagated without CM.

Propagation of the folate overproducer for 56 generations in the absence of CM led to a dramatic drop in folate pools, starting at 3.2 mg folate /L per OD_{600} unit and reaching only 0.083 mg folate /L per OD_{600} unit after 56 generation (Fig 2B). In the presence of CM this drop in folate pools was less pronounced. After 56 generations, still 0.5 mg folate/L per OD_{600} was detected. In summary, propagation of the folate overproducer for 56 generations resulted in a 40-fold and 6.6-fold drop in folate pools in the absence and presence of CM, respectively. These experiments clearly show that 80 mg/L CM is needed to maintain the folate-overproduction phenotype for a prolonged period of time. In the absence of CM the phenotype of folate-overproduction is rapidly lost.

The copy number for the control and folate-overproduction plasmid was determined by qPCR. The plasmid copy number was correlated to the chromosome, thus reflecting a relative copy number (Fig. 3). Remarkably, the copy number of the control plasmid increased in *L. plantarum* WCFS1 when cultivated on CDM both in the presence and absence of CM. In the absence of CM, the relative copy number increased from 36 copies per chromosome to 84 copies per chromosome at generation number 48. In the presence of CM the relative copy number increased from 36 copies per chromosome at the start to 69 copies per chromosome at generation 48 (Fig. 3A). The average copy number in the culture of the folate overproducing strain dropped dramatically when cultivated for 56 generations in the absence of CM, the relative copy number started at 68 copies and reached 3 copies per chromosome at generation 56. The drop in copy number was much lower when the folate overproducing strain was propagated in the presence of CM; the relative copy number started again at 68 copies and reached 21 copies per chromosome after propagation for 56 generations (Fig. 3B). So in the folate overproducing strain the drop in copy number was 23-fold and 3.2-fold in the absence and presence of CM, respectively. In summary, it can be concluded that for cultivation of the control strain no CM is needed for plasmid maintenance. However, the presence of CM was found to be essential for plasmid maintenance in the folate overproduction strain.

At the onset of the propagation experiment (generation 0), the folate production per plasmid, was calculated to be 0.047 mg/L per OD_{600} unit. At generation number 56, the folate production per plasmid per OD_{600} unit has dropped to 0.025 mg/L both in the presence and absence of CM. From this it can be concluded that propagation of the folate overproducing strain leads to a decrease in the folate production per plasmid, possibly caused by a lower expression level, or by structural rearrangements within the plasmid. The folate overexpression plasmid (pNZ7026) was retrieved from a single colony isolate and partially sequenced to investigate the mechanism of decreased folate production per plasmid. The complete plasmid was amplified by six individual PCR reactions; all fragments had the predicted size except for the fragment containing *folB* and *folK*, which displayed, in addition to the predicted band, an additional band of smaller size, suggesting structural plasmid rearrangements. Sequencing of the band with the reduced size showed that 714 base-pairs were missing from the plasmid; *folB* was deleted completely (357 base-pairs) and in *folK* partially (357 base-pairs). This shows that the overexpression of the folate gene cluster is associated with structural plasmid rearrangements. Since the tested single colony isolate contains two types of plasmids, it is expected that the folate production per plasmid depends on the presence the copy number of each plasmid.
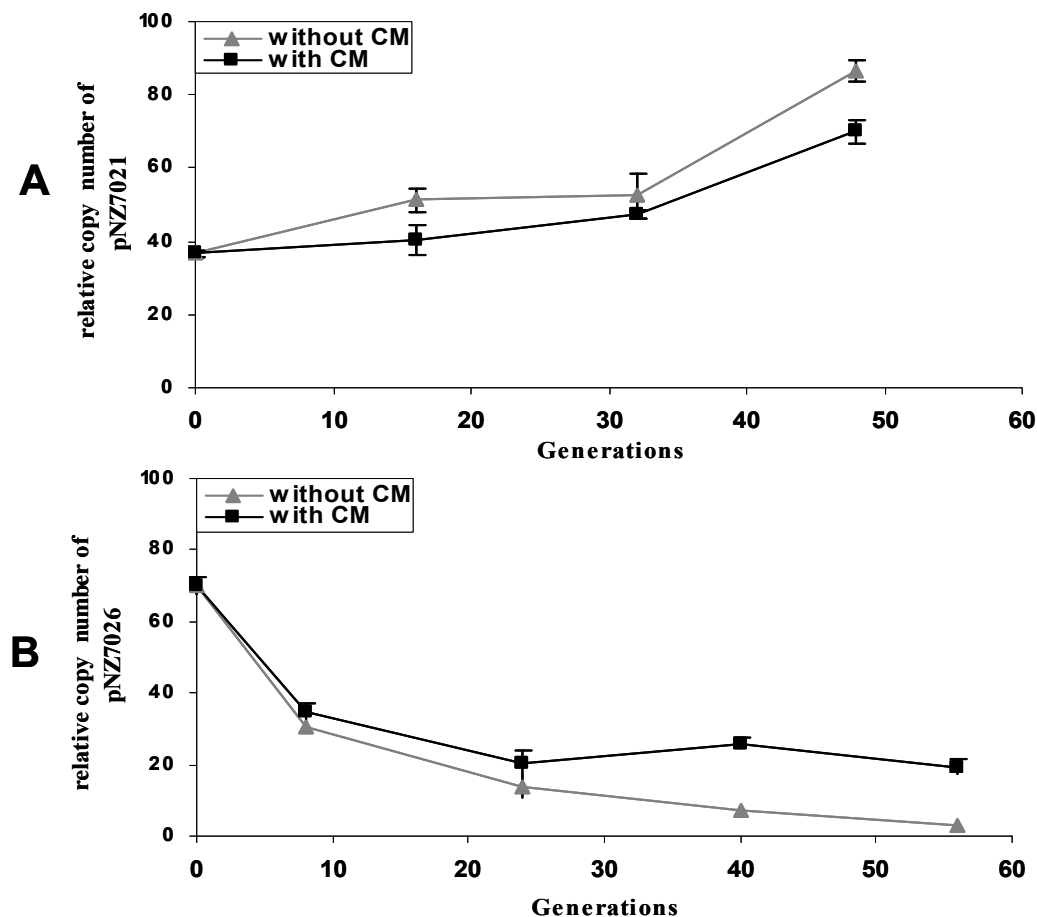
FIG 3: The relative copy numbers for the control and folate overproducing strain were determined both in the absence (black line) and presence (grey line) of CM. The relative copy numbers in the control strain were determined at generation 0, 16, 24 and 48 (A) and the relative copy number of the folate overproducing strain was determined at generation 0, 8, 24, 40, and 56 (B). The standard deviation of each measuring point is indicated in the graph.

Correlation between the growth rate, folate overproduction and copy number.

We have observed that the folate overproducing strain displays a 20-25% reduction in growth rate when compared to the control strain. To test whether low folate producing cells can out-compete high folate overproducing cells, the correlation between folate production, copy number and growth rate was determined for single colony isolates. The single colony isolates were obtained as follows; the folate overproducing strain was cultivated for 56

generations in CDM both in the presence and absence of CM. Aliquots of these full grown cultures were plated on CDM plates with and without CM. From these plates a total of 90 colonies were randomly selected. These single colony isolates were cultivated again on CDM and the total folate pools were determined for each isolate. The folate production capacity of 90 single colony isolates showed a large variation. From this panel of 90 single colony isolates, a total of 10 were sub-selected; three single colonies produced low folate levels and seven single colonies were high folate producing isolates. These 10 selected colonies were cultivated in CDM, subsequently the folate levels, copy number and growth rate were determined (Fig. 4).

The three single colony isolates with low folate pools display a low copy number and a high growth rate, while the 7 high folate producing isolates display a relative high copy number and a reduced growth rate. This experiment suggests that cells which over-express the folate gene cluster at a lower level can out-compete cells with a high copy number. Consequently, in a competitive propagation experiment, the phenotype of folate overproduction is rapidly lost.
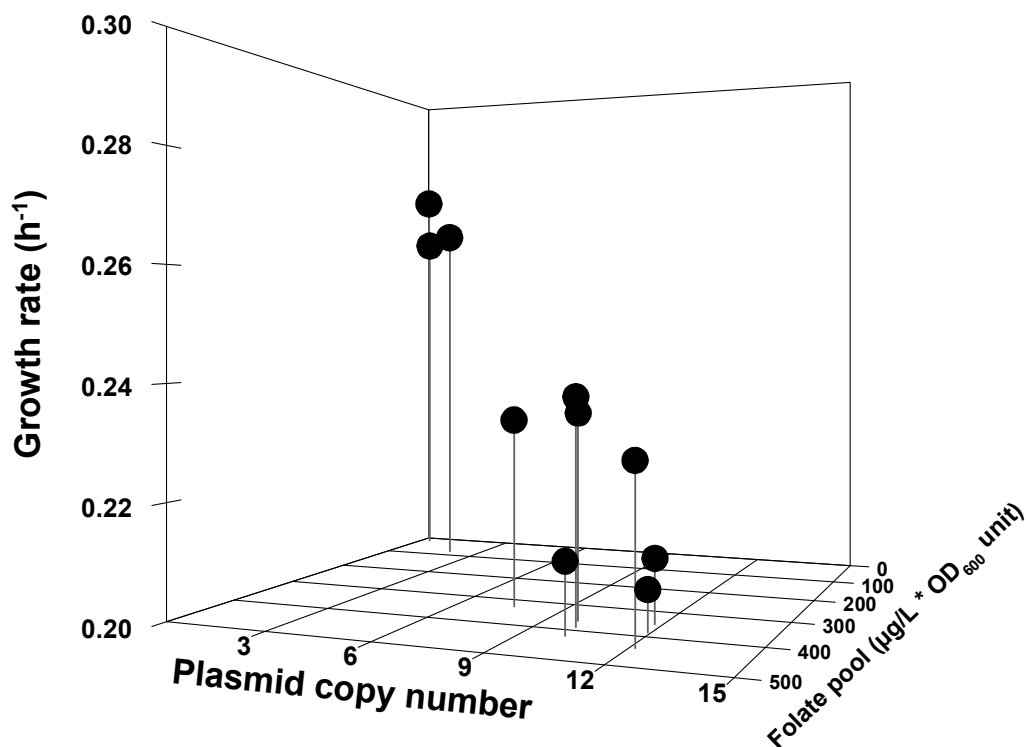

FIG 4: The relation between relative copy number (X-axis), maximum growth rate (μ_{max}, Y-axis) and folate production in ng/ml per OD_{600} unit (Z-axis) for 10 single colony isolates. Three colonies were indicated as low folate producing isolates (group A), and seven colonies were characterized as high folate producing isolates (group B).

Discussion

  High folate production levels can be achieved in *Lactobacillus plantarum* WCFS1 upon the homologues overexpression of the folate biosynthesis gene cluster of *L. plantarum* WCFS1. This gene cluster contains 6 genes, *folB*, *folK*, *folE*, *folC2*, *xtp2*, and *folP*. Interestingly, *xtp-2* can not directly be linked to the folate biosynthesis pathway, and was annotated as a gene coding for xanthosine triphosphate pyrophosphatase. Moreover, the enzyme for the conversion of dihydroneopterin triphosphate into dihydronepterin monophosphate, was still missing in our reconstruction of the folate biosynthesis pathway in *L. plantarum* WCFS1. For this reaction a triphosphate pyrophosphohydrolase is needed and since *xtp2* is annotated as xanthosine triphophate pyrophosphatase, we hypothesize that this particular gene codes for the missing enzyme. The gene cluster organization of *L. plantarum* WCFS1 looks very similar to that of *L. reuteri* JCM-1112 and *L. reuteri* 100-23 and all three folate gene clusters have an *xtp2*. However, the folate gene clusters of *L. sake* subsp. *sakei* 23 K, *L. delbrueckii* subsp. *bulgaricus* ATCCBAA 365, and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 do not have the *xtp2* homologue. Instead a gene called *mutT* (of the nudix protein family) was found in the folate gene cluster. *MutT* is highly homologues to *folQ*, a gene that contains a nudix-motif and is involved in the folate biosynthesis pathway in converting dihydroneopterin triphosphate into dihydronepterin monophosphate in *Lactococcus lactis* and *Arabidopsis thaliana* (5). The involvement of a gene with putative triphophate pyrophosphatase activity in the folate gene cluster suggests that *xtp2* and *mutT* are involved in the folate biosynthesis pathway in *L. plantarum* WCFS1, *L. reuteri* JCM-1112, *L. reuteri* 100-23, *L. sake* subsp. *sakei* 23 K, *L. delbrueckii* subsp. *bulgaricus* ATCCBAA 365, and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842.

  Besides x*tp-2*, the folate gene cluster of *L. plantarum* contains another interesting gene, *folC2*, which codes for one of the two folyl-polyglutamate synthetases present on the genome *of L. plantarum* WCFS1. All 15 *Lactobacillus* species have a separate *folC* encoded on the chromosome, the six species with the intact folate gene cluster possess the additional *folC* as part of the gene cluster. Since *folC* is essential for all organisms, it can be reasoned that the folate gene cluster with the additional *folC* is recently acquired. In addition, none of the lactobacilli in our analysis possesses the genes coding for *p*ABA production. Recently, we have demonstrated that folate and *p*ABA production in *L. lactis* are tightly coupled (29). This supports our observation that *L. plantarum* is incapable of autonomous folate production, which underpins the assumption that the folate gene cluster was recently acquired.

  The folate-overproduction plasmid that was constructed for this study was based on the pNZ7021 plasmid; this plasmid contains the constitutive *pepN* promoter of *Lactococcus lactis*, the *repA* and *repC* replication genes, the CM-resistance marker and the complete folate gene cluster. The replication genes are involved in the replication and copy number control of this rolling-circle plasmid (7). Repetitive propagation of the control strain in the absence and presence of CM resulted in an increase in relative copy number. This shows that CM is not needed for maintenance of the control plasmid as such. However, the increase in copy number is unexpected and the reason remains obscure. Fitzwater *et al* have shown that some plasmids have increased copy number in stationary phase (12). Therefore, some bias in the relative

copy numbers can be introduced since we have determined the copy number from cells isolated from stationary phase.

Overexpression of the folate gene cluster results in a drop in copy number both in the presence and absence of CM when propagated repeatedly. In addition, structural rearrangements have been observed in the folate overexpression plasmid. Plasmids have been isolated of which large parts of the folate gene cluster were missing. Moreover, it was determined that folate overproduction results in a 20-25% reduced growth rate. In previous studies it was determined that overexpression of genes may lead to a metabolic burden for the cell. In *E. coli* for example, the overexpression of methyltransferase, *Msp1*, resulted in a 33% growth reduction upon the induction with IPTG. This growth rate impairment was suggested to be caused by a higher maintenance coefficient (2).

L. plantarum WCFS1 carrying the folate-overproduction plasmid, was cultivated in continuous culture at a dilution rate of 0.25 h^{-1} with 10 mg/L CM. Subsequent plating on MRS plates with 10 mg/L CM revealed that only 10% of the folate overproducing cells still had the CM resistant phenotype. This was unexpected since both media contain the same antibiotic concentration. The difference is that growth on the MRS plates is supposed to be close to the maximum specific growth rate, whereas in continuous culture the growth rate is constrained to 0.25 h^{-1}. Therefore it is expected that growth rate reduction allows the cells to drop the copy number without problem. Increasing the CM concentration 8-fold (to 80 mg/L) in continuous culture assured that all folate overproducing cells kept the normal levels of CM resistance when analyzed on MRS plates with CM. In *Bacillus stearothermophilus* it was demonstrated that overexpression of penicillinase resulted in a drop in copy number, specifically at low dilution rates (19).

In single colony isolates, the relation between folate-overproduction levels, copy number and growth rate was determined. In the folate-overproducing strain a high copy number correlates with a low growth rate. Consequently, folate-overproducing cells that have lowered the copy number are expected to out-compete cells with a high copy number. Overexpression of the folate gene cluster can be considered as gratuitous overexpression, since folate production of the wild-type strain is sufficient to support optimal growth. In a similar experiment, the overexpression of a gratuitous truncated elongation factor, Δ*tufB*, in *E. coli* resulted in a drop in growth rate (10). This is similar to what we have observed upon the overexpression of the folate genes in *L. plantarum* WCFS1. Currently we are investigating the cause and the metabolic implications of the growth impairment at a physiological level.

High copy number plasmids can be used for the production of metabolic enzymes associated with biosynthesis for all sorts of primary and secondary metabolites like alanine, riboflavin and folate (6, 14, 29). We have shown that strains containing the control plasmid can be cultivated for 56 generations without plasmid loss. However, the overexpression of the folate genes resulted in a rapid plasmid loss when propagated in the absence of antibiotics. It was demonstrate that cells that produced lower folate levels have a higher growth rate and these can out-compete cells that produce higher amounts of folate.

The **abbreviations** used are: PCR, polymerase chain reaction; *p*ABA, *para*-aminobenzoic acid; THF, tetrahydrofolate; CDM, Chemically Defined Medium; CM, chloramphenicol; qPCR, quantitative polymerase chain reaction; C_t, critical threshold; N.D., not detected.

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Chapter 7

Physiological Responses to Folate Overproduction in *Lactobacillus plantarum* WCFS1

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Abstract

Using a functional genomics approach we addressed the impact of folate overproduction on metabolite formation and gene expression in *Lactobacillus plantarum* WCFS1. We first determined the effect of adding the precursor *para*-aminobenzoic acid (*p*ABA) on folate overproduction and observed that the overexpression of the folate gene cluster in the absence of *p*ABA resulted in intracellular accumulation of 6-hydroxymethylpterin and no folate overproduction. The addition of *p*ABA to the growth medium restored folate production in the folate-overproducing *L. plantarum* and, simultaneously, the intracellular 6-hydroxymethylpterin pools were found to drop to control levels. Next, the metabolite formation and gene expression were determined in the folate-overproducing and wild-type strains in the presence of *p*ABA. Differential metabolomics analysis of intracellular pools after cultivation in continuous culture, using LC-MS based metabolite fingerprinting, indicated that the pool sizes of 18 metabolites differed significantly between both strains. One component, 10-formyl folate was the metabolite with the highest relative abundance (more than 100-fold). The gene expression profile was determined for both strains in continuous culture and batch culture. Apart from the expected overexpression of the 6 genes of the folate gene cluster, the expression of no other genes were found to be affected in either continuous or batch cultures. However, it was found in continuous and batch culture that 8 and 11 genes (excluding the 6 folate genes), respectively, responded specifically to secondary effects of the overexpression of the folate gene cluster, which included a 25 % reduced growth rate, Remarkably, in the absence of *p*ABA the growth rate was also 25% reduced, showing that the growth rate reduction is not caused by high folate pools per se. Other possible mechanisms for the growth rate reduction were addressed experimentally and the most likely possibilities were found to be precursor drainage and metabolic burden.

Introduction

Microorganisms are often used as cellular factories to produce a wide range of metabolites and proteins. Metabolic engineering is a suitable method to increase the production levels of these desired compounds. Feasibility studies with lactic acid bacteria have been performed in which strains were constructed with increased production of metabolites such as alanine, sorbitol, riboflavin, and folate (7, 21, 55). In *Lactococcus lactis*, overproduction of alanine dehydrogenase in a lactate dehydrogenase (LDH) deficient background resulted in exclusive alanine production (21). In another case, overexpression of the complete riboflavin gene cluster in *L. lactis*, resulted in a high riboflavin producing *L. lactis* strain (7). A third example is the combined overexpression of the folate gene cluster and the *p*-aminobenzoic acid (*p*ABA) gene cluster in *L. lactis* which resulted in a high folate producing strain (55). The latter strain was able to produce 100-fold more folate (total folate levels) when compared to control strains, thereby no *p*ABA needed to be added to the growth medium. The folate biosynthesis proceeds via the conversion of GTP in eight consecutive steps to the biological active cofactor tetrahydrofolate (THF). The biosynthesis of THF includes two condensation reactions. The first is the condensation of *para*-aminobenzoic acid (*p*ABA) with 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine to produce dihydropteroate. Subsequently, glutamate is attached to dihydropteroate to form dihydrofolate (18). 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 (34, 45).

The model organism *Escherichia coli* is commonly used for recombinant overexpression of proteins (2). This micro-organism has a long history in application for the production of a vast range of proteins for example, for insulin, growth hormones or interferon (33, 43, 56). A problem with overexpression of recombinant or homologous proteins on high copy plasmids is that the desired phenotype may be rapidly lost when propagated for prolonged periods of time (15). One cause for the instable phenotype is a metabolic burden (3, 19), meaning that the overproduction of proteins reduces the growth rate of the protein overproducing strain. In *E. coli*, for example, the overproduction of a non-functional elongation factor EF-*Tu* leads to a reduced growth rate of the strain (30). It is evident that this EF-*Tu* overproducing strain is handicapped because of the production of a non-functional protein. In this case the production of functional proteins is reduced since the functional and non-functional proteins compete for resources of the translation machinery.

Lactobacilli are commonly used to ferment food products like meat, vegetables and dairy products (52). *Lactobacillus plantarum* is a well-characterized lactic acid bacterium and strain WCFS1 was the first in the genus *Lactobacillus* for which the entire genome sequence became publicly available (28). Previously, a high folate-producing *L. plantarum* WCFS1 strain was constructed that produced more than 1000-fold increased folate pools, when compared to the control strain (54). Remarkably, this strain exhibited a 20-25% reduction in growth rate. In a serial propagation experiment the folate overproducing capacity was rapidly lost when the culture was propagated in the absence of selection marker (54).

It remains unclear whether high production of specific secondary metabolites such as folate can provoke a large cellular response. This paper describes the impact of metabolic engineering of folate production on the overall performance of the cell. Functional genomics tools, including transcriptomics, proteomics and metabolomics, were used to elucidate global effects of folate overproduction. Leads from this analysis were used to help explaining the growth rate reduction upon the overexpression of the folate gene cluster.

Materials and methods

Bacterial strains, media and culture conditions.

Lactobacillus plantarum WCFS1 and derivatives thereof (see Table 1 for a complete list of used strains and plasmids) were cultivated at 37ºC on Chemically Defined Medium (CDM), as described before (49).

TABLE 1: List of strains, constructed plasmids, and primers used in this study.

Material	Relevant features	Source of reference
Strains		
L. lactis; NZ9000	MG1363 *pepN:nisRK*, Cloning host	(29)
L. plantarum WCFS1	Cloning host, genomic DNA isolation	(28)
L. plantarum NZ7100	WCFS1 :*nisRK*, Cloning host	(38)
Plasmids		
pNZ7021	Cm^R, pNZ8148 derivative, nisin promoter replaced by pepN promoter	(55)
pNZ7026	Cm^R, pNZ7021 derivative containing the *folB, folP, folK, folE, xtp2* and *folC2* gene cluster of *L. plantarum* WCFS1	(54)
pNZ8148	Cm^R	(29)
pNZ7030	Cm^R, pNZ8148 derivative containing *folB, folP, folK, folE, xtp2* and *folC2* gene cluster of *L. plantarum* WCFS1 in the sense orientation	(this study)

pNZ7031	Cm^R, pNZ8148 derivative containing *folB*, *folP*, *folK*, *folE*, *xtp2* and *folC2* gene cluster of *L. plantarum* WCFS1 in the antisense orientation	(this study)
Primers		
LpfBnco-F	CTGGGATACCCATGGGCATGATTC	
LpfPkpn-R	CGTCAAAAGGTACCGGACTAATCATTATTCG	
pNis-F	TAGTCTTATAACTATACTGAC	
LpfB-R	CTTGCCATTCGGCGTCCCCTCCACCTCAATTTCC	
LpfBatg-F	ATGGGCATGATTCGAATTAATAATTTACG	
LpfP-xbatest	GAATTTAATTATTTGCGACGCCCAAT	
FQPCRfolBS	CCTATCGAAACCAAGGTTCAACA	
RQPCRfolBS	ACAAATTCATCGACCACGTTACG	
FQPCRfolBAS	TCAACTTGTATGAATGGGTCGTTACA	
RQPCRfolBAS	CGTTCACGAGACCATCAATTACG	
FQPCRFPS	CATTATTAACGATGTGAACGCCTTT	
RQPCRFPS	CGCGACTGTCAGCCATCAAT	
FQPCRfPAS	CTAACAGCGTAATCAATTGCTTGGT	
RQPCRfPAS	CTTAAGGGTGGCCGGATCA	
groES-fo(2)	CCCAAAGCGGTAAGGTTGTT	
groES-re(2)	CTTCACGCTGGGGTCAACTT	
pfk-fo1	TCCAGGGACGATCGATAATGA	
pfk-re1	GCTTGCACGTTGGTGTTGAAC	

Unless stated otherwise, CDM is complete. In a number of specific batch experiments *p*ABA was omitted or added, thereby using a concentration of 10 mg/L. Cultivation of *L. plantarum* harboring pNZ7021 and pNZ7026 was performed in non-pH regulated batch culture using 56 mM glucose as main substrate. *L. plantarum* harboring pNZ7021 and pNZ7026 was cultivated in a pH-regulated batch fermentor and in chemostat culture on CDM supplemented with 25 mM glucose. A concentration 80 mg/L chloramphenicol (CM) was used in batch and continuous culture. For the construction of genetically modified strains MRS broth and agar was used (Difco, Surrey, U.K.). For selection on MRS plates 10 mg/L CM was applied to the agar. *Lactococcus lactis* was grown at 30ºC on CDM supplemented with 56 mM glucose as described previously (37, 41). Transformed *L. lactis* strains were cultivated and selected on M17 broth (48) and agar using 10 mg/L CM . For nisin induction a concentration of 25 ng/ml nisin was used, nisin induction itself proceeds as described previously (38).

Construction of genetically engineered strains.
        Genomic DNA of *L. plantarum* WCFS1 was isolated using established procedures (4). PCR was performed using PFX (Invitrogen, Breda, The Netherlands), applying PCR cycles of 94ºC for 30 sec denaturation, 43ºC for primer annealing for 30 sec, and 68ºC for elongation (1 min per Kb). DNA ligation was performed using T4 DNA ligase (Invitrogen) by overnight incubation at 16ºC. DNA fragments were mixed with a 5:1 insert: vector ratio. Two nisin inducible vectors were constructed, based on pNZ8148 (29). In one vector the folate gene cluster of *L. plantarum* was cloned under the control of the nisin promoter in the sense orientation and, in the other, in the antisense orientation. The folate gene cluster was amplified in the sense orientation by PCR using LpfBnco-F and LpfPkpn-R as forward and reverse primers, respectively. Both primers were modified to introduce a restriction site for cloning of the DNA fragments (modified bases underlined in Table 1). The insertion plasmid pNZ8148 and the amplified DNA were digested with KpnI and NcoI. Both fragments were mixed and used for T4 DNA ligation. The DNA mix was transferred to *L. lactis* NZ9000 for transformation by electroporation, using established procedures (11). The transformed *L.*

lactis strain was plated and grown for 40 h at 30ºC. Chloramphenicol (CM) resistant colonies were checked for the presence of plasmid by PCR with pNis-F and LpfB-R as forward and reverse primer, respectively. Positive colonies were grown and plasmid DNA was extracted and then isolated using Jetstar columns (Genomed GmbH, Bad Oeynhausen, Germany). The restriction profile of the plasmid was determined; the plasmid with the expected restriction profile was named pNZ7030. The antisense vector was made by amplification of the folate gene cluster using, LpfBatg-F and LpfPkpn-R as the forward and reverse primers, respectively. The amplified linear fragment of DNA was digested with KpnI, and pNZ8148 was digested with KpnI and PmlI. The digested PCR product and digested plasmid were mixed and used for T4-DNA ligation. The DNA mix was transferred *L. lactis* NZ9000 for transformation as described above and plated on M17 plates with CM. After 40 h of growth, CM resistant colonies were checked for the correct plasmid by PCR; pNis-F and LpfP-xbatest were used as forward and reverse primer, respectively. Positive colonies were grown and plasmid DNA was extracted and then isolated using Jetstar columns. The restriction profile of the plasmid was determined and the plasmid with the expected restriction profile was named pNZ7031. The plasmids pNZ8148, pNZ7030 and pNZ7031 were used for transformation of *L. plantarum* NZ7100 (38) by electroporation using established procedures (25), and plated on MRS with CM. CM-resistant colonies were checked for the proper plasmid by PCR, using the primers as described above. Colonies with the proper plasmid were grown on CDM with the 80 mg/L CM and stored at -80ºC in glycerol stocks waiting for further use.

Continuous culture.

Chemostat cultures was performed in a 1-L reactor (Applikon Dependable Instruments, Schiedam, The Netherlands) containing 0.5 L CDM. Temperature was controlled at 37ºC. *L. plantarum* harboring pNZ7021 and pNZ7026 were inoculated in the reactor; first exponential growth of the culture was allowed until the maximal turbidity at 600nm was reached. Next, the dilution rate of both cultures was set at 0.25 h^{-1}. Steady state was assumed after 5 volume changes. A stable pH of 5.5 was maintained by titration with 5 M NaOH, the pH was monitored by an ADI 1020 fermentation control unit (Applikon Dependable Instruments, Schiedam, The Netherlands). Anaerobic conditions were obtained by flushing the headspace of the reactor with nitrogen gas.

Folate, *p*ABA and pterin analyses.

Folate was quantified using the microbiological assay, including enzymatic deconjugation of polyglutamate tails (22, 46). Pterin pools were determined (after oxidation to the aromatic forms) by HPLC in the intracellular and extracellular fractions of *L. plantarum* WCFS1 cultures using the procedures described by Klaus et al. (27). The 6-hydroxymethylpterin standard for HPLC was purchased from Schircks (Jona, Switzerland).

Transcriptome analysis.

Cultures of *L. plantarum* WCFS1 strains were quenched using the cold methanol method (40). Total RNA was isolated and extracted as described before (42). The RNA concentration was determined with the ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The quality of the isolated RNA was checked using the 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA); a ratio of 23S over 16S RNA of ≥1.6 was taken as satisfactory. For cDNA synthesis, 5 μg RNA was used. Indirect labeling was performed with

the CyScribe first-strand cDNA labeling kit (Amersham, United Kingdom) according to the manufacturer's protocol. The cDNA samples were labeled with cyanine 3 or cyanine 5. After labeling, the cDNA concentration and the labeling-efficiency were determined using the ND-1000 spectrophotometer. Each microarray was hybridized with 0.5 µg labeled Cy3 and Cy5 cDNA. A total of 12 custom designed microarrays (Agilent Technologies) were used for the comparison between the *L. plantarum* harboring pNZ7021 and pNZ7026 in continuous culture. Both strains were also cultivated in pH regulated batch culture on CDM with and without *p*ABA; for this experiment 21 microarrays were used. Microarrays were hybridized and washed according to the manufactures protocol. Slides were scanned with a ScanArray Express scanner (Perkin-Elmer), using a 10-µm resolution. Images were analyzed with the ImaGene 4.2 software (BioDiscovery, Inc.).

The fraction of folate mRNAs as part of the total mRNA pool was determined as follows. First the signals from the control spots, which are needed for validation purposes, on the custom designed Agilent DNA-micro-arrays were removed from the raw data set, assuring that only 8012 *L. plantarum* probes, representing 2792 genes (91.5% of the genome), were measured. From each probe the intensity of the foreground-signal and background-signal was measured separately for Cy3 and Cy5 signals. The pure probe signal was determined by subtracting the background from the foreground signal. Total signal was determined by summing the raw probe signal of all 8012 probes, the folate signal was determined by adding-up the raw probe signals of the 18 folate probes.

Microarray hybridization scheme's was made for the continuous culture experiment and the batch experiment performed in the presence and absence of *p*ABA. The continuous culture scheme consisted of a loop design with 12 microarrays with the following samples hybridized on one array and labeled with Cy3 and Cy5, respectively: C1 and F1, F1 and C3, C3 and F2, F2 and C2, C2 and F3, and F3 and C1, C1 and C2, F2 and F1, C4 and F4, C2 and C4, F4 and F1, and F4 and C3. Here, C1, C2, C3, and C4 represent fourfold biological replicates from *L. plantarum* harboring pNZ7021. F1, F2, F3, and F4 represent fourfold biological replicates of *L. plantarum* harboring pNZ7026. The experimental scheme for the batch experiment performed with and without *p*ABA, consisted of a loop design with 21 microarrays with the following samples hybridized on one array and labeled with Cy3 and Cy5, respectively: C1+P and F1+P, F1+P and C2+P, C2+P and F3+P, F3+P and C3+P, C3+P and F2+P, F2+P and C1+P, C1+P and C2+P, F2+P and F3+P, C1-P and F1-P, F1-P and C2-P, C2-P and F3-P, F3-P and C3-P, C3-P and F2-P, F2-P and C1-P, C1-P and C2-P, F2-P and F3-P, C3-P and F1+P, F2+P and C1-P, C2+P and F3-P, F1-P and C3+P, and F2+P and F1-P. Here, C1+P, C2+P, C3+P, F1+P, F2+P, and F3+P represent threefold biological replicates of the *L. plantarum* harboring pNZ7021 and pNZ7026, respectively, when grown in batch in the presence of *p*ABA. The C1-P, C2-P, C3-P, F1-P, F2-P, and F3-P, represent the *L. plantarum* harboring pNZ7021 and pNZ7026, respectively, when grown in batch in the absence of *p*ABA.

Microarray data were analyzed as described previously (42). The statistical significance of differences was calculated from variation in biological replicated, using the eBayes function in Limma (cross-probe variance estimation) and Holmes determination of significance. Only genes with a log2 ratio of -1 and +1 and a Holmes value less than 0.1 were considered significant.

The microarray platform and microarray data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession numbers (pending).

Metabolome analysis.

The complete metabolome of *L. plantarum* WCFS1 harboring pNZ7021 and pNZ7026 from continuous cultivation, in three independent replicates, was quenched using the sodium chloride-method as described previously (14). After dissolving in water, the intracellular metabolites were profiled in an untargeted manner on a reversed phase HPLC-MS system with a high resolution accurate mass detector (QTOF Ultima MS) as described before (10). A Synergi Hydro-RP column, 250 x 2.0 mm and 4 μm pore size (Phenomenex, USA), and a gradient of 0 to 35% acetonitril in water (acidified with 0.1% formic acid) during 45 min were used to separate the metabolites. Full scan accurate mass data (m/z 80-1500) were collected in both positive and negative electrospray ionization mode, using leucine enkephalin as a lock mass. Hereafter the mass signals exceeding three times the local noise were extracted, and mass profiles of both strains were compared using MetAlign™ software (1, 10, 51). This program is designed to determine significant differences in the relative abundance of mass signals originating from metabolites. Based on their accurate masses and MS/MS fragmentation patterns, metabolites were annotated using the PubChem DB metabolite database. (www.ncbi.nlm.nih.gov).

Determining the relative copy number of the pNZ derived plasmids.

The relative copy number was determined as described previously (54).

RT-qPCR

Cells of *L. plantarum* WCFS1 cultures were quenched using the cold methanol method as described above. RNA was extracted, quantified, and checked for quality as described above. Primers were used to convert specific mRNA molecules into cDNA using a first-strand cDNA synthesis kit (Amersham, United Kingdom). In *L. plantarum* harboring pNZ8148 and pNZ7030 the following primers were used for cDNA synthesis: groES-re(2), pfk-re1, RQPCRfolBS, and RQPCRFPS. In *L. plantarum* harboring pNZ7031 the following primers were used for cDNA synthesis: groES-re(2), pfk-re1, RQPCRfolBAS, and RQPCRfPAS. The sequence of the primers can be found in Table 1. All cDNA samples were diluted 100-fold to allow accurate quantification by qPCR. Sybr Green (ABI, Cheshire, UK) was used as fluorescent dye for determining the level of amplification. For qPCR on *groES*, *pfK*, *folBS*, *folBAS*, *folPS*, and *folPAS* the following primers-sets were used: groES-fo(2) and groES-re(2), pfk-fo1 and pfk-re2, FQPCRfolBS and RQPCRfolBS, FQPCRfolBAS and RQPCRfolBAS, FQPCRFPS and RQPCRFPS, and FQPCRfPAS and RQPCRfPAS, respectively. The Critical threshold number (C_t) was determined using ABI Prism 7500 Fast Real-Time PCR system and software. The C_t value was used to calculate the relative gene expression ($N_{relative}$) using the formula $N_{relative} = 2^{((C_{tRF} - C_{tRN})-(C_{tEF}-C_{tEN}))}$. In this formula, C_{tRF} and C_{tRN} represent the C_t value in the reference strain for the folate gene and normalizing gene, respectively. C_{tEF} and C_{tEN} are the C_t value for the tested strain for the folate and normalizing gene, respectively.

SDS-PAGE and protein quantification.

Protein was isolated as described previously (6). To determine the level of protein overexpression, SDS-PAGE was performed as described previously (29). The level of protein overexpression was quantified using ImageJ (http://rsb.info.nih.gov/ij/). The program ImageJ

has a package for the conversion of protein bands into peaks, each peak can be quantified by determining the area.

Results

Folate production in the absence of *p*ABA

Previously it was shown that the homologous overexpression of the folate gene cluster of *L. plantarum* results in large production of folate. Remarkably, these increased folate production pools were only obtained when *para*-aminobenzoic acid was supplied to the growth medium (54). Here we determine which pterin intermediate of the folate biosynthesis pathway accumulates when the folate biosynthesis pathway is blocked upon the overexpression of the folate gene cluster in the absence of *p*ABA. *L. plantarum* harboring pNZ7021 (control vector) and pNZ7026 (folate gene cluster overexpression plasmid) were cultivated in batch in the presence and absence of *p*ABA. Folate and pterin pools were analyzed by the microbiological assay and HPLC in the intra- and extracellular fractions, respectively (Table 2).

TABLE 2. Intracellular and extracellular concentration of 6-hydroxymethylpterin and folate in *L. plantarum* harboring pNZ7021 and pNZ7026 in the presence and absence of *p*ABA.

L. plantarum harboring	μ_{max} h^{-1}	6-Hydroxymethylpterin (nmol/50-ml culture)		Folate µg/L per OD$_{600}$ unit	
		Intracellular	Extracellular	Intracellular	Extracellular
pNZ7021	0.61 (0.02)	0.1	ND[a]	ND[a]	ND[a]
pNZ7021 + *p*ABA	0.60 (0.02)	0.1	ND[a]	3.93 (1)	8.56 (3)
pNZ7026	0.45 0.02)	3.0	1692	ND[a]	ND[a]
pNZ7026 + *p*ABA	0.44 (0.01)	0.2	217	216 (29)	3020 (202)

[a] ND, not detectable

Note: The standard deviation of the folate assay is shown between brackets**.**

High intracellular pterin pools were only detected in *L. plantarum* harboring pNZ7026 in the absence of *p*ABA. The principal pterin was identified as 6-hydroxymethylpterin from its chromatographic properties, and was detected extra- and intracellularly. In the folate biosynthesis pathway, 6-hydroxymethylpterin (in its dihydroform) is activated by pyrophosphorylation and then condensed with *p*ABA to form dihydropteroate, which is then glutamylated to yield folate. In conclusion, *L. plantarum* WCFS1 is unable to convert 6-hydroxymethylpterin into folate in medium lacking *p*ABA.

Previously, it was found that the growth rate of *L. plantarum* harboring pNZ7026 was found to be 25% reduced, when compared to *L. plantarum* harboring pNZ7021 in the presence of *p*ABA (54) It remained unclear, however, whether high folate pools are of influence on the growth rate reduction. Therefore the growth rate was determined for both strains in the presence and absence of *p*ABA (Table 2). Remarkably, the growth rate was reduced regardless whether *p*ABA was added to the medium, showing that high folate pools itself do not explain the growth rate reduction.

Metabolite formation upon folate overproduction.

From the folate production pools (Table 2) it can be seen that there is almost 60-fold more folate in *L. plantarum* harboring pNZ7026 when compared to the control strain. Next, a differential metabolomics method was used to determine whether specific metabolites differed significantly in pools size between *L. plantarum* harboring pNZ7021 and pNZ7026. Both strains were cultivated in continuous culture in the presence of *p*ABA, at steady state cells were harvested, quenched and extracted for metabolome analysis by LC-MS/MS. In total 18 metabolites with differential abundance were detected (see Table 3).

TABLE 3: Metabolites that differ significantly in relative abundance between *L. plantarum* WCFS1 harboring pNZ7026 and pNZ7021. The table shows the putative compound name, the relative abundance of the metabolite, apparent mass of the compound, and the deviation of the apparent mass compared to the expected mass.

Putative compound name	ratio pNZ7026/pNZ7021	apparent mass [M+H]$^+$	Ppm Δ mass
10-formyl folate	117.2	470.1431	2.6
10-formyl folate isomer	33.6	470.1493	15.8
Novel $C_{17}H_{14}O_3$	20.4	267.1007	-5.3
Novel folate $C_{24}H_{23}N_7O_5$	19.4	490.1796	-7.9
1-[(2-methoxyphenyl)methyl]-5-nitro-2H-indazol-3-one	11.6	300.1000	7.1
$C_{20}H_{22}N_5O_2S$	5.7	396.1491	0.3
Unidentified	5.7	728.2331	
2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic methyl ester	4.9	222.0674	23.5
Unidentified	3.8	254.0952	
Adenosine	2.8	268.1066	-2.8
$C_{18}H_{32}O_{16}$	2.6	505.1852	17.4
5-methylthioadenosine	2.4	298.1034	10.9
$C_4H_{10}N_4OS$	2.2	163.0651	1.09
$C_{12}H_{27}N_7O_{14}P_2$ e.g. nicotinamide arabinoside adenine dinucleotide	2.1	556.116	12.8
10-formyltetrahydrofolate	2.1	474.1813	7.3
Thymidine	0.6	243.0938	9.4
$C_{10}H_{14}N_6O_5$ e.g. 1-amino guanosine	0.6	299.1132	15.1
3-dehydroshikimate	0.5	173.0471	14.3

Of this group, 15 metabolites were significantly more abundant in *L. plantarum* harboring pNZ7026 and 3 metabolites were significantly less abundant. Five of the 15 metabolites, that were more abundant in *L. plantarum* harboring pNZ7026, could be linked directly to folate biosynthesis. The metabolite assigned as 10-formyl folate (Fig. 1a) showed the largest difference in relative abundance; this molecule was 117-fold more abundant in *L. plantarum* harboring pNZ7026 as compared to the control strain (pNZ7021). We also detected a 33-, 19- and 2.1-fold increase in abundance of a 10-formyl folate isomer, of a novel folate with a chemical formula of $C_{24}H_{23}N_7O_5$ (Fig. 1b) (this molecule appears structurally related to folate, and has the following systematic name 1-[(4-{[(2,4-diaminopyridino[2,3-d]pyrimidin-6-

yl)methyl]amino}naphthyl) carbonylamino]propane-1,3-dicarboxylic acid), and 10-formyl tetrahydrofolate (Fig. 1c), respectively. One metabolite, 2-amino-1,4-dihydro-4-oxo-6-pteridinecarboxylic methyl ester, is a known breakdown product of folate. When folate is exposed to light it decomposes into the latter compound and 2-amino-4-hydroxypteridine (53). The other 10 metabolites can not be linked directly to the folate biosynthesis pathway and their involvement remains to be investigated. Only 3 metabolites were present in a significantly lower abundance (less than 2-fold) in *L. plantarum* harboring pNZ7026; these components were putatively annotated as thymidine, 3-dehydroshikimate and 1-amino guanosine. In conclusion, the overexpression of the folate gene cluster leads to a massive accumulation in 10-formyl folate and other folate related metabolites. However, the global impact of folate overproduction on metabolite accumulation is relatively low with only 18 significantly differently abundant metabolites.
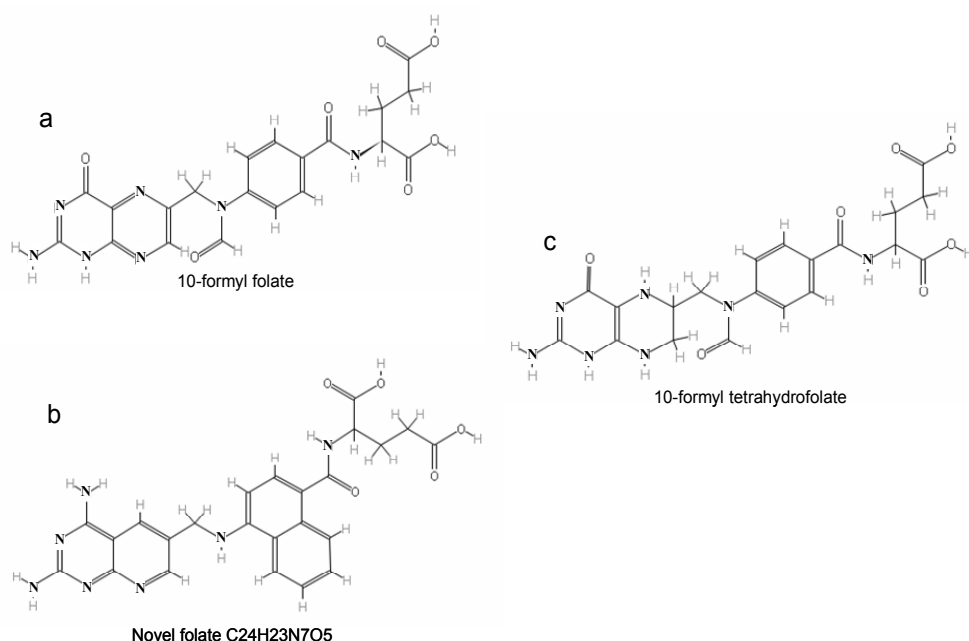
FIG. 1: The structure of 10-formyl folate (a), a novel folate (b) and 10-formyl tetrahydrofolate (c).

Transcriptional profiling of folate overproducing cells.

Using DNA microarrays it was analyzed whether the presence of high intracellular folate pools results in differentially expressed genes. We have selected two different cultivation conditions (continuous and batch culture) to make a distinction between high folate pools specific gene expression and to secondary effects of the overexpression of the folate gene cluster, e.g. differences in growth rate. It is expected that any similarity in gene expression between both cultivation conditions is due to the production of folate or the high folate pools. All genes which are significantly up- or down-regulated are presented in Table 4. From this table it can be seen that the only genes that were differentially expressed both in batch and continuous culture are the 6 genes of the folate biosynthesis cluster. Because these

genes were constitutively overexpressed on a high copy plasmid, the observed response is obviously expected. This analysis shows that high folate pools or the elevated synthesis of folate does not lead to a global transcriptional response. Instead, it was found that 8 and 11 genes responded specifically to secondary effects of the overexpression of the folate cluster in continuous and batch culture, respectively (Table 4).

TABLE 4: Overview of genes that are differentially expressed in the *L. plantarum* strain harboring pNZ7026 when compared to the control strain (pNZ7021).

| Synonyms | Sub class | Continuous culture | | Batch culture | |
		log2 ratio[a]	Holmes sign.	log2 ratio	Holmes sign.
rhe1	ATP dependent RNA helicase	0.38	1.00	1.59	0.07
mtsC	Cations	-1.33	0.00	0.01	1.00
mtsB	Cations	-1.43	0.00	0.02	1.00
mtsA	Cations	-1.46	0.00	0.07	1.00
pyrP	Nucleoside, purines and pyrimidines	-0.19	1.00	-1.89	0.02
Lp_2696	Conserved: membrane proteins	-0.04	1.00	-1.32	0.07
pyrE	Pyrimidine ribonucleotide biosynthesis	-0.15	1.00	-2.84	0.07
pyrF	Pyrimidine ribonucleotide biosynthesis	-0.14	1.00	-2.73	0.01
pyrD	Pyrimidine ribonucleotide biosynthesis	-0.14	1.00	-2.75	0.00
pyrAB	Pyrimidine ribonucleotide biosynthesis	-0.11	1.00	-2.74	0.00
pyrC	Pyrimidine ribonucleotide biosynthesis	-0.14	1.00	-3.04	0.00
pyrB	Pyrimidine ribonucleotide biosynthesis	-0.08	1.00	-2.88	0.00
pyrR1	Other	0.06	1.00	-2.01	0.00
ansB	Glutamate familiy	0.16	1.00	1.37	0.08
mntH2	Cations	-1.28	0.00	-0.45	1.00
folP	Folate biosynthesis	5.50	0.00	5.86	0.00
Xtp2	Folate biosynthesis	5.31	0.00	6.28	0.00
folC2	Folate biosynthesis	5.85	0.00	6.44	0.00
folE	Folate biosynthesis	5.36	0.00	6.41	0.00
folK	Folate biosynthesis	5.80	0.00	6.47	0.00
folB	Folate biosynthesis	5.54	0.00	6.21	0.00
Lp_3412	Cell surface proteins: other	1.53	0.01	0.28	1.00
Lp_3413	Cell surface proteins: other	1.93	0.00	0.37	1.00
Lp_3414	Cell surface proteins: other	2.10	0.00	0.43	1.00
Lp_3415	Other	1.17	0.00	0.72	0.72

[a] Negative and positive numbers indicates down-regulation and up-regulation of genes, respectively.

In continuous culture the 8 differentially expressed genes are involved in cation uptake or belong to a cell surface cluster which is predicted to be involved in the uptake of complex carbohydrates (44). The biological relevance of down-regulation of these genes is unclear. In the batch experiment a total of 11 genes were significantly regulated upon the overexpression of the folate gene cluster. One gene cluster, involved in pyrimidine biosynthesis, appears to

respond specifically to the growth rate reduction (Table 4). Remarkably, this gene cluster was also down-regulated when the folate gene cluster was overexpressed in the absence of *p*ABA (data not shown). The pyrimidine biosynthesis gene cluster is composed of 9 genes, from lp_2697 (*pyrE*) until lp_2704 (*pyrR1*), including a gene upstream of the pyrimidine gene cluster, lp_2696 and a pyrimidine transporter *pyrP*, lp_2371. Two additional genes, *ansB* and *rhe1*, are up-regulated upon the overexpression of the folate gene cluster in batch culture. AnsB (E.C. 3.5.1.1) is involved in the conversion of L-aspargine into L-aspartate. Rhe1 is involved in the unwinding of RNA-helices. The biological relevance of the differential expression of these genes under those conditions remains unclear. From these experiments it can be concluded that the reduced growth rate does not trigger a large transcriptional response, instead a few genes could potentially be linked to the growth rate reduction. Moreover, none of the genes of *L. plantarum* appears to respond specifically to high folate pools, or the increased biosynthesis of folate.

Determination of causes for the reduced growth rate.

Functional genomics tools such as transcriptomics and metabolomics showed that folate overproduction as such has a low impact on global transcription and metabolite formation, whereas growth is severely reduced. From these functional genomic approaches several leads were investigated to explain or eliminate causes for the reduced growth rate; i) determining the drain of GTP precursor for folate production, ii) cost for mRNA synthesis and plasmid synthesis, iii) increased pools of mRNA and/or protein of the transcription/translation machinery.

The drain of folate production on GTP pools.

Apart from being a precursor in folate biosynthesis, GTP is also used for the synthesis of DNA, RNA and proteins. The drain on the GTP-pool due to excessive folate production is calculated in *L. plantarum* harboring pNZ7026. Based on the biomass composition of *L. plantarum* WCFS1 (50), it was determined that 0.10 mmol/g dry weight (DW) GTP is stored in DNA and RNA. In *L. plantarum* harboring pNZ7026 approximately 0.04 mmol/g DW GTP is stored in folate. In view of the fact that in *L. lactis* the free GTP pool is 0.55 mM (39) and taking into account the internal bacterial cell volume, the free GTP pool is assumed to be in the order of magnitude of 10^{-6} mol/g DW and therefore negligible. Based on these numbers it was estimated that about 30% of the GTP in *L. plantarum* harboring pNZ7026 is directed into folate (or pterins). For *L. plantarum* harboring pNZ7021 this is less than 0.03%. Surprisingly, the large drain on GTP did not provoke a transcriptional response with respect to purine expression in *L. plantarum* harboring pNZ7026. In addition, from the differential metabolomics experiment it was observed that 1-amino guanosine was less abundant in *L. plantarum* harboring pNZ7026, suggesting that this might be a result of the high drain of converting GTP into folate. The large flux of GTP into folate might be a cause in the reduced growth rate of the folate gene cluster overexpressing strain .

Effect of increased mRNA and plasmid production on the growth rate.

It was determined whether the growth rate reduction could be explained by increased mRNA or plasmid production. From the microarray analysis (Table 4) it was found that the folate genes (a total of 18 probes on the microarray) are the highest expressed genes on the entire microarray. In *L. plantarum* harboring pNZ7021 and pNZ7026 the folate mRNAs are

on average 0.16% and 8.3% of the total mRNA pool, respectively, showing that the constitutive overexpression of the folate gene cluster leads to a massive accumulation of folate mRNAs. This high level of mRNA production might be a cause as well in the growth rate reduction of *L. plantarum* harboring pNZ7026. Moreover, another factor that could explain the growth rate reduction is the difference in size of pNZ7021 and pNZ7026, with 3.3 and 7.7 Kb, respectively, resulting in a higher specific plasmid synthesis cost. The growth performance, mRNA synthesis and plasmid copy numbers were determined for *L. plantarum* harboring pNZ8148 (empty vector), pNZ7030 (folate gene cluster in sense orientation downstream of the nisin inducible promoter P_{nisA}) and pNZ7031 (folate gene cluster in antisense orientation downstream of the nisin inducible promoter P_{nisA}). Using these strains we can distinguish between the effect of mRNA synthesis alone (*L. plantarum* harboring pNZ7031) and the combined effects of synthesis of mRNA and protein synthesis (*L. plantarum* harboring pNZ7030). *In silico* analysis using MEME and MAST predicted no putative functional ribosome binding sites on the folate gene cluster in the antisense orientation. The growth rate and the folate pools produced are presented in Table 5. The growth rate of *L. plantarum* harboring pNZ7030 was reduced regardless whether cells were induced with nisin or not. However, the growth rate of *L. plantarum* containing pNZ8148 and pNZ7031 was unaffected.

TABLE 5: Growth rates, and folate pools in the uninduced and induced cell culture of *L. plantarum* harboring pNZ8148, pNZ7030, and pNZ7031.

L. plantarum	0 ng/ml nisin		25 ng/ml nisin	
Harboring	Folate µg/L per OD_{600} unit	μ_{max} h^{-1}	Folate µg/L per OD_{600} unit	μ_{max} h^{-1}
pNZ8148	6 (0.6)	0.40 (0.04)	6 (0.4)	0.369 (0.01)
pNZ7030	783. (63)	0.31 (0.02)	1736 (211)	0.24 (0.03)
pNZ7031	35 (3)	0.41 (0.02)	31 (4)	0.44 (0.01)

Note; standard deviation is give between brackets.

By RT-qPCR it was determined that *L. plantarum* strains harboring pNZ7030 and pNZ7031 were able to produce the anticipated mRNAs (see Table 6). The relative expression level in *L. plantarum* harboring pNZ8148 is arbitrarily set at 1 and the gene expression values in the two other strains were related to this strain. The overexpression of the folate genes in the sense and antisense orientations resulted in a vast increase in the expected mRNAs, but only in *L. plantarum* harboring pNZ7030, a reduced growth rate was observed, suggesting that the mRNA production itself is not responsible for the growth impairment. The relative plasmid copy number of *L. plantarum* harboring pNZ8148, pNZ7030 and pNZ7031 before and after nisin induction is shown in Table 7. What is obvious from this analysis (see table 7) is that relative copy number varies between the different constructs.

TABLE 6: Relative expression of *folB* and *folP* in *L. plantarum* harboring pNZ8148, pNZ7030, and pNZ7031 after 20 minutes and 4 hours following nisin induction and in the uninduced cultures. Expression values of the two folate genes, *folB* and *folP*, are normalized to *groES*, and are indicated as average expression *folB-folP*

time minutes	*L. plantarum* harboring	0 ng/ml nisin Average expression *folB-folP*	25 ng/ml nisin Average expression *folB-folP*
20	pNZ8148 sense	1	1
20	pNZ7030 sense	64	584
20	pNZ7031 antisense	84	2864
240	pNZ8148 sense	1	1
240	pNZ7030 sense	1	38
240	pNZ7031 antisense	3	11

TABLE 7: Relative copy number for pNZ8148, pNZ7030, and pNZ7031 in *L. plantarum* determined in the induced and uninduced cultures.

L. plantarum harboring	0 ng/ml nisin Relative copy number	25 ng/ml nisin Relative copy number
pNZ8148	218 (2)	228 (17)
pNZ7030	2245 (197)	801 (51)
pNZ7031	2058 (171)	387 (17)

Note: The standard deviation is presented between brackets, and is calculated from two independent measurements.

The strain with the highest copy number is *L. plantarum* harboring pNZ7030, suggesting that increased plasmid synthesis could explain the growth rate reduction. However, a 5-fold increase in relative copy number for *L. plantarum* harboring pNZ7031 in the induced and uninduced condition did not result in a growth rate reduction, showing that relative copy numbers may vary between strains, but increased synthesis of plasmid does not result in a reduced growth rate. Therefore, we conclude that the growth rate reduction is not caused by the increased cost for mRNA or plasmid synthesis.

Analysis of mRNA and protein pools upon overexpression of the folate gene cluster.
Another explanation for the growth rate reduction by the overexpression of the folate gene cluster might be competition for the transcription/translation machinery by synthesis of useless transcripts and proteins. In *L. plantarum* harboring pNZ7026 it was determined that the transcripts derived from the folate genes constitute 8.3% of the total mRNA pool. Since the growth rate of *L. plantarum* harboring pNZ7030 was also reduced, the same analysis was performed on the mRNA pools of this strain. It was estimated that the folate specific mRNA pool constitutes 33% of the total mRNA pool. Consequently, the overexpression of the folate gene cluster results in an enormous accumulation of folate specific mRNA's.

Also, the folate protein content was determined by SDS-PAGE for *L. plantarum* harboring pNZ7021, pNZ7026, pNZ8148, pNZ7030, and pNZ7031 (in pNZ8148, pNZ7030, and pNZ7031 with and without nisin induction) (Fig. 2).
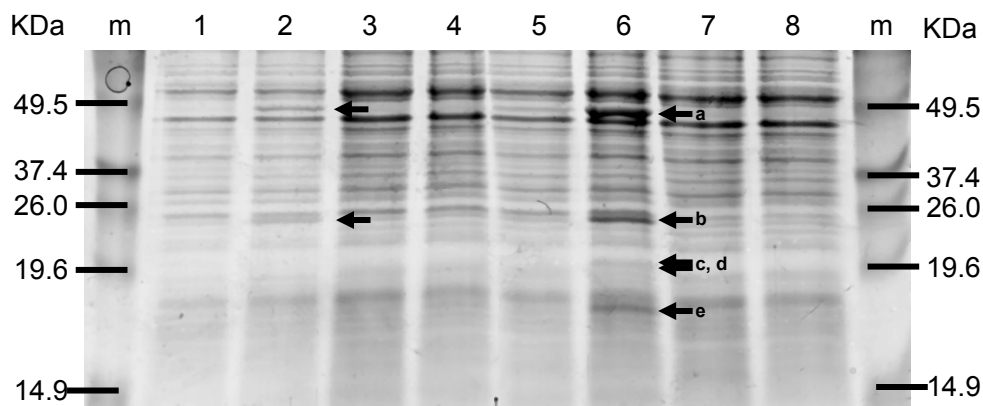
FIG. 2: SDS-PAGE gel showing a standard protein marker with the indicated molecular weights (in kDa) on both outside lanes of the gel. Lane 1 till 8 shows the protein content of *L. plantarum* harboring pNZ7021, pNZ7026, pNZ8148 (0 ng/ml nisin), pNZ8148 (25 ng/ml nisin), pNZ7030 (0 ng/ml nisin), pNZ7030 (25 ng/ml nisin), pNZ7031 (0 ng/ml nisin) and pNZ7031 (25 ng/ml nisin), respectively. In lane 6 five bands are indicated as: a) (FolC2, 50.4 KDa), b) (FolP, 29.2 KDa), c) (Xtp2, 21.7 KDa), d) (FolE, 21.0 KDa), and e) (FolK, 18.9 KDa). In lane 2 the bands a) (FolC2, 50.4 KDa) and b) (FolP, 29.2 KDa) were detected.

The protein band patterns on the SDS-PAGE gel were quantified using ImageJ. The total area under the peaks (total protein content) and the area under the peaks of folate biosynthesis proteins were determined. Clear folate protein peaks could be distinguished in the *L. plantarum* harboring pNZ7030 that matched with the expected protein sizes (5 of the 6 proteins were detected, 1 protein was too small to be detected), in *L. plantarum* harboring pNZ7026 the two largest proteins were identified (Fig. 2). The folate protein content in *L. plantarum* harboring pNZ7021, pNZ8148 and pNZ7031 were set at 0% folate proteins. In the *L. plantarum* containing pNZ7026 and pNZ7030 (after nisin induction) the folate proteins constitute 4 and 10% of the total protein pool, respectively. Another factor that can be of influence on the growth rate reduction is the codon usage upon the overexpression of the folate gene cluster. It was found that three codons (tRNA^{Arg} (AGG), tRNA^{Cys}, (UGC), and tRNA^{Ile} (AUA)) were 5-fold less abundant in the genome when compared to the folate gene cluster (data not shown). From this it can be suggested that the availability of these rare codons combined with the high level of folate specific transcripts and enzymes can indeed have a large effect on the transcription/translation machinery. It appears that the transcription/translation machinery proceeds normally, however, the production of these useless mRNA's and proteins dilutes the synthesis of growth related mRNA's and proteins. This observation matches well with the lack of response from the DNA microarrays.

Discussion

Previously a folate-overproducing strain was constructed that was dependent on supplemented *p*ABA in order to make folate in high amounts (54). In the absence of *p*ABA, the folate pathway appeared to be blocked, resulting in accumulation of 6-hydroxymethylpterin. Based on the topology of the folate biosynthetic pathway it was expected that 6-hydroxymethyl-7,8-dihydropterin pyrophosphate would accumulate, since the

latter component is the metabolite that condenses with *p*ABA to make pterorate. However, the HPLC system that was used could not detect these phosphorylated pterins since they elute in the void volume. The pterin, 6-hydroxymethylpterin, was also found to accumulate in tomatoes upon overexpression of GTP cyclohydrolase I (12, 23). In these experiments it was observed that availability of *p*ABA limits folate overproduction. The combined overexpression of GTP cyclohydrolase I with the *p*ABA precursor aminodeoxychorismate synthetase led to a 25-fold increase in folate production (13). The large efflux of 6-hydroxymethylpterin into the medium might be caused by leakage. This leakage can be prevented when glutamate is attached to this molecule. Sybesma *et al*. (46) described that leakage of folates can be prevented progressively by addition of multiple glutamate moieties to the folate molecule.

The metabolomics data indicated that only a few metabolites were significantly affected in abundance in *L. plantarum* harboring pNZ7026. One metabolite, 10-formyl folate, was 117-fold more abundant in *L. plantarum* harboring pNZ7026. This was unexpected since it is assumed that the reduced derivative, 10-formyl tetrahydrofolate, is made by *L. plantarum* harboring pNZ7026. In *L. lactis,* for example, using HPLC with PDA detection, 10-formyl tetrahydrofolate was detected as the most dominant type of folate (47). Since tetrahydrofolate derivatives are known to be unstable (9, 35, 36) this component may have been converted to the oxidized form (folate) in the bacterial cells or during metabolite extraction or LC-MS analysis. It is known that 10-formyl folate is biological inactive, (5). However, it is demonstrated that 10-formyl folate can be used by the indicator strain in the microbiological assay, henceforth the growth of this strain on the high folate production levels produced by *L. plantarum* harboring pNZ7026.

It was predicted that 30% of the synthesized GTP is directed into folate, suggesting that the growth rate reduction can be caused by GTP shortage. This high drain on GTP pools can also have implication for protein synthesis, since GTP hydrolysis for protein synthesis alone accounts for more than 32% of the energy turnover of a lactic acid bacterium (8, 26). From the transcriptome analysis, however, no differential expression of the purine genes was observed, suggesting that either there is no GTP shortage, or GTP shortage does not provoke a transcriptional response to the purine genes. In other bacteria the free pool of GTP is considered to be linked with the growth rate. In *Bacillus subtilis* there is a positive correlation between free GTP pools and the growth rate of this strain (31). The GMP-synthetase inhibitor, decoyinine, could reduce the free GTP pool 2-fold, thereby reducing the growth rate of *L. lactis* (39). We were unable to detect a reduced relative abundance of GMP, GDP or GTP in our metabolome analysis. The only metabolite that could be linked to GTP shortage is 1-amino guanosine. However, it remains unclear whether this component can be phosphorylated, since not many nucleoside kinases are known in lactic acid bacteria (26, 32).

It was found that high folate pools or the increased production of folate itself did not lead to expression of other genes than the folate genes itself. However, the overexpression of the folate gene cluster results in the regulation of several genes, which appear to be caused by secondary effect of the overexpression of the folate gene cluster. The low transcriptional response to a 25% growth rate reduction is quite remarkable. One gene cluster that reacts specifically to the reduced growth is the pyrimidine gene cluster. It is known that the pyrimidine gene cluster is down-regulated in the presence of high CTP pools (24), however, we have not measured the pool sizes of this metabolite in batch culture. The reduced growth rate of *L. plantarum* harboring pNZ7026 suggests a kind of stress, but besides the down-

regulation of the pyrimidine gene cluster no generic stress response was provoked. Applying stress to a microorganism often leads to slower growth. In *E. coli*, for example, the transcriptional response was determined in a strain carrying a plasmid for overexpression of chloramphenicol acetyltransferase in comparison with a wild-type strain (19). From this experiment it was evident that overexpression of chloramphenicol acetyltransferase provoked stress to the cell, as indicated by the large number of stress-response and growth related genes that were differentially expressed. This is clearly different from the data presented here. One possible explanation is that we have compared the gene expression of two plasmid bearing strains. Metabolic burden is associated with the production of specific proteins which lead to a reduction in growth rate (17, 30). In *E. coli* it was demonstrated that the amount of ribosomes capable of protein synthesis is dependent on the growth rate (20). At a growth rate of $1.2.h^{-1}$ it was found that 70% of the ribosomes were actually synthesizing proteins, showing that most of the ribosomes are translating mRNA into protein. From this point it can be reasoned that if the fixed amount of ribosomes are faced with 8% gratuitous mRNA, all mRNAs are translated into proteins, leading to dilution of the growth related proteins. Since the process of transcription and translation is tightly coupled it is impossible to distinguish between mRNA or protein burden. Another factor involved in the growth rate reduction in some organisms is the demand for rare codons in the protein to be synthesized. In *E. coli* it was observed that the overexpression of tryptophanase (EC 4.1.99.1) resulted in a growth rate reduction mainly because it led to a shortage of a specific tRNA (16). Three rare codons are over-represented in the folate gene cluster of *L. plantarum*.

Overexpression plasmids are often used for the synthesis of commercially interesting components (i.e. proteins or metabolites). The impact of overproduction of these components on the overall performance of the cell is not very well understood. The data obtained from functional genomics studies can help to elucidate these effects. However, it appears to be crucial for long-term stable overproduction phenotypes that the growth rate reduction caused by the overproduction is minimized. To achieve this it is important to gain insight in the causes of this growth rate reduction.

Acknowledgement

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Chapter 8

Development of a Minimal
Growth Medium for
Lactobacillus plantarum WCFS1

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Abstract

A medium was developed delivering the minimal requirements for growth of *Lactobacillus plantarum* WCFS, this was done by repetitive single omission experiments. The biomass yield on this minimal medium (Plantarum Minimal Medium; PMM5) did not exceed 35 mg/L dry weight (DW) (equivalent to OD_{600} of 0.1). The addition of pyridoxamine, cysteine and ammonium citrate to PMM5 increased the final biomass yield to 350 mg/L DW (OD_{600} of 1.0). This medium was named PMM7. The growth performance of *L. plantarum* on PMM5 and PMM7 were compared to established growth media (Chemically Defined Medium (CDM) and MRS). The specific growth rate of *L. plantarum* on PMM7 was found to be 50% and 63% lower when compared to growth on CDM and MRS, respectively. Using a genome-scale metabolic model of *L. plantarum*, it was predicted that PMM7 would not support growth since several vitamins and also the biosynthesis of *para*-aminobenzoic acid were predicted to be essential. The discrepancy in simulated growth and experimental growth on PMM7 was further investigated. The growth performance and folate production capabilities were determined on PMM7 in the presence and absence of *p*ABA. It was found that a 12,000- fold reduction in folate pools exerted no influence on formation of biomass or growth rate of *L. plantarum* cultures, showing that high folate pools are not essential for growth.

Introduction

Mankind has used lactic acid bacteria, such as Lactobacilli and Lactococci, for thousands of years, principally for preventing spoilage of food products like meat, vegetables, fruits, and dairy products. Lactic acid bacteria convert carbohydrates rapidly into mainly lactic acid but also other organics acids, which cause acidification of the food product. Prolonged cultivation of these bacteria in industrial settings resulted in adaptation to these new niches. During this adaptation period genes may be gained or lost. It has been suggested that virulence factors, which are abundant in many Streptococci, were lost or inactivated during prolonged propagation of *Streptococcus thermophilus* on milk (2). Analysis of the genetic diversity between two dairy strains and one plant-derived *Lactococcus lactis* strain, showed that several genes needed for growth on plants were absent in dairy species (28, 31). In general, lactic acid bacteria have multiple amino acid and vitamin auxotrophies, and it is believed that evolution on rich and complex media have developed specific auxotrophies which are closely correlated to their biotope (3). The lactic acid bacterium *Lactobacillus plantarum* WCFS1 was the first Lactobacillus for which the genome became publicly available (11). In addition, this strain can ferment several types of complex-media ranging from plant material (24) to liquid media like MRS (5), or Chemically Defined Medium (CDM) (25). CDM contains a total of 18 amino acids, 10 vitamins, 7 nucleobases/nucleosides, glucose, phosphate buffer, acetate, citrate, ascorbic acid and a total of 9 minerals (16, 18, 19). Using this medium it was established that *L. plantarum* WCFS1 has a large number of auxotrophies with respect to the requirement for amino acids and vitamins (25). Some of these auxotrophies were also established for *L. plantarum* ATCC 8014 by using a minimal medium with respect to amino acids and vitamins (14). Noteworthy, the latter species is distinct from *L. plantarum* WCFS1 (NCIMB8826), showing that there seems to be a discrepancy between the minimal medium designed by Morishita *et al* (14) and the predicted minimal growth

requirements for *L. plantarum* WCFS1. In fact, the amino acid and vitamin requirements of several *L. plantarum* species may vary remarkably for different subspecies (14, 20, 25).

In this paper we describe the minimal requirements for growth of *L. plantarum* WCFS1. Moreover, based on the composition of minimal medium an experimentally suitable medium was developed. The biomass-yield and growth rate on these media were compared to existing media for *L. plantarum*. The genome-scale metabolic model for *L. plantarum* was used to predict growth behavior on the developed media. The results of these predictions were used to investigate the ability to produce folate in the absence of *p*ABA on the minimal medium.

Materials and methods

Bacterial strains and medium composition.

Cultivation of *Lactobaciilus plantarum* WCFS1 harboring pNZ7021 (empty vector) was performed at 37°C on MRS, Chemically Defined Medium (CDM) (16, 18, 19), and PMM based media developed in this paper. CDM contains the following medium components: 1 g/L $K_2HPO_4 \cdot 1\ H_2O$, 5 g/L $KH_2PO_4 \cdot 1\ H_2O$, 1 g/L sodium acetate, 0.6 g/L ammonium citrate, 11 g/L glucose·1 H_2O, 0.5 g/L ascorbic acid, 0.25 g/L L-tyrosine, 0.001 g/L Ca-(D)-(+)-pantothenate, 0.0025 g/L D-biotin. 0.001 g/L 6,8-thiotic acid, 0.001 g/L nicotinic acid, 0.005 g/L pyridoxamine HCl, 0.002 g/L pyridoxine HCl, 0.001 g/L riboflavin, 0.001 g/L thiamine HCl, 0.001 g/L vitamin B12, 0.005 g/L inosine, 0.005 g/L thymidine and 0.005 g/L orotic acid, 0.2 g/L $MgCl_2 \cdot 6\ H_2O$, 0.05 g/L $CaCl_2 \cdot 2\ H_2O$, 0.016 g/L $MnCl_2 \cdot 4\ H_2O$, 0.003 g/L $FeCl_3 \cdot 6\ H_2O$, 0.005 g/L $FeCl_2 \cdot 4\ H_2O$, 0.005 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.0025 g/L $CoSO_4 \cdot 7\ H_2O$, 0.0025 g/L $CuSO_4 \cdot 5\ H_2O$, 0.0025 g/L $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 0.24 g/L alanine, 0.125 g/L arginine, 0.42 g/L aspartic acid, 0.13 g/L cysteine HCl, 0.50 g/L glutamic acid, 0.175 g/L glycine, 0.15 g/L histidine, 0.21 g/L isoleucine, 0.475 g/L leucine, 0.44 g/L lysine, 0.125 g/L methionine, 0.275 g/L phenylalanine, 0.675 g/L proline, 0.34 g/L serine, 0.225 g/L threonine, 0.05 g/L tryptophan, and 0.325 g/L valine, 0.01 g/L guanine, 0.01 g/L adenine, 0.01 g/L uracil, and 0.01 g/L xanthine. When alterations on the designed media were made, the components were either removed or re-added in the concentrations described above.

Cultivation conditions

The *L. plantarum* WCFS1 strain was cultivated routinely in batch culture without specific precautions. When indicated, special measures were taken to compare the effect of air and strict anaerobic conditions on growth of *L. plantarum*. Prior to inoculation, the precultures were washed twice with 0.85% sodium chloride. To prevent carryover of medium components *in situ* the cultures were first pre-grown in the desired media and subsequently propagated on a medium with the same composition. Independent from the final optical density, the growth medium was always inoculated with 0.2% (v/v) washed culture. The growth rates were determined in 96 wells microtiter plates by turbidity measurements at 600 nm using the Spectra Max384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A plasmid-bearing *L. plantarum* WCFS1 strain (pNZ7021) was used for inoculation of all

media, to prevent contamination. To assure plasmid maintenance a chloramphenicol concentration of 20 mg/L was added to each medium.

Growth prediction on the developed media using genome-scale metabolic models

  Recently, a genome-scale metabolic model for *L. plantarum* was developed (26). The model was developed within the Simpheny^{TM} software environment (Genomatica Inc., San Diego, CA, USA). The model was used to predict and simulate growth of *L. plantarum* on CDM and the other developed media (PMM).

Folate analyses.

  Folate was quantified using a microbiological assay on samples which were pretreated with γ-glutamyl carboxypeptidase (E.C.3.4.19.9) for deconjugation of polyglutamate tails (9, 22). Determination of folate levels on 500-fold concentrated biomass extracts were performed as described previously (29).

Bacterial strain identification

  Diluted aliquots of overnight culture of *L. plantarum* WCFS1 harboring pNZ7021 were plated on MRS plates. After overnight growth at 37°C, two clones per plate were checked by Random Amplification of Polymorphic DNA (RAPD)-analysis. The RAPD analysis was performed as described previously (12).

Results

Development of a minimal medium for *L. plantarum* WCFS1.

  Recently, the auxotrophies of *L. plantarum* WCFS1 were determined based on single omissions experiments (see Table 1) (25).

TABLE 1: Final OD_{600} of the developed media upon single omission.

Name of media	CDM	PMM3	PMM5
	OD_{600}	OD_{600}	OD_{600}
All components (indicated in white below)	2.56	1.85	0.10
Single omission	CDM	PMM3	PMM5
Basic medium			
Phosphate	ND	ND	0.01
Glucose	ND	ND	0.03
Sodium acetate	ND	ND	0.01
Ammonium citrate	ND	ND	
Ascorbic acid	ND	ND	ND
Amino acids	CDM	PMM3	PMM5
Alanine	2.18		
Arginine	0.70	0.14	0.01
Aspartic acid	2.29		
Cysteine	1.48	0.99	
Glutatmate	0.04	0.01	0.00

Amino acids	CDM	PMM3	PMM5
Glycine	2.30		
Histidine	1.76		
Isoleucine	0.23	0.35	0.00
Leucine	0.03	0.01	0.00
Lysine	2.38		
Methionine	0.25	0.24	0.01
Phenylalanine	0.06	0.00	0.01
Proline	2.32		
Serine	2.33		
Threonine	2.62	0.01	0.01
Tryptophane	0.07	0.00	0.00
Tyrosine	0.13	ND	0.03
Valine	0.04	0.00	0.00
vitamins	CDM	PMM3	PMM5
6,8-thiotic acid	2.09		
Biotin	2.27	0.96	
Nicotinic acid	0.08	0.01	0.00
Panthothenic acid	0.06	0.01	0.00
Para-aminobenzoic acid	2.00		
Pryidoxamine	1.59	0.95	
Pyridoxine	2.36		
Riboflavin	0.07	0.00	0.00
Thiamine	2.10		
Vitamin B12	2.30		
Purines	CDM	PMM3	PMM5
Adenine	2.43		
Guanine	2.38		
Inosine	2.27		
Xanthine	2.10		
Pyrimidines	CDM	PMM3	PMM5
Orotic acid	2.71		
Thymidine	2.78		
Uracil	2.28		
minerals	CDM	PMM3	PMM5
$MgCl_2$	ND	0.01	0.01
$CaCl_2$	ND		
$MnCl_2$	ND	0.01	0.02
$FeCl_3$	ND		
$FeCl_2$	ND		
$ZnSO_4$	ND		
$CoSO_4$	ND		
$CuSO_4$	ND		
$(NH_4)_6Mo_7O_{24}$	ND		

The grey cells are no part of the indicated media, ND; not determined.

The assumption for single omission experiments is that components, for which the biosynthetic capability is present, can be omitted without complete abolishment of growth. Based on this single omission experiments it can be seen that *L. plantarum* has auxotrophies for nicotinic acid, panthotenic acid, riboflavin, arginine, glutamate, isoleucine, leucine, valine, phenylalanine, tryptophan and tyrosine (Table 1). Based on these auxotrophies PMM (Plantarum Minimal Medium) was designed (see Table 2).

TABLE 2: Medium composition of CDM, PMM, PMM1, PMM2, PMM3, PMM4, PMM5, and PMM7.

Medium component	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
Final OD$_{600}$	2.56	0.00	2.15	1.98	1.85	0.90	0.10	1.07
Predicted growth-yields expressed in %[a]	100	0	0	0	0	0	0	0
Basic medium	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
Phosphate								
Glucose								
Sodium acetate								
Ammonium citrate								
Ascorbic acid								
Amino acids	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
Alanine								
Arginine								
Aspartic acid								
Cysteine								
Glutatmate								
Glycine								
Histidine								
Isoleucine								
Leucine								
Lysine								
Methionine								
Phenylalanine								
Proline								
Serine								
Threonine								
Tryptophane								
Tyrosine								
Valine								
vitamins	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
6,8-thiotic acid								
Biotin								
Nicotinic acid								
Panthothenic acid								
Para-aminobenzoic acid								
Pryidoxamine								
Pyridoxine								
Riboflavin								
Thiamine								
Vitamin B12								

Purines	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
Adenine								
Guanine								
Inosine								
Xanthine								
Pyrimidines	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
Orotic acid								
Thymidine								
Uracil								
minerals	CDM	PMM	PMM1	PMM2	PMM3	PMM4	PMM5	PMM7
$MgCl_2$								
$CaCl_2$								
$MnCl_2$								
$FeCl_3$								
$FeCl_2$								
$ZnSO_4$								
$CoSO_4$								
$CuSO_4$								
$(NH_4)_6Mo_7O_{24}$								

[a] these growth yield are calculated using the genome-scale metabolic model for *L. plantarum*. The predictions were made using the medium composition as indicated with the white cells. Grey cells show the absence of components in the indicated medium.

Inoculation of *L. plantarum* WCFS1 on PMM did not lead to detectable growth, showing that some of the omitted amino acids or vitamins are required for growth. To design a modified PMM medium, which supports growth of *L. plantarum* WCFS1, specific medium components: cysteine, methionine, threonine, pyridoxamine, biotin and *para*-aminobenzoic acid (*p*ABA) were added to the PMM medium. The resulting medium was labeled PMM1 (see Table 2). The inability of *L. plantarum* WCFS1 to grow on PMM suggests that *L. plantarum* WCFS1 has more auxotrophies than can be deduced from the single omission experiment alone.

To design a minimal medium that supports growth of *L. plantarum*, all medium components in PMM1 which are thought to be non-essential for growth (see list above) were stepwise omitted from PMM1. First, it was determined which minerals are essential for growth of *L. plantarum* WCFS1. To test this, all minerals (except Na^+, K^+ and Cl^-) were removed from PMM1, which abolished growth completely. The combined re-addition of Mg^{2+} and Mn^{2+} assured growth of *L. plantarum*, showing that both minerals are essential for growth of *L. plantarum* (data not shown). The medium that met the mineral requirements for *L. plantarum* was named PMM2 (see Table 2). Based on PMM2 it was noticed that *p*ABA could be omitted from the latter medium without a reduction in final optical density. This medium is indicated as PMM3. To determine which of the remaining medium components could still be removed from PMM3, a second round of single omission experiments were performed on this medium (see Table 1). From these experiments it was noticed that removal of the following components did not abolish growth: pyridoxamine, biotin, cysteine, and ammonium citrate. Next, we determined whether the simultaneous omission of pyridoxamine and biotin still yielded a medium that supports growth. Interestingly, it was found that *L. plantarum* was able to reach a final OD_{600} of 0.90 on PMM3 lacking pyridoxamine and biotin.

The resulting medium was called PMM4. Subsequently, cysteine and ammonium citrate were removed from PMM4, which led to limited but still detectable growth (a final OD_{600} of 0.10 was obtained). This medium was called PMM5. It was determined that a final OD_{600} 0.10 is equivalent to a biomass of 35 mg/L dry weight for *L. plantarum* WCFS1, whereby the correlation between final turbidity (OD_{600}) and biomass was found to be linear (data not shown).

Finally, it was tested whether PMM5 is a truly minimal medium. A single omission experiment was performed on each of the medium components in PMM5 (see Table 1). It was found that removal of any of the remaining medium components abolished growth completely, showing that PMM5 is a minimal medium for *L. plantarum* WCFS1.

For experimental purposes, a biomass yield of 35 mg/L DW on PMM5 is not very useful. Therefore, a medium was developed that allowed efficient growth to higher optical densities. The following components were added to PMM5: cysteine, pyridoxamine and ammonium-citrate, and the resulting medium was named PMM7. These three components were chosen based on their effect on the biomass reduction upon removal of the components from the PMM3 and PMM4. Growth of *L. plantarum* on PMM7 resulted in a biomass yield of 350 mg/L DW, which is sufficient for a vast range of purposes. The sole addition of pyridoxamine, cysteine and ammonium-citrate to PMM5 increased the biomass from 35 to 123, 158, and 70 mg/L DW, respectively, showing the impact of the three components on increasing the final biomass yield. Noteworthy, it was found that ammonium and not citrate was responsible for the increase in optical density. The addition of ammonium chloride to PMM5 could also increase the optical density to 70 mg/L DW. Random Amplified Polymorphic DNA-analysis (RAPD) was performed to confirm the identity if cells isolated from the PMM5 and PMM7 cultures. All tested single colony isolates displayed an identical PCR-profile when compared to *L. plantarum* wild-type cells.

The impact of aerobic and anaerobic atmosphere on the growth performance of *L. plantarum* on PMM5 and PMM7 is of great interest, since some experiments require absence or presence of oxygen, for example, investigation of a respiratory phenotype in *L. plantarum*. The impact of these two atmospheric conditions on biomass formation was determined on PMM5 and PMM7. Under aerobic conditions *L. plantarum* is unable to grow on PMM5, while under strict anaerobic conditions a biomass yield of 35 mg/L DW was found. These experiments show that the presence of oxygen hampers growth of *L. plantarum* on PMM5. Aerobic and anaerobic conditions did not influence biomass formation of *L. plantarum* on PMM7: with both conditions a biomass yield of 350 mg/L DW was reached. Furthermore, it was observed that the removal of ammonium did not result in growth reduction in aerobic conditions, indicating that the presence of cysteine and pyridoxamine increases the tolerance for growth in the presence of oxygen.

Comparing growth of *L. plantarum* on PMM5 and PMM7 with other growth media.

The growth performance of *L. plantarum* on PMM5 and PMM7 was compared to that on established laboratory media such as CDM and MRS. *L. plantarum* WCFS1 was inoculated on the four types of media; in 96-wells microtiter plates and in screw capped tubes. All four media contained equal amounts of glucose (2%). The final biomass yield (OD_{600}) and

growth rates were determined in 96-wells microtiter plate experiments, (Table 3) that clearly showed that, cultivation of *L. plantarum* on MRS leads to the highest specific growth rate and final OD_{600}.

TABLE 3: Growth performance of *L. plantarum* WCFS1 on PMM5, PMM7, CDM and MRS. The growth performance is expressed as final OD_{600} (corrected for background) and growth rate (μ_{max}) for microtiter plates, and final OD_{600} for screw capped tubes.

Microtiter plate	PMM5	PMM7	CDM	MRS
Final OD_{600}	0.02	0.24	1.11	1.52
μ_{max} (hour^{-1})	0.00	0.15	0.29	0.40
Screw cap tubes	PMM5	PMM7	CDM	MRS
Final OD_{600}	0.10	1.18	3.36	7.92

The growth performance of *L. plantarum* on PMM5 in microtiter plates did not result in detectable growth and therefore the growth rate could not be determined. In screw-capped tubes, however, *L. plantarum* is able to grow to a final OD_{600} of 0.1 on PMM5, showing a clear distinction in growth performance in 96-wells microtiter plates and screw-capped tubes. On PMM7 a specific growth rate of 0.15 h^{-1} was obtained which is 50% and 62.5% lower when compared to growth on CDM and MRS, respectively. These experiments show that PMM7 is practical for growth experiments of *L. plantarum* in growth tubes and 96-well plates both under aerobic as well as anaerobic conditions.

Growth predictions using the genome-scale metabolic model.
The genome-scale metabolic model of *L. plantarum* (26) was used to predict growth of *L. plantarum* WCFS1 on the various media described in this study (CDM, PMM till PMM6, and PMM7). As expected, the model predicts good growth of *L. plantarum* on CDM (Table 2). However, we were not able to simulate growth of *L. plantarum* on any of the designed PMM media. The reason for this discrepancy is that the reconstructed metabolic network of *L. plantarum* WCFS1 contains incomplete pathways for biosynthesis of *p*ABA, biotin, and pyridoxamine.

Folate production in the absence of *p*ABA on PMM7.
The metabolic model for *L. plantarum* WCFS1, predicts that growth on PMM7 is not possible. This prediction is based on the notion that folate can not be synthesized on this medium because *para*-aminobenzoic acid (*p*ABA) is essential for folate production, is not present in PMM7. Moreover, the model embodies the assumption that folate is absolutely required for growth. The genes for *p*ABA biosynthesis have not been identified on the genome of *L. plantarum* (29). Based on these findings, we investigated whether folate could be produced by *L. plantarum* on PMM7 in the presence or absence of *p*ABA (Table 4).

TABLE 4: Folate production and final OD_{600} of *L. plantarum* WCFS1 when cultivated on PMM7 and CDM in the presence and absence of *p*ABA.

	PMM7 - *p*ABA	PMM7 + *p*ABA	CDM - *p*ABA	CDM + *p*ABA
Final OD_{600}	1.18	1.17	3.36	3.67
Folate pools µg/L	0.0013	16	Not Detected	18

In the presence of *p*ABA, *L. plantarum* was able to produce 16 µg/L folate per OD_{600} unit on PMM7. In the absence of *p*ABA, the folate production pools remained below the detection threshold of our bioassay (2 µg/L folate). To determine the folate pool in these cultures, the cell extract was concentrated 500-fold. In this sample a folate pool of 1.3 ng/L per OD_{600} unit was measured (Table 4). Interestingly, the presence or absence of *p*ABA did not affect growth rate or biomass formation at all (data not shown). From these experiments it can be concluded that a 12.000-fold reduction in folate production pools still allows proper growth of *L. plantarum* WCFS1, showing that high folate pools are not essential for growth. Simulations of growth using stoichiometric metabolic models are valuable for pinpointing complex minimal medium requirements of lactic acid bacteria.

Discussion

Most of the natural biotopes of lactic acid bacteria (e.g. plant material, milk, gastrointestinal tract and meat) are rich in amino acids and vitamins. Therefore, it can easily be explained why auxotrophies are abundant amongst lactic acid bacteria. Recently, it was established, based on single omission experiments, that *L. plantarum* has 11 and 3 auxotrophies with respect to amino acid and vitamin requirements, respectively (25). A minimal medium for *L. plantarum* WCFS1 has been developed that contains 10 amino acids, 3 vitamins, 2 minerals, glucose, phosphate buffer, sodium acetate, ammonium citrate, and ascorbic acid (this medium is called PMM5). The medium composition of the developed minimal medium matches well with the single omission experiments on CDM with only one exception: cysteine. Reconstruction of the metabolic network of *L. plantarum* indicated that the cysteine biosynthesis pathway is incomplete and is expected to be essential (25, 26). However, we hypothesize that methionine (still present on PMM5) can be converted into cysteine. In *Bacillus subtilis* it was found that methionine could be used as sole sulfur source. In this organisms, methionine is converted into homocysteine through S-adenosylmethionine synthetase, (EC 2.5.1.6), after which the reverse transsulfuration pathway is used to convert homocysteine into cysteine (10). We have found that the ability for form biomass in the absence of cysteine is fairly low; suggesting that flux from methionine to cysteine will be low. The sole addition of cysteine, pyridoxamine and ammonium to PMM5 resulted in an increased biomass. The fact that ammonium could increase the formed biomass in PMM5, suggests that ammonium becomes limiting for the formation of amino acids. In *Escherichia coli* and *B. subtilis*, ammonium can be used as sole nitrogen source (7, 17). Pyridoxamine, is involved in the interconversion of amino acids, specifically, transamination and decarboxylation reactions (27). Since fewer amino acids are present in the minimal medium, transamination and decarboxylation reactions by pyridoxamine become relatively more important. The minerals Mg^{2+} and Mn^{2+} are essential for growth of *L. plantarum*. Recently, it was found that Mn^{2+} is essential for biomass formation in *L. plantarum* (8). In this paper it was suggested that high Mn^{2+} concentration helps the cell to deal with reactive oxygen species and serves as an alternative for the absence of a gene for the superoxide reductase. It is well established that Mg^{2+} is essential for a vast number of enzymatic reactions. Noteworthy, the mineral content of PMM7 is similar to a well described Defined LActobacillus medium (DLA) (4).

In the presence of oxygen, growth on PMM5 was found to be severely reduced, whereas on PMM7 this was not observed. The presence of cysteine, or pyridoxamine increased the resistance to aerobic conditions. Cysteine is crucial for the biosynthesis of glutathione and proteins like thioredoxin. Glutathione and/or thioredoxin play a role the oxidative stress response (6). Recently, it was found that overproduction of thioredoxin reductase improves the resistance towards oxidative stress in *L. plantarum* WCFS1 (21).

Bacterial growth depends on the biosynthesis and/or uptake of medium components required for the formation of biomass. *L. plantarum* WCFS1 grows 50% slower on PMM7 when compared to CDM, showing that the removal of all redundant components decreased the growth rate drastically. This growth rate reduction reflects to the fact that more energy is invested in the *de novo* synthesis of proteins, needed for the synthesis or interconversion of, for example, amino acids. *Escherichia coli* can grow on minimal media (M9) as well as rich media (Luria Broth, LB). The difference in medium composition reflects to the difference in growth rate for *E. coli* on both types of medium. The specific growth rates of *E. coli* K12 on M9 and LB, are 0.4 h^{-1} and 1.6 h^{-1}, respectively (1, 17). A reduced growth rate (when compared to rich broth) on minimal medium implicates that all building blocks required for biomass formation must be synthesized.

Based on the reconstructed metabolic network of *L. plantarum*, it was predicted that folate, biotin and pyridoxamine are essential for growth. However, growth experiments showed that removal of these vitamins did not abolish growth of the microorganism. Biotin is essential for fatty acid synthesis in lactic acid bacteria (32). It was also predicted that 2 out of 13 pyridoxamine requiring reactions in *L. plantarum*, can not occur on minimal medium. These reactions are i) the conversion of 26dap-M (2,6-meso-diaminopimelate) into lysine, mediated by LysA (diaminopimelate decarboxylase, E.C. 4.1.1.20), and ii) serine into glycine, which is catalyzed by GlyA (glycine hydroxymethyltransferase, E.C. 2.1.2.1), lp_2375. Growth in the absence of biotin, pyridoxamine, and folate suggests that i) trace amounts of these compounds are present in the medium, or ii) alternative reactions occur that do not require these compounds. For the folate case, it can be expected that molecules, such as formate, can be used in the purine biosynthesis pathway and for the initiation of protein synthesis. In *Escherichia coli*, for example, the purine synthesis and initiation of protein synthesis are not strictly dependent on formyl-tetrahydrofolate for its synthesis, instead unbound formate can be used in both pathways (13, 15). The use of minimal media combined with genome-scale metabolic models can be very powerful for finding leads of novel or alternative pathways. A clear disadvantage of using rich media like MRS or CDM is that specific knockouts do not show their expected phenotype. Detailed knowledge on the metabolic network (a metabolic model) helps to predict the phenotype of certain knock-outs strains on rich and minimal medium. The *Lactococcus lactis* *p*ABA knockout strain, for example, is still able to grow on CDM lacking *p*ABA (30). It was found that nucleobases and nucleosides present in the CDM could circumvent the need for folate, thereby surpassing the requirement for *p*ABA (30). Minimal media can reduce this problem, since there are less medium components that can surpass the need for specific cofactors. The PMM7 medium lacks all components needed for folate production or folate-dependent metabolite formation. Growth of *L. plantarum* WCFS1 on this medium was therefore unexpected.

On undefined media, several folate producing bacteria were able to synthesize folate pools ranging from 2 to 214 μg/L folate per OD_{600} unit (23). The folate production levels of these bacteria are in between 3 to 5 orders of magnitude higher than the folate levels that are produced by *L. plantarum* WCFS1 on PMM7. This was unexpected and suggests that high levels of folate production are not essential for growth of *L. plantarum*. Our experiments suggest that wild-type cells of *L. plantarum* produce a surplus of folate that exceeds the requirement of its own metabolism. Finally, these results do not exclude the presence of alternative cofactor for the one-carbon donation. This paper highlights the importance of developing a minimal medium, such a medium can be combined with a metabolic models. Predictions based on this minimal medium will help to reveal inconsistencies in between the genomic context and growth behaviour. In addition, this medium can also help to reveal phenotypes, such as growth behaviour, of deletion-strains that would be difficult to recognize in a rich media.

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Chapter 9

Folate Overproduction in
Lactobacillus plantarum WCFS1
Causes Methotrexate Resistance

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Abstract

Previously, a folate overproducing *Lactobacillus plantarum* strain was constructed (give ref). Using this strain we tested the hypothesis whether folate overproduction can be used as a mode of resistance against the folate antagonist methotrexate (MTX). Growth of the control strain was decreased with increasing MTX concentrations; at the highest tested MTX concentration growth was completely impaired. Interestingly, growth of the folate overproducing strain was unaffected by MTX. By cell plating it was observed that nearly all folate overproducing cells were able to grow on CDM plates supplemented with MTX, whereas only 1 in every $3*10^5$ wild-type cells was able to grow on these plates, showing that folate overproduction is a potential mechanism for MTX resistance. More than 500 of the naturally MTX resistant colonies were analyzed for increased folate pool in growing cultures. Interestingly, one of these isolated colonies produced 70% more folate when compared to wild-type isolates. By random mutagenesis it was attempted to deregulate folate biosynthesis in *L. plantarum* WCFS1. A total of 1920 of these mutagenized MTX resistant colonies were isolated and checked for increased folate production; a total of 2 single colonies produced increased folate levels when compared to the wild-type. However, propagation of these isolates caused that the folate overproduction phenotype is rapidly lost. In conclusion, we have shown that folate overproduction is a mode of resistance against growth inhibition by MTX but this mechanism is not a common response towards the folate antagonist MTX.

Introduction

Folate antagonists like methotrexate (MTX) are chemically produced compounds with a broad use in therapeutics and medicine. For example, MTX is used as medicine against malaria to prevent growth of the parasite *Plasmodium falciparum* (21). Often folate antagonists are added to animal-feed to protect cattle against bacterial infections (18). Moreover, it has been demonstrated that MTX has specific activity against some types of cancer; in cancer cells MTX is taken-up more readily than folate, thereby slowing down the proliferation of the malignant cells (31). On the enzyme level MTX competes with dihydrofolate for the binding site of dihydrofolate reductase (DHFR, EC 1.5.1.3). In rat liver cells it was found that MTX binds with a 20.000-fold higher affinity to DHFR as compared to the natural substrate dihydrofolate (42). This results in depletion of the cellular tetrahydrofolate pools. Tetrahydrofolate and its derivatives are essential for the synthesis of purines, thymidylate, some amino acids and formylation of tRNA[Met] (16, 20, 37). In *Escherichia coli* it was demonstrated that the presence of the folate-dependent metabolites (purines, thymidine, glycine, methionine, and panthotenic acid) in the growth medium, could diminish the growth rate reduction by the folate antagonist trimethoprim (TRM) (16, 39). However, it was noted that a moderate growth rate reduction could not be prevented, since formylated tRNA[Met] can not taken up from the growth medium (19).

In lactic acid bacteria the susceptibly to folate antagonists varies; *Lactococcus lactis* is known be insensitive to TRM (24), whereas *Lactobacillus plantarum* and *Lactobacillus casei* are sensitive to folate antagonists (6). In *L. lactis* it was determined that the gene coding for DHFR determined the resistance toward TRM (24). Prolonged exposure of bacterial cells, *Plasmodium falciparum* or cancer cell to folate antagonists like MTX and TRM leads to

resistance to these agents. In cancer cells many modes of resistance to MTX have been observed: reduction in the folylpolyglutamate synthetase activity, amplification of the gene coding for DHFR, or decreasing the binding affinity for MTX on the DHFR (35). MTX is an important agent for treatment of bacterial infections, malaria and cancer. Therefore the pharmaceutical industry continuously searches for improved folate antagonists with increased affinity towards DHFR (13).

In a previous study, we have constructed a folate overproducing *Lactobacillus plantarum* strain by metabolic engineering. This strain was able to produce more than 200-fold higher folate levels in comparison with a wild-type control strain (40). MTX competes with dihydrofolate for the binding site of DHFR, therefore it can be assumed that increased folate pools may reduce the toxicity of MTX. In this paper we have tested the hypothesis that elevated folate production leads to MTX resistance. Furthermore, it was tested whether folate overproduction is a common feature in natural and randomly mutagenized MTX resistant colonies.

Materials and methods

Strains and cultivation conditions

Lactobacillus plantarum WCFS1 wild-type and *L. plantarum* engineered strains harboring pNZ7021 (control vector, (41)) and pNZ7026 (folate gene cluster expression vector (40), were grown at 37°C on Chemically Defined Media (CDM) as described previously (36). For specific experiments, a modified CDM was used; this medium lacks folate-dependent metabolites (glycine, inosine, orotic acid, thymidine, guanine, adenine, uracil and xanthine) and/or *p*ABA. CDM-plates were made as follows: two-fold concentrated CDM was mixed with 1% dissolved agarose (Invitrogen, Paisley, UK). Three strains; *L. plantarum* wild-type, control, and folate overproducing strain were cultured on CDM without folate-dependent metabolites either in the presence and absence of *p*ABA. Hereafter, serial dilutions were made (from $1*10^0$ till $1*10^6$ times diluted) of the three cultures. The undiluted and $1*10^6$ times diluted cultures were plated on CDM plates (without folate-dependent metabolites either in the presence and absence of *p*ABA) with and without 2.5 mg/L MTX. The number of colony forming units were determined for all three cultures. All experiments were performed without assuring anaerobic conditions. A concentration of 80 mg/L chloramphenicol (CM) was used for plasmid maintenance. Methotrexate (amethopterin, Sigma, Steinheim, Germany) was used at concentrations ranging from 0 to 2.5 mg/L.

Folate analysis using the microbiological assay

Folate was quantified using a microbiological assay on samples which were pretreated with γ-glutamyl carboxypeptidase (E.C.3.4.19.9) for deconjugation of polyglutamate tails (17, 33). A qualitative folate assay was developed and used for high throughput analysis of the samples. This assay is similar to the original microbiological assay except for the following modifications. Instead of quantifying folate levels using a calibration curve with standard folate samples, folate levels of MTX resistant isolates of *L. plantarum* strains were correlated to the folate level of the wild-type. Prior to the quantitative folate

analysis, remaining MTX traces were removed as follows. The single colonies were isolated from the MTX plates, spread on fresh CDM plates lacking MTX and incubated overnight at 37°C. This procedure was repeated an additional time. Single colonies from these plates were selected and transferred to microtiter plates in which quantitative folate analysis was performed. A part of this culture was mixed with glycerol and frozen at -80°C prior to further analysis. Cultures of MTX resistant isolates that displayed a significant higher folate pool in comparison to the wild-type strains, were selected from the glycerol stocks in 96 wells plates and propagated twice. Subsequently folate pools were quantified.

Growth rate analysis

Specific growth rates of cultures of *L. plantarum* were determined in batch cultures and in 96-wells microtiter plates by turbidity measurements at 600 nm using the Spectra Max384 spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, USA). The growth was tested for *L. plantarum* WCFS1 harboring pNZ7021 and pNZ7026 on CDM with and without folate-dependent metabolites, using the following MTX concentrations: 0, 0.31, 0.63, 1.25 and 2.50 mg/L.

Random mutagenesis

L. plantarum WCFS1 (wild-type) was treated with MNNG (N-methyl-N'-nitro-N-nitrosoguanidine, Sigma Chemical Co., St. Louis, MO, USA), thereby employing the following procedure. The *L. plantarum* WCFS1 was cultivated overnight on MRS (8). The overnight culture was serially diluted to obtain exponential growing cells after overnight cultivation. Hereafter, the exponential growing culture was incubated in fresh MRS till a final optical density (600 nm) of 0.7 units was reached. The cell pellet was two times washed with 100 mM citrate buffer (pH 5.0). Hereafter, the pellet was resuspended in citrate buffer and mixed at a 1:1 ratio with 1 mg/ml MNNG and subsequently incubated for 120 min. Aliquots were taken at 0, 15, 30, 45, 60, 90, and 120 minutes to determine the viable count (in colony forming units) on MRS plates. Residual MNNG was removed by washing with 100 mM potassium phosphate buffer (pH 7.0). Subsequently, mutagenized cells were incubated for 2 hours on MRS. Hereafter, the cells were washed twice with 0.85% (w/v) sodium chloride, and subsequently inoculated for 8 hours on CDM lacking folate-dependent metabolites. The inoculation and washing procedure was repeated again, and finally these cells were stored in 15% glycerol (v/v) at -80°C for further use.

Results

Folate overproduction as mechanism for MTX resistance.

The aim of this study was to determine whether elevated folate pools in *L. plantarum* can protect the organism from the inhibitory action of MTX. Previously a plasmid was constructed for elevated folate production (40). The complete folate biosynthesis gene cluster of *L. plantarum* WCFS1 was cloned under the control of the constitutive *pepN* promoter of *L. lactis*. Transferring this plasmid (pNZ7026) to *L. plantarum* resulted in a strain that was able to produce 200-fold increased folate pools in comparison with *L. plantarum* harboring the

control plasmid (pNZ7021) (40). This 200-fold increased folate production levels were obtained on CDM containing the folate-dependent metabolites. In several reports it was found that the bacterial growth inhibiting effect of MTX is abolished in a growth medium that contains folate-dependent metabolites (16, 39). For testing the hypothesis that folate overproduction leads to MTX resistance, it is crucial that folate overproduction is achieved on a growth medium that lacks the folate dependent metabolites. Therefore, the folate overproducer was cultivated on CDM lacking folate-dependent metabolites and analyzed for folate production (Table 1).

TABLE 1: The total folate levels of *L. plantarum* WCFS1 harboring pNZ7021 and pNZ7026 after growth on CDM in the presence and absence of folate-dependent metabolites.

	with folate-dependent metabolites	without folate-dependent metabolites
L. plantarum harboring	folate mg/L per OD_{600} (SD)	folate mg/L per OD_{600} (SD)
pNZ7021 (empty vector)	15 (1)	43 (2)
pNZ7026 (folate overproduction plasmid)	2903 (91)	2258 (248)

The overproducing strain was found to have 52-fold elevated folate pools on this growth medium when compared to the control strain. Moreover, it can be seen that folate production of the control strain on CDM lacking folate-dependent is 3-fold higher when compared to folate production on growth medium containing folate-dependent metabolites, showing that folate production can be modulated by the presence of the folate-dependent metabolites. In short, folate overproduction is possible on medium lacking the folate-dependent metabolites (Table 1).

To test the hypothesis that folate overproduction leads to reduced susceptibility towards MTX, the growth rates of the control and folate overproducing strain were determined on CDM supplemented with and without the folate-dependent metabolites and MTX in concentrations ranging from 0 to 2.5 mg/L (see Table 2). In the presence of the folate- dependent metabolites the growth rate of both the control and folate overproducing strain was unaffected by MTX, showing that tetrahydrofolate depletion by MTX can be bypassed if folate-dependent metabolites are present in the medium. Moreover, in the absence of MTX, the folate overproducing strain showed a 21% reduced growth rate on the complete CDM which contains all the folate-dependent metabolites. This observation is in agreement with previously reported effects of folate overproduction (40). Increasing MTX concentrations reduced the growth rate difference between the control and folate overproducing strain; at 2.5 mg/L MTX the folate overproducing strain only grows 10% slower than the control strain, showing that at higher MTX concentrations the folate overproducing strains has a clear growth benefit on this medium. On CDM lacking the folate-dependent metabolites, growth of the control strain was severely reduced in presence of MTX, while under these conditions the growth rate of the folate overproducing strain was not significantly affected (Table 2).

TABLE 2: The growth rate of *L. plantarum* WCFS harboring pNZ7021 and pNZ7026 on CDM in the presence and absence of folate-dependent metabolites as a function of the concentration of MTX (between brackets standard deviations).

Concentration MTX mg/L	With folate-dependent metabolites μ_{max} pNZ7021 (SD)	with folate-dependent metabolites μ_{max} pNZ7026 (SD)	Without folate-dependent metabolites μ_{max} pNZ7021 (SD)	without folate-dependent metabolites μ_{max} pNZ7026 (SD)
0	0.31 (0.03)	0.25 (0.01)	0.21 (0.01)	0.16 (0.01)
0.31	0.32 (0.02)	0.26 (0.01)	0.12 (0.01)	0.17 (0.01)
0.63	0.32 (0.04)	0.26 (0.01)	0.13 (0.01)	0.18 (0.02)
1.25	0.31 (0.02)	0.26 (0.01)	0.01 (0.00)	0.15 (0.00)
2.5	0.28 (0.04)	0.25 (0.02)	0 (N.A.)	0.14 0.01)

Growth of the control strain was already reduced by 40% at an MTX concentration of 0.31 mg/L. At an MTX concentration of 2.5 mg/L growth was completely diminished. These experiments support the hypothesis that folate overproduction can serve as a mode of resistance towards MTX.

Spontaneous MTX resistant mutants

As reported in the previous paragraph, growth of the control strain was completely blocked on CDM lacking the folate-dependent metabolites at a MTX concentration of 2.5 mg/L. However, incubation for an additional 24 hours showed clear growth, suggesting that spontaneous MTX resistant mutants were enriched in the culture. The frequency of these MTX resistant colonies was determined for the *L. plantarum* wild-type, control and folate overproducing strain by CFU counts on CDM plates supplemented with MTX. On plates containing 2.5 mg/L MTX, spontaneous resistant mutants derived from the wild-type culture were isolated with a mutation frequency of about 3×10^{-5}. Nearly all folate overproducing cells were able to grow on the CDM plates supplemented with MTX, confirming our previous observations.

Recently it was demonstrated that folate overproduction in *L. plantarum* was only possible on a growth medium that contains a significant amount of *para* aminobenzoic acid (*p*ABA) (40). To test whether high folate pools or other secondary properties of the folate overproducer were responsible for MTX resistance, the folate overproducer was spread on CDM plates with and without *p*ABA and MTX. To avoid carry-over effects, the folate overproducer was pre-cultured on CDM without *p*ABA and folate-dependent metabolites. Aliquots of overnight cultures were serially diluted and transferred to the CDM plates (without folate-dependent metabolites) in the presence and absence of *p*ABA and MTX. It was found that folate overproducing cells could be quantitatively recovered on CDM plates with *p*ABA. However, in the absence of *p*ABA only 1 in every 4×10^{5} cells was able to grow on CDM plates with MTX, showing that resistance towards MTX is directly linked to the ability to produce high intracellular folate pools.

Folate overproduction in the spontaneous MTX resistant single colony isolates.

We have found that in populations of *Lactobacillus plantarum*, natural resistance towards MTX occurs at a relatively high frequency of 3×10^{-5}. We have analyzed 576 single colony isolates of these resistant strains for the size of their folate pool. All methotrexate resistant cells were obtained by plating *L. plantarum* WCFS1 wild-type on CDM plates with MTX. The qualitative folate assay was used to screen the selected single colony isolates for elevated folate production. One of the analyzed colonies displayed a 70% elevated folate pool when compared to the wild-type strain (Fig. 1), showing that natural folate-overproducers can be obtained by this method.
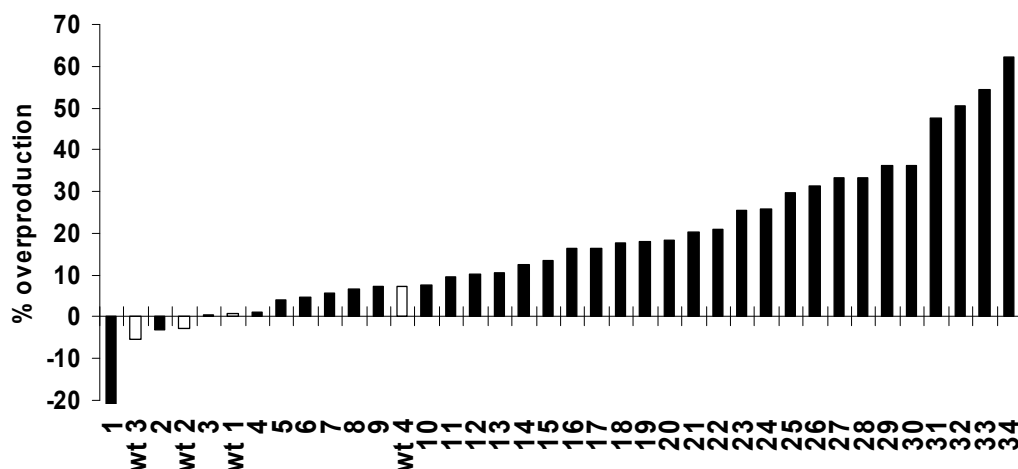
FIG 1: The percentage of folate overproduction of a total of 34 MTX resistant single colony isolates (black bars). The average folate production pools of 4 wild-type colonies (white bars) was correlated with the MTX resistant colonies. The production levels of the MTX resistant mutants were compared with the wild-type levels to calculate the percentage of overproduction.

Continued propagation of this single colony isolate led to a drop in folate production pools, suggesting that folate production phenotype is lost after repetitive propagation.

Folate overproduction in random mutagenized *L. plantarum* WCFS1.

We assume that folate biosynthesis is a tightly regulated process. Therefore, random chemical mutagenesis was applied to obtain *L. plantarum* strains with elevated or deregulated folate biosynthesis. A culture of *L. plantarum* WCSF1 (wild-type) was treated with the mutagenic agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 120 minutes to obtain 10% survival. Commonly, a high death-rate is associated with a high mutation frequency. First it was determined whether the MNNG treated survivors in the *L. plantarum* culture were on average more resistant towards MTX. This was done by plating the overnight culture

undiluted on CDM plates supplemented with 2.5 mg/L MTX. We found that almost 10 times more of the MNNG treated *L. plantarum* cells were resistant to MTX in comparison to the untreated wild-type cells. In this vast pool of MTX resistant colonies a total of 1920 colonies were screened for increased folate pools, using the qualitative folate assay. Interestingly, a total of 2 isolates displayed a higher folate production pool when compared to the control strain, these pools were 30% higher when compared to wild-type folate production pools. Next, the accurate folate production pools were determined for these 2 isolates and 8 randomly selected wild-type isolates. For this procedure, the cultures were propagated in the absence of MTX. The quantitative folate assay showed that the increased folate levels in the mutants were lost upon propagation in the absence of MTX.

This study shows that folate overproduction can lead to MTX resistance but it certainly is not a common mechanism to cope the growth inhibitory action of the anti-folate MTX.

Discussion

This study has demonstrated that folate overproduction in *Lactobacillus plantarum* WCFS1 causes resistance towards folate antagonist methotrexate (MTX). Numerous studies have demonstrated one or combinations of the following mechanisms of resistance towards MTX in cancer cells: (i) gene amplification of DHFR (EC 1.5.1.3) (2); (ii) mutation to produce DHFR with decreased affinity to MTX (14); (iii) reduced polyglutamate synthesis (22); (iv) increased activity of folate conjugase (26); (v) increased activity of folylpolyglutamate synthetase (E.C. 6.3.2.17) (27); (vi) decreased transport of MTX into the cell (3); and (vii) increased expression of multidrug transporter (30). To our knowledge, folate overproduction itself was never reported as a mechanism for MTX resistance. For human cancer cells and protozoa, folate overproduction can not be a mechanism for MTX resistance since these cells do not possess all the genes for a complete folate biosynthesis pathway (10, 29). Plants, some fungi and some bacteria have the ability to produce folate (5, 12, 15). *L. plantarum* is a folate producing bacterium and the genome was found to encode the complete folate gene cluster (*folB*, *folK*, *folE*, *folC2*, *xtp2*, and *folP*) (34, 40). However, folate production in *L. plantarum* could only be achieved on growth media supplemented with *p*-aminobenzoic acid (*p*ABA), this was expected since the genes for *p*ABA biosynthesis were found to be absent (40). In the absence of *p*ABA no folate production was detected. These observations match with data obtained in this study; the engineered folate overproducer is unable to produce folate in the absence of *p*ABA and under these conditions the strain is sensitive towards MTX. In the presence of *p*ABA the engineered folate overproducer was found to be resistant towards MTX, showing that elevated folate pools and not secondary properties of the overproduction strain are responsible for the resistance to MTX.

Tetrahydrofolate is synthesized from dihydrofolate by DHFR. The general view is that this enzyme is found in all organisms (38). However, the genomes of many bacterial species seem to lack DHFR coding genes (7). As a consequence these organisms that lack DHFR are endogenously resistant to folate antagonists such as TRM (11). Some organisms are naturally resistant to inhibitors of DHFR. It was found that DHFR of *L. lactis* is insensitive towards trimethoprim (TRM). This was demonstrated by converting a TRM

sensitive bacterium into a TRM resistant bacterium by cloning and expression of the *L. lactis* gene coding for DHFR into the host strain (24). Within the population of *L. plantarum* it was found that roughly 1 in every 300.000 cells is resistant towards MTX. We have demonstrated that in these resistant strains, natural overproduction of folate is not the most abundant mechanism for MTX resistance. Spontaneous mutations in *L. plantarum* have been reported to occur with high frequencies (1). Similar frequencies of natural resistant cells were also reported for resistance of *L. lactis* towards the toxic dipeptide L-alanyl-β-chloro-L-alanine (32).

We have observed that growth inhibition of *L. plantarum* by MTX does not take place on medium rich in folate-dependent metabolites. The bacterium *Streptococcus faecalis* R, for example, is unable to produce folate, but the addition of the folate-dependent metabolites to the growth medium could assure growth of the latter strain (28). Previously, it was reported that growth of *L. plantarum* could be suppressed by TRM, however, the susceptibility to TRM appeared to be dependent on the medium composition (6). On a rich broth medium, like MRS, no growth inhibition was detected, whereas on Folic Acid Casei Medium (FACM) a minimal inhibitory concentration value of 0.19 mg/L was recorded. The MRS broth contains folate, purines and pyrimidines whereas FACM lacks these components, showing that in the presence of folate-dependent metabolites growth inhibition by TRM is reduced. The only folate-dependent metabolite that can not be taken up by the cell is formylated tRNA^{Met} (16). Therefore, depletion of formylated tRNA^{Met} can have an impact on the growth rate, independent from supplying folate-dependent metabolites to the growth medium. However, in *E. coli* it was shown that initiation of protein synthesis can take place with unformylated tRNA^{Met} at high Mg^{2+} (higher than 10 mM) (23, 25). Folate antagonists are often used as pharmaceutical and clinical agent to prevent growth of bacterial cells. In nutrient rich environments, these compounds are expected to have limited effect.

We have demonstrated that folate overproduction causes resistance of bacteria against MTX. An explanation for the mechanism of MTX resistance is that high intracellular folate pools allow better competition with MTX for binding at DHFR. To explore if increased folate production is a common mechanism against MTX resistance, we analyzed spontaneous and mutagenized MTX resistant isolates for their ability to produce elevated folate pools. A total of 3 (from more than 2500) single colonies isolates displayed increased folate pools; however, these levels did not exceed 30% increased folate pools. Albeit, these elevated folate pools were significantly higher, we wonder whether this small increase in folate production can compete with MTX for the binding of DHFR. In addition, we observed that the increased folate production phenotype was rapidly lost upon further propagation. Based on the propagation experiments we have calculated that the MTX resistant strains were cultured for 30 generation in the absence of MTX, prior to the qualitative folate assay. Next, these cells were cultured for an additional 20 generations in the absence of MTX. Therefore, it can be concluded that the high folate production pools rapidly decline in absence of the selection pressure applied by MTX. Propagation of these strains in the absence of MTX was essential, since the indicator strain of the folate assay is sensitive to MTX (6). We hypothesize that these folate overproducing single colony isolates have reverted into wild-type cells, thereby,

losing the folate overproduction phenotype. Phenotype instability resulting in the disappearance of mutant phenotypes is a common phenomenon (4, 32).

An efficient way to boost folate production in *L. plantarum* is the homologous overexpression of the folate gene cluster which resulted in a culture of which nearly all cells were able to grow on CDM plates with high levels of MTX. Many metabolic engineering attempts were performed to boost folate production (9, 33, 43), however, no method was as successful as the overexpression of the complete gene cluster in both *L. lactis* and *L. plantarum* (40, 41).

Resistance to folate antagonists like MTX can have many causes. For that reason new and improved folate antagonists with increased specificity or activity towards DHFR and other targets are continuously being synthesized. This study has shown that folate overproduction can lead to MTX resistance but it certainly is not a common mechanism to cope the growth inhibitory action of the MTX antagonist.

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Chapter 10

Combined Production of B12 and Folate by *Lactobacillus reuteri* JCM1112 for the Natural Enrichment of Fermented Foods

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Abstract

We observed that *Lactobacillus reuteri* JCM1112 produces vitamin B12 and folate. However, the folate/B12 ratio found was far below that desired for human consumption (~170:1). We used metabolic engineering by applying genetic and physiological approaches to improve this ratio and developed a generic and natural process that significantly increases folate production.

Introduction.

Humans have an auxotrophic requirement for vitamin B12 and folate, with a recommended nutrient intake for healthy adults of 2.4 and 400 μg/day, respectively (6). Deficient intake of either of these compounds has been linked to cardiovascular disease, neuropathy, birth defects, cancer, and different types of anemia, amongst other pathologies (4). Remarkably, the onset of vitamin B12 deficiency symptoms is often delayed by the increased intake of folate (18). This masking of B12 deficiency has resulted in the restriction of folate intake levels and prevented folate fortification in many countries (6). Strict vegetarian dietary regimes tend to be poor in vitamin B12 and rich in folic acid, increasing the risk of vitamin B12 deficiency masking. This has resulted in the increased popularity of fortifying vegetarian foodstuffs with B12 (1).

Coenzyme B12 is synthesized by a few members of bacteria and archaea (13). *In situ* microbial B12 production is a convenient strategy to achieve the natural enrichment of fermented foods, notably from vegetable sources. *Lactobacillus reuteri* is a gram-positive, heterofermentative lactic acid bacterium with a long history of safe use by the food industry (10). It is able to ferment 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 vitamin B12 (16, 21) and the draft genome sequence of strain JCM1112 (accession no. CP000705) (9) suggests the capacity to produce folate, as well as, vitamin B12. In this study, we investigated the possibility of using *L. reuteri* for the combined production of both vitamins in a ratio desired for human consumption, ~170:1 (w/w).

In silico analysis of the folate biosynthesis genes of L. reuteri JCM1112.

The predicted product of each folate biosynthesis gene of *L. plantarum* WCFS1 was used to search the genome of *L. reuteri* JCM1112 using the BLAST algorithm (3). Sequence identity of the bi-directional best hit was calculated on a nucleotide and amino acid level. Gene order was analyzed using the ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) (15). Both clusters are very similar as expected from the close phylogeny of their hosts (Table 1).

TABLE 1: Identification of a folate biosynthesis gene cluster in the genome of *L. reuteri* JCM1112 by homology searches with *L. plantarum* WCFS1

L. plantarum WCFS1				*L. reuteri* JCM1112			
				Orthologue		Identity (%)	
ORF #	Name	Length (aa)*	Assigned function	ORF #	Length (aa)*	Amino acid	Nucleotide
lp3299	folB	122	Dihydroneopterin aldolase (EC 4.1.2.25)	Lreu1280	111	48	56
lp3298	folK	170	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)	Lreu1279	170	43	53
lp3297	folE	189	GTP cyclohydrolase I (EC 3.5.4.16)	Lreu1278	192	56	60
lp3296	folC2	454	Folylpolyglutamate synthase (EC 6.3.2.17) / Dihydrofolate synthase (EC 6.3.2.12)	Lreu1277	419	38	50
lp3295	xtp2	195	Xanthosine triphosphate pyrophosphatase (EC 3.6.1.-)	Lreu1276	195	35	49
lp3294	folP	263	Dihydropteroate synthase (EC 2.5.1.15)	Lreu1275	387	36	40

* Length based on the number of amino acid residues (aa) predicted in the gene product

Sequence identity is high on both the amino acid and nucleotide level, on average 43% and 51%, respectively. Gene order is completely conserved throughout the entire extension of the approximately 4.5 kb cluster composed of six genes.

Characterization of vitamin B12 and folate production in CDM by L. reuteri JCM1112 and derivatives.

The human isolate *L. reuteri* JCM1112[T] was obtained from the Japanese Collection of Microorganisms (Riken, Japan). It was cultured at 37° C in chemically defined medium CDM) lacking vitamin B12 and folic acid (22). Folate was quantified from stationary phase cultures as described before (7), with a bioassay using *L. casei* ATCC 7469 as the indicator strain, and including an enzymatic deconjugation of polyglutamate tails (20). Vitamin B12 content was determined according to the Official Methods of Analysis of AOAC International, using the *L. delbrueckii* subsp. *lactis* ATCC 7830 vitamin B12 assay (8). Cell extracts of stationary phase cultures for vitamin B12 analysis were prepared as previously described (16). *L. reuteri* JCM1112 produces in CDM around 20 µg/L per OD_{600} unit of folate, approximately on a 1:1 ratio (w/w) with vitamin B12 (Fig. 1). We implemented a metabolic engineering strategy as proof of principle for the possibility to modulate the ratio of production of these two vitamins. We aimed at increasing folate production through the overexpression of the complete folate biosynthesis gene cluster, as described previously for other lactic acid bacteria (24), leaving unchanged the native vitamin B12 production. *L. reuteri* was transformed by electroporation as described elsewhere (23) with plasmids pNZ7021 (empty vector) and pNZ7026 harboring the folate biosynthesis gene cluster of *L.*

plantarum WCFS1 under control of the *pepN* promoter (25). These derivative strains of JCM1112 were cultured and analyzed for folate and vitamin B12 content in a similar fashion as the parent strain. Chloramphenicol was used as a selection marker in a final concentration of 10 μg/ml.
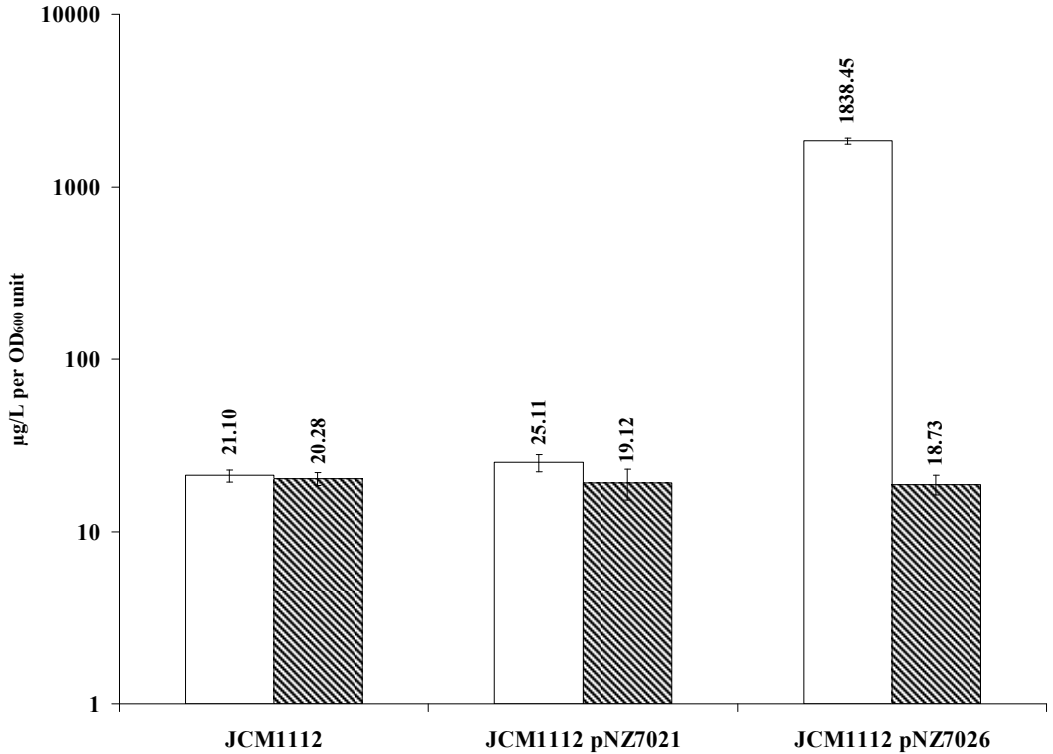

FIG. 1. Folate (white bars) and B12 (diagonally striped bars) production in CDM by *L. reuteri* JCM1112 and derivative strains containing the empty plasmid pNZ7021 and plasmid pNZ7026 with the folate biosynthesis gene cluster *L. plantarum*

     The constitutive overexpression of the folate biosynthesis genes of *L. plantarum* WCFS1, in cultures of *L. reuteri* JCM1112 pNZ7026 resulted in an increment of almost 100-fold in folate levels (Fig 1), while the control (*L. reuteri* JCM1112 pNZ7021) did not show any change in folate and vitamin B12 production. The overproduction of folate was found to have a very small effect on vitamin B12 production (<10% reduction) resulting in a folate/B12 ratio of approximately 100:1 (w/w), stable over five consecutive transfers (data not shown).

Characterization of vitamin B12 and folate production in fruit fermentations.

We assessed the applicability of the principle of modulating folate/B12 ratios through genetic engineering to different 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 over 50% in less economically developed nations (5, 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 known to be low in folate and deficient in vitamin B12 (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 (Moulinex, Masterchef 370, France) and the resulting paste was squeezed through a cotton cloth. The flow-through was centrifuged twice at 10.000 RPM for 10 min using a Sorvall centrifuge (Newton, Connecticut, USA). The supernatant was stored at -20ºC until further use. Before inoculation, the melon juice was diluted with a 0.5 M potassium-phosphate buffer (pH 5.8) in a 4:1 ratio (v/v). The final pH was adjusted to 6.0 and the melon juice medium was forced through a 0.22 μm filter to assure sterility. Cucumber juice medium was prepared from intact cucumber (*Cucumis sativus*) using the procedure described for melon with the following modifications: *(i)* an additional filtration step using a cellulose filter (0.15 mm) was implemented before centrifugation; *(ii)* the cucumber juice was diluted in 1 volume of 0.2 M potassium-phosphate buffer (pH 5.8). When appropriate, both media were supplemented with 10 mg/L *para*-aminobenzoic acid (*p*ABA) and, if necessary, 10 μg/ml chloramphenicol.

Folate and vitamin B12 content were determined from cultures of *L. reuteri* transformed with pNZ7026 and pNZ7021. Background folate levels in melon and cucumber media were found to be 22.5 ± 0.9 and 10.0 ± 0.4 μg/L, respectively. No vitamin B12 could be detected in these media. The overexpression of the folate biosynthesis cluster of WCFS1 in *L. reuteri* JCM1112 pNZ7026 leads to high production of folate (2518.2 ± 182.1 μg/L per OD_{600} unit) and a folate/B12 ratio of about 250:1 (w/w), but only when *p*ABA is added (Fig. 2). *p*ABA availability has been shown to limit folate biosynthesis in several lactic acid bacteria (25). The control experiment using *L. reuteri* JCM1112 with the empty vector (pNZ7021) resulted in the production, in melon media, of 131.7 ± 5.5 μg/L per OD_{600} unit of folate, which is much higher than in CDM. In cucumber media, folate production by JCM1112 pNZ7021 was negatively affected in comparison to CDM, regardless of the addition of *p*ABA (data not shown). The overexpression of the folate biosynthesis genes has a similar affect as described for CDM, but the final folate/B12 ratios remain one order of magnitude lower than desired. The 2-fold reduction in vitamin B12 production observed for the melon juice fermentation can be attributed to the amount of sugars present (~1.5% glucose and ~2% fructose as determined by HPLC analyses performed as described elsewhere (19)). Such concentrations have been shown in previous studies to repress vitamin B12 biosynthesis at the transcriptional level (2, 17).
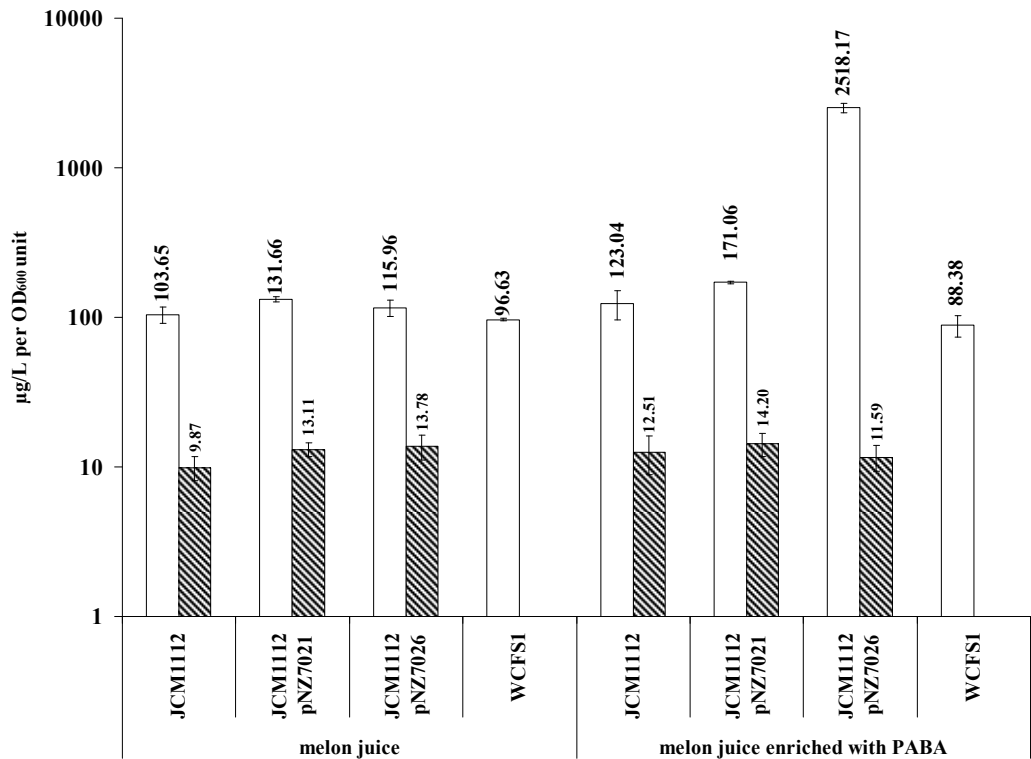
FIG. 2. Folate (white bars) and B12 (diagonally striped bars) production by the different *L. reuteri* JCM1112 constructs and by *L. plantarum* WCFS1 in melon juice medium.

The remarkable feature about melon fermentation in comparison to CDM and cucumber is the 5-10 fold higher production of folate for the strain carrying the empty plasmid (pNZ7021). To establish the unique ability of melon juice to induce high folate production, 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 of folate production in melon juice media compared to CDM (Fig. 2).

Conclusion.
In this study, we have demonstrated the possibility of combining the production of folate and vitamin B12 in *L. reuteri*. We implemented, as proof of principle, a metabolic engineering strategy to modulate 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 previously published. The findings reported here may lead to the development of (fermented) foods based on perishable fruits, such as melons, with an extended durability and

higher nutritional value. A well-tasting melon juice or melon squash, containing high folate and vitamin B12 could be the start of a longer shelf-life product line specially targeting vitamin deficient populations.

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Chapter 11

Summary and Concluding Remarks

Arno Wegkamp

Several approaches can be taken to elevate the intake of folate by consumers: i) fortification of foods with folate (2, 3), ii) intake of folate in a relatively pure form as tablets, pills and the like (16), iii) consumption of specific cultivars of plants with elevated folate content (12), or iv) food products with increased folate content by so called fermentation fortification (6), whereby the folate content of the food products is increased by the fermentation process. Essential for applying fermentation fortification in food processes is to have knowledge on the genes and pathways that are involved in folate production. We have focused our research on modulation of folate biosynthesis by a number of lactic acid bacteria: *Lactobacillus plantarum* (7), *Lactobacillus reuteri*, *Lactobacillus gasseri* (1) and *Lactococcus lactis* (19). Several methods have been applied to alter folate production in these bacteria: i) identification of genes involved in folate biosynthesis, ii) overexpression of genes involved in folate production, iii) modulation of folate production by medium components, iv) use of a folate antagonist to select a natural folate-overproducing strain and v) selection of novel food sources that can be used for fermentation fortification. Finally, we have investigated the impact of increased folate production in *L. plantarum* on the cellular physiology. Here we give a summary and concluding remarks of the each of the chapters in this thesis. Moreover, a future outlook is given.

Chapter 1 presents the objectives of the study described in this thesis. Furthermore, a general introduction on the topic of folate and folate biosynthesis as well as a brief outline of the thesis is given. C**hapter 2** provides a review on the role of folate and folate-dependent metabolites in Bacteria and Archaea. It is well established that folate is an important cofactor for the synthesis of purines, pyrimidines, amino acids and the formylation of $tRNA^{Met}$. However, there are many papers describing biochemical reactions that are not depending on folate as one-carbon donor. In Archaea, for example, folate is not used in the one-carbon metabolism, instead, these organism use methanopterin in the one-carbon metabolism. Several bacterial species and all Archaea are able to formylate methionyl-tRNA in the absence of folate. In lactic acid bacteria it has been reported that supplementation of the folate-dependent metabolites (purines, pyrimidines, and some amino acids) in the growth medium could circumvent the need for folate.

Comparative genomics has shown that bacterial species, such as *Rickettsia rickettsii* and *Borrelia burgdorferi*, lack some of the folate biosynthesis genes which were considered to be essential, *folA* and *thyA*. One strain *Mycoplasma hyopneumoniae* appeared to be completely independent of folate for the synthesis of purines, pyrimidines, amino acids and formylated $tRNA^{Met}$. All these bacteria, however, are intracellular pathogens and are likely to obtain nucleotides and other building blocks from the host. Disruption of *folA* and *thyA* in *Escherichia coli*, have resulted in alternative pathways that could support the synthesis of folate-dependent metabolites. Future research on new folate biosynthesis genes can help to extent our knowledge of the folate biosynthesis pathway, and may also help to reveal the existence and operation of alternative one-carbon donors.

A missing step in the folate biosynthesis pathway of bacteria and plants is described in **Chapter 3**. The folate biosynthesis pathway is composed of eight enzymatic steps, whereby GTP is converted into tetrahydrofolate (Fig. 1).
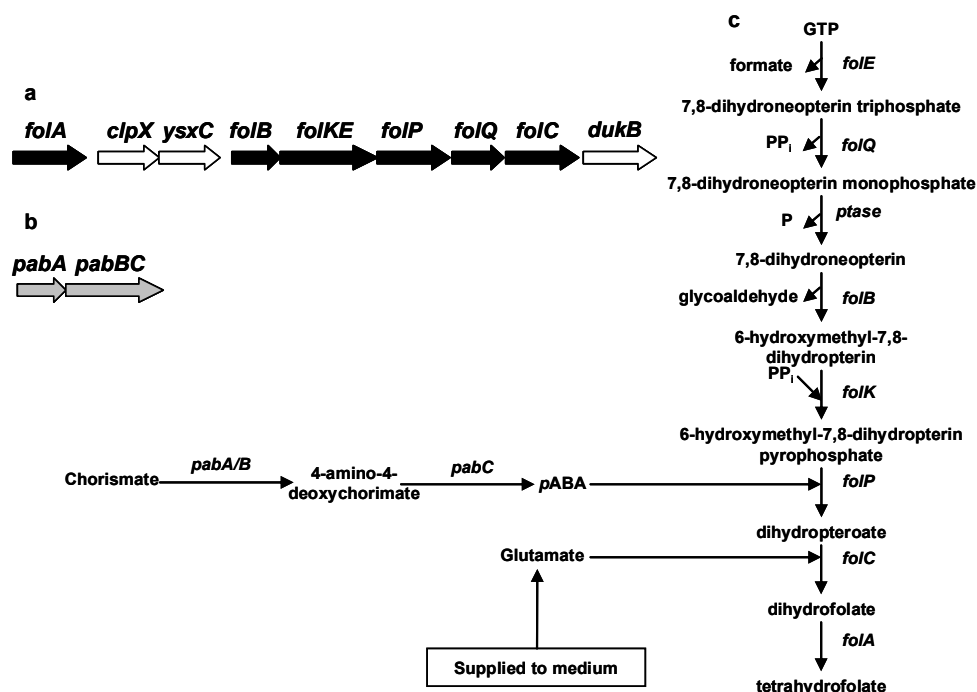
c

GTP

formate ⤹ *folE*

7,8-dihydroneopterin triphosphate

PP$_i$ ⤹ *folQ*

7,8-dihydroneopterin monophosphate

P ⤹ *ptase*

7,8-dihydroneopterin

glycoaldehyde ⤹ *folB*

6-hydroxymethyl-7,8-dihydropterin

PP$_i$ ↘ *folK*

6-hydroxymethyl-7,8-dihydropterin pyrophosphate

↓ *folP*

dihydropteroate

↓ *folC*

dihydrofolate

folA

tetrahydrofolate

a

folA *clpX* *ysxC* *folB* *folKE* *folP* *folQ* *folC* *dukB*

b

pabA *pabBC*

Chorismate —*pabA/B*→ 4-amino-4-deoxychorimate —*pabC*→ *p*ABA ———→

Glutamate ————————→

Supplied to medium

FIG. 1. Folate biosynthesis gene cluster of *Lactococcus lactis* (a; black arrows) and *p*ABA biosynthesis gene cluster (b; grey arrows). In the folate biosynthesis pathway GTP is converted via 8 enzymes into tetrahydrofolate (c).

All genes needed for tetrahydrofolate synthesis were identified, except that coding for a dihydroneopterin triphosphate pyrophosphohydrolase. Such a gene was never annotated before. In a search for this gene we carefully studied the folate gene cluster in *Lactococcus lactis*; this gene cluster contains a gene with predicted nucleoside diphosphatase function. To test this hypothesis, *ylgG* (*folQ* in Fig. 1) was disrupted and the enzyme was purified and characterized. Interestingly, the *ylgG*-deletion strain produced 75% lower folate pools when compared to the wild-type strain, suggesting involvement in the folate biosynthesis pathway. By HPLC it was determined that dihydroneopterin triphosphate (DHNTP) accumulated in the deletion strains, showing blockage of the folate biosynthesis pathway. The purified enzyme displayed cleavage of DHNTP into dihydroneopterin monophosphate and pyrophosphate with a 1 to 1 ratio. At 1 mM of Mg^{2+} the enzyme activity was at its maximum, with a K$_m$ for DHNTP of 2.1 μM and a V$_{max}$ of 0.65 μmol min^{-1} x mg^{-1}. In this way, we have established that *ylgG* is the missing gene in the folate biosynthesis pathway of the lactic acid bacterium *Lactococcus lactis*. For consistency reasons *ylgG* was renamed *folQ* (Fig. 1).

A BLAST search of *folQ* on the genome *Arabidopsis thaliana* showed that 20 genes contain the nudix-motif (which is typical for *folQ*). Five of these genes were found to have sufficient overall homology, but *At1g68760* displayed the most identity with *folQ*. The protein At1g68670 was overexpressed, purified and characterized. The purified enzyme was able to

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convert DHNTP into DNMP and pyrophosphate with a 1 to 1 ratio, the enzyme was also dependent on Mg^{2+} for the conversion, showing that this gene is involved in the folate biosynthesis pathway of plants. One distinction between the two purified enzymes is that At1g68670 is able to cleave pyrophosphates from other components than DHNTP, showing that this enzyme is less specific. This lower specific activity is also reflected in the high K_m and lower V_{max} for At1g68670 on DHNTP. In summary, we have identified the missing gene of the folate biosynthesis pathway of bacteria and plants. A methodology that is presented in this chapter can be used for the identification of novel genes, specifically for uncharacterized genes, which are present in a gene cluster. One such an example would be the uncharacterized gene, which is annotated as *xtp2* in the folate gene cluster of *L. plantarum*.

We describe the role of the *para*-aminobenzoic acid in the production of folate by *Lactococcus lactis* in **Chapter 4**. On the genome of *Lactococcus lactis*, only two of the three genes for *p*ABA production were identified. The gene, *pabC*, coding for 4-amino-4-deoxychorsimate lyase, was initially not detected. Remarkably, *pabB*, one of the two annotated *p*ABA genes of *Lactococcus lactis*, appeared longer than *pabB* genes found in other species. By BLAST analysis it was shown that *pabB* of *Lactococcus lactis* is most likely a fusion-gene, whereby, *pabB* and *pabC* are fused (Fig. 1). Therefore, we hypothesized that *Lactococcus lactis* possesses all genes needed to synthesize *p*ABA. The presence, activity, and physiological role of the *p*ABA gene cluster (Fig. 1) were evaluated by disruption and overexpression of the latter gene cluster. The disruption of the *p*ABA gene clusters led to complete abolishment of folate production. In the absence of *p*ABA, nucleobases and nucleosides, growth of the *p*ABA-deletion strain was severely impaired, showing that under these conditions, folate production is essential for growth. The addition of nucleobases and nucleosides could restore the growth of the *p*ABA-deletion strain, showing that folate production is not required when nucleobases and nucleosides are supplied to the growth medium. The overexpression of the *p*ABA gene cluster resulted in intracellular *p*ABA pools of 7.8 mg/L per OD_{600}, showing that this gene cluster indeed contains all genes needed for the synthesis of *p*ABA. In addition, folate pools remained unaffected by *p*ABA overproduction, suggesting that the flux through the folate pathway becomes limiting for high folate production. Previously, a folate overproduction strain was constructed (Fig. 1, from *folB* to *folC*). For increased folate production this strain was dependent on supplementation of *p*ABA to the growth medium. In this study we have constructed a strain that simultaneously overexpressed the *p*ABA and folate gene cluster. The resulting strain was able to produce 2.8 mg/L folate per OD_{600} in the absence of exogenous *p*ABA. In conclusion, the sole overexpression of the *p*ABA and folate gene cluster can not boost folate production. Instead, when the genes for both pathways are overexpressed simultaneously, high folate levels can be obtained. From this it can be reasoned that the expression of both gene clusters appears to be balanced in the wild-type strains of *Lactococcus lactis*. We believe that *p*ABA uptake is mediated by passive transport via the membrane in undissociated form. The genome of *L. plantarum* appears to lack the genes for *p*ABA production. An attempt to transfer the *p*ABA gene cluster to *L. plantarum* did not result in detectable production of *p*ABA. However, it was observed that folate production pools were slightly increased. These results led us to the conclusion that the production of *p*ABA precursors in *L. plantarum* is not sufficient to produce *p*ABA in high amounts. This chapter also shows the importance of *p*ABA for the production of folate.

We described the conversion of a folate-consuming bacterium into a folate-producing bacterium in **Chapter 5**. The five genes for folate biosynthesis in *Lactococcus lactis* were cloned on a broad-host-range lactococcal vector and were transferred to the folate auxotroph *L. gasseri*. The resulting strain was able to grow in the absence of folate and produce a detectable amount of folate. This principle can be used to increase folate levels in many fermented food products. Moreover, consumption of this folate producing probiotic strain could help to increase the folate status in humans, specifically when the produced folate is released during passage in the gastrointestinal tract. This is important since folate-mal intake is associated with a number of health problems (4, 5, 11, 15).

In **Chapter 6**, we have evaluated the effect folate overproduction on plasmid maintenance in *L. plantarum*. First, the folate gene clusters of several lactobacilli were compared with the known folate gene cluster of *Lactococcus lactis*. The folate gene cluster of *L. plantarum* does not posses a gene homologous to *folQ* (chapter 3). Instead, *xtp2* coding for xanthosine triphosphate pyrophosphatase was identified. Since it is predicted that Xtp2 has nucleotide pyrophosphatase activity (just as FolQ) and the gene is located in the folate gene cluster, we hypothesized that *xtp2* is involved in folate production in *L. plantarum*. The folate gene cluster of *L. plantarum* was cloned on a high copy plasmid vector and transferred to *L. plantarum*. This resulting strain was able to produce high folate levels (3.29 mg/L folate per OD_{600} unit) when compared to the control strain (9.6 µg/L folate per OD_{600} unit). Similar to what has been described in **Chapter 4**, high folate production levels could only be achieved in the presence of exogenous *p*ABA. The control strain and the folate overproducer were unable to produce folate when *p*ABA was omitted from the growth medium. This coincides with the notion that the *p*ABA biosynthesis genes are not present in genome of *L. plantarum*. Remarkably, it was noticed that the overproduction of folate leads to a 25% reduction in growth rate. Propagation of the folate overproduction strain in the absence of chloramphenicol results in a rapid drop in copy number of the plasmid. In the presence of chloramphenicol, the drop in plasmid copy number was significantly lower. In the control strain (empty vector), no drop in copy number was detected, suggesting that overexpression of the folate gene cluster causes the decline in plasmid copy number. The correlation between plasmid copy number, folate production and growth rate was monitored for single colony isolates. It was found that low folate-producing single colony isolates displayed an increased growth rate and a reduced copy number. This contrast with the high folate-producing single colony isolates: that have a reduced growth rate and a high plasmid copy number. These experiments clearly show that in competition experiments high folate-producing cells will be rapidly outgrown by low folate-producing cells. Sequence analysis of the plasmids in low folate -producing single colony isolates has shown that a large part of the plasmid was lost, probably through plasmid rearrangements. To prevent folate-production-loss in, for example, an industrial application, it is essential that the number of generations by the master-inoculant is kept low. Especially, if the production of specific metabolites results in a growth rate reduction. The use of alternative, food-grade selection markers would help to reduce plasmid loss and supports the maintenance of the desired phenotype for more generations. Alternatively, chromosomal integration of genes with efficient promoters can reduce the instability issues addressed here, however, the protein expression levels will be lower by this approach.

The physiological response to folate overproduction in *L. plantarum* is described in **Chapter 7**. In Chapter 6, we have described the homologous overexpression of the folate gene cluster in *L. plantarum* leading to a large increase in folate pools. By a functional genomics approach we have evaluated the impact of folate overproduction on the global gene expression profile and metabolite production in *L. plantarum*. The differential metabolome showed that 18 metabolites were significantly differentially abundant in the folate-overproducing strain. Five of these metabolites could directly be linked to the production of folate. The highest differentially produced metabolite was 10-formyl folic acid. Overexpression of the folate gene cluster in the absence of exogenous *p*ABA was found to block the folate biosynthesis pathway and resulted in accumulation of 6-hydroxymethylpterin.

DNA microarray analysis of the mRNA transcripts showed that none of the genes of *L. plantarum* were differentially expressed in a significant way in response to the high folate pools. However, it was found that in continuous culture and batch culture 8 and 11 genes, respectively, were significantly up- or downregulated not because of the high folate pools, but as a result of overexpression of the folate gene cluster. This distinction is made since it was found that the overexpression of the folate gene cluster had a severe impact on the growth rate.

We were quite puzzled by the low transcriptional and metabolome response, since the growth rate of the folate-overproducing strain is reduced by 25%. Using the leads from the functional genomics analysis we could pinpoint several potential causes for the growth rate reduction. With use of the genome scale metabolic model it was predicted that nearly 30% of the GTP of the cell is stored as folate, showing that folate production imposes a enormous drain on GTP demand. We believe this can be a factor in explaining the growth rate reduction. Another factor that could explain the growth rate reduction of the folate gene cluster overexpression strain is the accumulation of amounts of folate specific mRNAs and proteins. In the folate gene cluster overexpression strain, the folate mRNA's constitute in between 8-30% of the total mRNA pools, whereas the folate biosynthesis proteins constitute 4-10% of the total protein content. We believe that the increased demand for GTP combined with the accumulation of folate specific mRNA's and proteins can have an effect on the growth rate.

The use of functional genomics tools such as metabolomics, transcriptomics and metabolic modeling has increased our understanding of the effect of high folate levels on the behavior of the entire micro-organism. Moreover, this approach gave us insight in potential mechanism to explain the growth rate reduction of the folate gene cluster overexpressing strain. This knowledge can be used for the development of strains that produce high folate levels without a growth rate reduction. These strains should have an increased production of GTP combined with an increased abundance of all components needed for the transcription/translation machinery.

Chapter 8 describes the development of a minimal growth medium for *L. plantarum*. The composition of the chemically defined medium (CDM) which has been used throughout this study has been chosen as the starting point for this the composition of the minimal medium. However, CDM is a rich medium containing a number of components that are not essential for growth of *L. plantarum*. All non-essential components were stepwise omitted from CDM and the resulting minimal medium was named PMM5. Single omission of each of the medium components in PMM5 abolishes growth completely. However, the biomass formation of *L. plantarum* on PMM5 is limited to a level of only 35 mg/L dry weight (DW)

(which corresponds with an OD_{600} of 0.1). Therefore, reproducible growth experiments in screw-capped tubes and 96-well plates can not be performed well using this medium. To design a practical growth medium, the following components were re-added to PMM5: pyridoxamine, cysteine and ammonium citrate. The resulting medium, PMM7, enabled proper growth of *L. plantarum*, thereby reaching a final biomass of 350 mg/L DW (OD_{600} of 1.0). The genome-scale metabolic model of *L. plantarum* predicted that no growth was possible on PMM5 and PMM7 since biosynthesis of folate and folate-dependent metabolites (nucleobases, nucleosides, glycine and serine) was expected to be impossible. To study this discrepancy, we analyzed the growth performance and ability to produce folate on this medium in the presence and absence of *p*ABA. The folate-production levels differed approximately 12000-fold after cultivation in the absence and presence of *p*ABA. At the same time, the growth performance was almost identical on both variants of the growth medium. From these experiment it was concluded that a large reduction in folate production pools has no impact on the growth performance of *L. plantarum*. However, it remains unclear how biosynthesis of purines, pyrimidines and certain amino acids proceeds with such low folate pools.

The described minimal media can have several applications, for example, for revealing specific phenotypes of knock-out mutant strains. Another application is to determine discrepancies between the presence of genes and the observed metabolic potential. Such an approach can support the discovery of new metabolic pathways or activities.

Folate overproduction in *L. plantarum* WCFS1 was found to lead to resistance to methotrexate (MTX) (**Chapter 9**). There are several ways for an organism to acquire resistance against the folate antagonist MTX. However, to our knowledge folate overproduction has never been proposed as a potential mechanism. As described in **Chapters 6 and 7**, a folate-overproducing strain of *L. plantarum* was constructed. The growth rate of the control and folate-overproducing strain were determined in the presence of MTX. The control strain was unable to grow in the presence of 2.5 mg/L MTX, whereas the folate-overproducing strain was not hampered by this concentration of MTX. Plating of the control and folate-overproducing strain on CDM plates with 2.5 mg/L MTX, revealed that all folate-overproducing cells were able to grow, whereas only 1 in every 500.000 cells were naturally resistant to 2.5 mg/L MTX. From these experiments we conclude that folate overproduction can serve as a mechanism against MTX resistance. We have isolated a total of 576 colonies that show resistance towards MTX. These cells were screened for increased folate production levels. Interestingly, one single colony isolate displayed a 70% increased folate pools, showing that MTX resistance can be used as a pre-screening method for natural folate-overproducing strains. A total of 1920 mutagenized MTX-resistant single colonies were screened for increased folate production pools. Two single colonies were able to produce 30% increased folate levels. Remarkably, these increased folate-pools were rapidly lost upon propagation, suggesting that a specific mutation may be acquired, which may have caused that the folate-overproduction phenotype is lost.

In summary, from these experiments it can be concluded that folate overproduction serves as a potential mechanism against MTX resistance. The selection of natural non-GMO folate overproducers can support the development of cultures that increase the folate content of fermented food products.

The combined production of folate and B12 by *Lactobacillus reuteri* is described in **Chapter 10**. In Europe is fortification of food products with folate not advised, since high folate intake can mask symptoms associated with B12-deficiency in humans (8). In this study, we have demonstrated the possibility for combined production of folate and B12 in *L. reuteri*. Based on the draft genome sequence of *L. reuteri* JCM1112, it was predicted that this organism appears to be capable to produce folate and B12. The biosynthesis route of B12 is complex and involves a total of 26 genes, which reflects to the complexity of the vitamin B12 molecule itself. The biosynthesis pathway of folate and B12 only share glutamate as common precursor (Fig. 2).

Strain-culture condition	OD_{600}	80% Melon+PABA	
		Folate (ug/L)	B12 (ug/L)
uninoculated	0.00	22	0
L. reuteri JCM1112	2.48	328	31
L. reuteri control	1.73	318	25
L. reuteri folate overproducer	2.95	7451	34
L. plantarum WCFS1	3.00	288	0
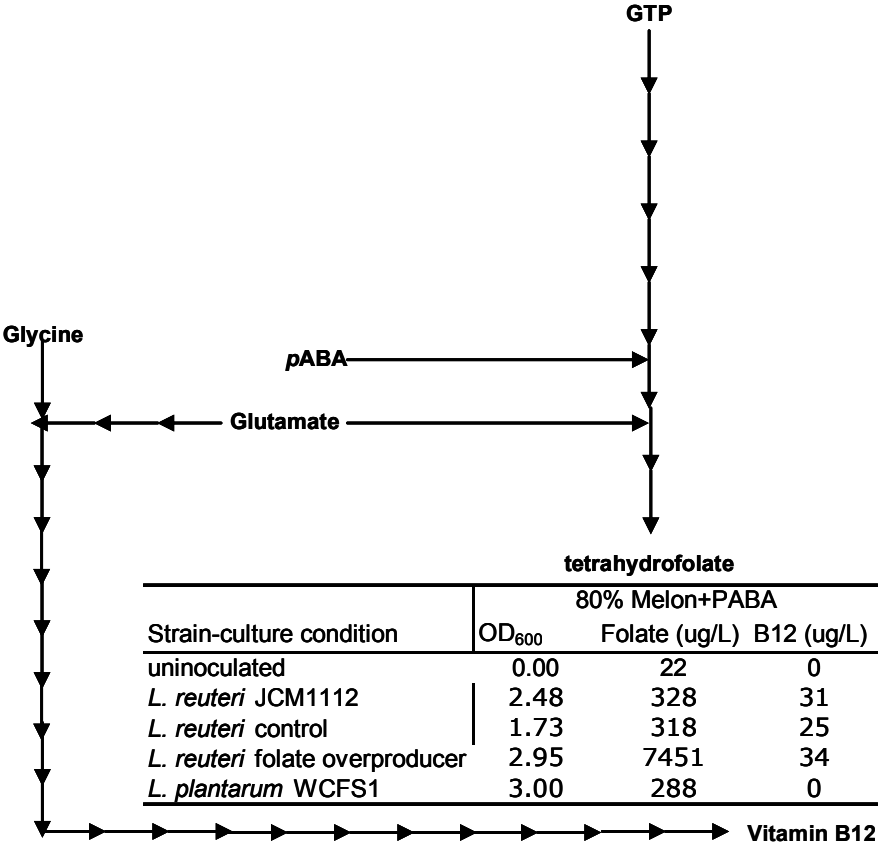
FIG 2. Simplified scheme of the folate and vitamin B12 biosynthesis pathway, with the precursors, insertions and end products of both pathways. Folate and vitamin B12 production levels are shown in µg/L in 80% melon juice supplied with *p*ABA.

On chemically defined media (CDM) the *L. reuteri* wild-type was able to produce folate and B12 in equivalent weight ratio (w/w). We implemented, as proof of principle, a metabolic engineering strategy to modulate the ratio of production of these two vitamins. Overexpression of the folate gene cluster of *L. plantarum* in *L. reuteri* was found to be a

suitable method to increase the folate/B12 ratio to 100:1 on CDM (this is close to the desirable RDA intake ratio of folate and B12 (170 to 1; w/w) for humans). Now that we were able to modulate the suitable folate/B12 production ratio, we assessed its applicability to fruit fermentations. On fermented melon juice, the engineered *L. reuteri* strain was able to achieve a folate/B12 ratio of 220:1, but provided that *p*ABA was supplied to the melon juice.

Remarkably, the *L. reuteri* wild-type is capable to produce 5 times more folate on melon juice when compared to chemically defined medium (CDM). This surprising finding was also observed for *L. plantarum*, this organism was capable to produce 10-fold higher folate levels on melon juice in comparison to CDM. Finally, it was tested whether increased folate levels could be achieved on other fruit juice, such as cucumber-juice. However, both tested lactobacilli were not able to produce more folate on cucumber juice. From these experiments we can conclude that melon juice contains components that can increase the folate production capabilities, and these components are absent from cucumber-juice.

The findings reported here may lead to the development of (fermented) foods based on perishable fruits, such as melons, with an extended durability and higher nutritional value. A well-tasting melon juice or melon squash, containing high folate and vitamin B_{12} could be the start of a longer shelf-life product line specially targeting vitamin deficient populations. Noteworthy, the *L. reuteri* wild-type strain can be directly implemented in food products, whereby no GMO strain is used.

Future outlook

Knowledge of the folate biosynthesis genes and the metabolic pathway in several folate-producing lactic acid bacteria enabled us to modulate the folate levels more than a million-fold, from 1 ng/L to 3.3 mg/L folate for *L. plantarum* (Table 1).

TABLE 1: Total vitamin levels (µg/L) for several media and strains.

Strain and plasmid	CDM	PMM7[a]	Melon	FACM	Other	Vitamin	Reference
L. plantarum WCFS1	18	0.001	270			Folate	(7) (Chapter 8,10)
L. plantarum pNZ7021	30					Folate	(Chapter 7)
L. plantarum pNZ7026	3300					Folate	(Chapter 7)
L. gasseri ATCC 33323				ND		Folate	(17)
L. gasseri pNZ7019				75		Folate	(17)
L. reuteri JCM1112	60		310			Folate	(Chapter 10)
L. reuteri pNZ7021	70		300			Folate	(Chapter 10)
L. reuteri pNZ7026	4500		7400			Folate	(Chapter 10)
Lactococcus lactis MG1363	100					Folate	(14)
Lactococcus lactis pNZ8148	100					Folate	(18)
Lactococcus lactis pNZ7019	6700					Folate	(18)
Lactococcus lactis CB010	1500					Riboflavin	(13)

[a], PMM7 without *p*ABA

Abbreviations used; CDM, chemically defined medium; PMM7, plantarum minimal medium7; FACM, Folic Acid Casei Medium; ND, not determinded.

Previously other vitamins such as riboflavin and B12 were also overproduced using metabolic engineering or random mutagenesis, respectively. The level of riboflavin overproduction (Table 1) is in the same order as folate overproduction. However, bacteria such as *Propionibacterium freudenreichii*, are able to produce B12 levels that are nearly two orders of magnitude higher than folate and riboflavin. Since vitamin B12 is very difficult to synthesize chemically and it is important for human health, much effort has been put in

increasing the B12 production levels by bacteria. Vitamins such as folate and riboflavin, on the other hand, are easy to synthesis chemically. However, recently, there is some debate on whether the intake of high amounts of synthetic folate can have adverse health effects (10). It was proposed that eating natural-folate-containing food-sources can be almost as efficient in improving the folate status as compared to folate-fortification of food products, but then without these presumed health problems (10, 20).

Essential for the ability to modulate folate pools is the presence or absence of *p*ABA and the level of expression of the folate gene cluster in these bacteria. For fermentation fortification it is desirable that *p*ABA is present in the food substrate, especially when the food product is inoculated with a *p*ABA auxotrophic strain. One presumed *p*ABA auxotrophic strain is *L. plantarum*. Remarkably, this strain was able to grow in the absence of *p*ABA. The level of folate production in the absence of *p*ABA is highly reduced. Therefore, it remains unclear how purines, pyrimidines and amino acids can be synthesized under conditions of low folate pools. Possibly these *p*ABA auxotrophic strains can produce an alternative one-carbon donor when cultivated in the absence of *p*ABA. These alternative one-carbon donors are still waiting to be discovered. One approach to do so is by using a functional genomics approach whereby the differential-metabolomics is combined with transcriptomics (similar to the approach described in Chapter 7). One could compare the formed metabolites and expressed genes on the minimal medium (PMM7; Chapter 9) in the presence and absence of *p*ABA.

The increased production of folate on melon juice is a nice example of fermentation modification. This example raises the question whether fruit-juices of different origin can also be used to increase the folate content. The use of functional genomics can be of great help to determine the cause for the increased folate production on melon juice. In this thesis we have been shown successful in modulation of folate production in several lactic acid bacteria. The data that is gathered from these experiments may pave the way for development of food-products that can increase the folate status of animals or humans.

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Appendix

Reactions for one-carbon metabolism and folate and *p*ABA production.

Enzyme	E.C. #	Functional Description	Reaction
FolE	3.5.4.16	GTP cyclohydrolase I	2 H2O + GTP = dihydroneopterin triphosphate + formate
FolQ	3.6.1.-	Dihydroneopterin triphosphate pyrophosphohydrolase	7,8-dihydroneopterin triphosphate = 7,8-dihydroneopterin monophosphate + PPi
		Aspecific phosphatase	7,8-dihydroneopterin monophosphate = 7,8-dihydroneopterin + P
FolB	4.1.2.25	Dihydroneopterin aldolase	dihydroneopterin = glycolaldehyde + 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine (ahhdhp)
FolK	2.7.6.3	2-Amino-4-hydoxy-6-hydroxymethyldihydropteridine diphosphokinase	ahhdhp + ATP = 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate (ahhdhpdp) + AMP
FolP	2.5.1.15	Dihydropteroate synthase	*para*-aminobenzoate + (ahhdhpdp) = 7,8-dihydropteroate + pyrophosphate
FolC1	6.3.2.12	Dihydrofolate synthase	L-glutamate + 7,8-dihydropteroate + ATP = 7,8-dihydrofolate + phosphate + ADP
FolC2	6.3.2.17	Tetrahydrofolylpolyglutamate synthase	L-glutamate + H4PteGlu(n+1) + ATP = H4PteGlu(n) + phosphate + ADP
FolA	1.5.1.3	Dihydrofolate reductase	NADP + THF = NADPH + 7,8-dihydrofolate
Fhs	6.3.4.3	Formate-tetrahydrofolate ligase	ATP + formate + THF = ADP + phosphate + 10-formyl-THF
MetF	1.5.1.20	5,10-Mmethylenetetrahydrofolate reductase	5-methyltetrahydrofolate + NAD(P)+ = 5,10-methylenetetrahydrofolate + NAD(P)H + H+
MetF2	1.7.99.5	5,10-Methylenetetrahydrofolate reductase (FADH2)	5-methyl-THF + NAD = 5,10-methylene-THF + NADH
FthC	6.3.3.2	5-Formyltetrahydrofolate cyclo-ligase	5-formyl-THF + ATP = 5,10-methenyl-THF + ADP + phosphate
FolD	3.5.4.9	Methenyltetrahydrofolate cyclohydrolase	H2O + 5,10-methenyl-THF = 10-formyl-THF
FolD	1.5.1.5	Methylenetetrahydrofolate dehydrogenase (NADP+)	5,10-methylene-THF + NADP = NADPH + 5,10-methenyl-THF
PurN	2.1.2.2	Phosphoribosylglycinamide formyltransferase	10-formyl-THF + 5-phospho-ribosyl-glycineamide = THF + 5'-phosphoribosyl-N-formylglycineamide
PurH	2.1.2.3	AICAR transformylase	10-formyl-THF + AICAR = THF + phosphoribosyl-formamido-carboxamide
Fmt	2.1.2.9	Methionyl-tRNA formyltransferase	10-formyl-THF + H2O + L-methionyl-tRNAmet = tRNAfmet + THF
ThyA	2.1.1.45	Thymidylate synthase	dUMP + 5,10-methylene-THF = dTMP + 7,8-dihydrofolate
ThyX	2.1.1.-	Thymidylate synthase thyX	5,10-methylenetetrahydrofolate + dUMP + FADH2 = dTMP + tetrahydrofolate + FAD
GlyA	2.1.2.1	Glycine hydroxymethyltransferase	L-serine + THF = 5,10-methylene-THF + L-glycine + H2O
MetH	2.1.1.13	N5-Methyltetrahydrofolate methyltransferase	homocysteine + 5-methyl-THF = L-methionine + THF
MetE	2.1.1.14	5-Nethyltetrahydropteroyltriglutamate-- homocysteine S-methyltransferase	homocysteine + 5-methyltetrahydropteroyltri-L-glutamate = L-methionine + tetrahydropteroyltri-L-glutamate
PabA	6.3.5.8	Para-aminobenzoate synthase glutamine amidotransferase component II	chorismate + L-glutamine = 4-amino-4-deoxychorismate + L-glutamate
PabB	6.3.5.8	Para-aminobenzoate synthetase component I	chorismate + L-glutamine = 4-amino-4-deoxychorismate + L-glutamate
PabC	4.1.3.38	4-Amino-4-deoxychorismate lyase	4-amino-4-deoxychorismate = pABA + pyruvate

Samenvatting en Conclusies

      Verscheidene methoden kunnen worden toegepast om de inname van foliumzuur door consumenten te verhogen. De mogelijkheden zijn onder meer: i) voedingsproducten fortificeren met foliumzuur, ii) inname van foliumzuur door middel van pillen vergroten, iii) planten consumeren met verhoogde foliumzuurniveaus, of iv) voedingsproduct verrijken met foliumzuur door deze met foliumzuur producerende schimmels of bacteriën te laten fermenteren, ook wel fermentatiefortificatie genoemd. Het belang van het onderzoek in dit proefschrift ligt hoofdzakelijk op het proces van fermentatiefortificatie. Voor het zinvol toepassen van fermentatiefortificatie is het belangrijk om inzicht te krijgen in de biosynthesegenen van foliumzuur, maar ook in de biosyntheseroute van foliumzuurproductie. In ons onderzoek hebben wij de productieniveaus van foliumzuur gemoduleerd in vier verschillende melkzuurbacteriën: *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus gasseri* en *Lactococcus lactis*. Verschillende methoden zijn gebruikt om de foliumzuurproductieniveaus van deze bacteriën te veranderen door: i) het identificeren van de genen die betrokken zijn bij het maken van foliumzuur, ii) foliumzuurgenen tot verhoogde expressie te brengen, iii) de samenstelling van het groeimedium variëren, iv) gebruik van methoden om natuurlijke overproducerende stammen van foliumzuur te selecteren, v) selectie van een nieuwe voedselmatrix. Daarnaast hebben wij ook gekeken naar de invloed van foliumzuur-overproductie op de fysiologie van *Lactobacillus plantarum*. Hieronder geven wij een samenvatting en conclusies op elk van de beschreven hoofdstukken en eindigen wij vervolgens met een kijk op de toekomst met betrekking tot dit onderzoek.

      In **hoofdstuk 1** wordt de achtergrond van dit onderzoek beschreven. Ook geven wij een algemene introductie over melkzuurbacteriën, foliumzuur en de productie van foliumzuur.

      Daarnaast worden de hoofdstukken in dit proefschrift kort beschreven. **Hoofdstuk 2** geeft een literatuuronderzoek over de rol van foliumzuur en de metabolieten die worden gemaakt met behulp van foliumzuur in bacteriën; tevens wordt hier de functie van de foliumzuurachtige component beschreven in Archaea. Het is goed gedocumenteerd in literatuur dat foliumzuur nodig is als C1-donor voor de biosynthese van purines, pyrimidines en aminozuren. Maar foliumzuur is ook nodig voor het initiëren van eiwitsyntheses. Er zijn verschillende artikelen die beschrijven dat sommige biochemische reacties echter plaats kunnen vinden in afwezigheid van foliumzuur. Veel van deze voorbeelden worden in dit literatuuronderzoek beschreven. In Archaea wordt foliumzuur niet gebruikt als C1-donor. Deze organismen gebruiken daarentegen methanopterin als C1-donor. Genoemde component is structureel gerelateerd aan foliumzuur, maar kan niet worden gebruikt door bacteriën die afhankelijk zijn van foliumzuur als C1-donor. Bepaalde bacteriën en alle Archaea zijn in staat om in afwezigheid van foliumzuur een formyl-groep te doneren aan methionyl-tRNA. In

melkzuurbacteriën kan de noodzaak voor foliumzuur omzeild worden als de purines, pyrimidinen en aminozuren worden toegevoegd aan het groeimedium.

Vergelijkend genoomonderzoek heeft aangetoond dat bacteriën zoals *Richettsia rickettsii* and *Borrelia burgdorferi* enkele foliumzuur biosynthesegenen missen die als essentieel worden beschouwd. Een andere stam blijkt totaal onafhankelijk te zijn van foliumzuur voor de synthese van purines, pyrimidines, aminozuren en formylatie van methionyl-tRNA. Al de bovengenoemde bacteriën zijn intracellulair pathogene bacteriën die waarschijnlijk instaat zijn bepaalde types foliumzuur opnemen. Het uitschakelen van *folA* en *thyA* in *Escherichia coli* heeft ertoe geleid dat alternatieve routes tot expressie worden gebracht die er voor zorgen dat groei kan plaats vinden, alhoewel foliumzuur eigenlijk niet gemaakt kan worden. Meer onderzoek naar nieuwe foliumzuur biosynthesegenen kunnen onze kennis van de biosyntheseroute van foliumzuur vergroten en kunnen tevens helpen bij de zoektocht naar alternatieve C1-donoren anders dan foliumzuur.

De ontbrekende enzymatische stap in de foliumzuur biosyntheseroute van bacteriën en planten wordt beschreven in **hoofdstuk 3**. De biosyntheseroute van foliumzuur vindt plaats via acht enzymatische stappen, waarbij GTP wordt omgezet in tetrahydrofolate (Fig. 1, pagina 151). Elk van de enzymen in de biosyntheseroute van het foliumzuur zijn geïdentificeerd, behalve het enzym wat codeert voor dihydroneopterin thiphosphate pyrophospholydrolase. Het gen wat codeert voor dit enzym was nog nooit eerder geïdentificeerd in welk organisme dan ook. In een zoektocht naar dit gen hebben wij het foliumzuur gencluster van *Lactococcus lactis* bestudeerd en een onbekend gen (*ylgG*) gevonden. Dit gen heeft een vermeend nudix-motief, wat indicatief is voor pyrophosphohydrolase-activiteit. Om dit gen te correleren aan foliumzuurbiosynthese, werd dit gen uitgeschakeld. Het resultaat was dat er 75% minder foliumzuur geproduceerd werd, een indicatie dat dit gen betrokken is bij de foliumzuurbiosynthese. Met behulp van HPLC is bepaald dat uitschakeling van *ylgG* leidt tot ophoping van dihydroneopterin trifosfaat (DHNTP), wat laat zie dat de foliumzuurroute net voor *ylgG* geblokkeerd is. Het gezuiverde enzym was in staat om DHNTP om te zetten in dihydroneopterin monofosfaat en pyrofosfaat; dit enzym had een K_m voor DHNTP van 2.1 µM en een V_{max} van 0.65 µmol min^{-1} x mg^{-1}. Hierdoor hebben wij bepaald dat *ylgG* het ontbrekende gen is in de foliumzuurroute van de melkzuurbacterie *Lactococcus lactis*. Om consistentieredenen is *ylgG* hernoemd tot *folQ*.

Door middel van een BLAST-analyse van *folQ* op het genoom van *Arabidopsis thaliana*, is gevonden dat dit genoom 20 genen bevat die ook een nudix-motief bezitten. Eén van deze genen, namelijk *Atlg68760,* is het meest homoloog aan *folQ*. Het eiwit Atlg68760 is tot overexpressie gebracht, gezuiverd en gekarakteriseerd. Het gezuiverde eiwit was in staat om DHNTP om te zetten in dihydroneopterin monofosfaat en pyrofosfaat. Echter, een klein verschil tussen FolQ en Atlg68760 is dat Atlg68760 in staat is om andere componenten dan DHNTP om te zetten (alleen maar nucleotide-trifosfaten). Samenvattend: het ontbrekende gen in de foliumzuurroute van bacteriën en planten is

ontdekt. Een methodologie die in dit hoofdstuk is beschreven kan gebruikt worden voor het identificeren van onbekende genen in genclusters. Een voorbeeld voor een niet-geïdentificeerd gen is een onbekend gen in het foliumzuurgencluster van *Lactobacillus plantarum*, *xtp2*.

In **hoofdstuk 4** wordt de invloed van *para*-aminobenzoinezuur (*p*ABA) biosynthese op het produceren van foliumzuur door *Lactococcus lactis* beschreven. Op het genoom van *Lactococcus lactis* zijn maar twee (*pabA* en *pabB*) van de drie genen voor *p*ABA-productie beschreven. Het gen, *pabC*, wat codeert voor 4-amino-4-deoxychorismate lyase, werd in eerste instantie niet gevonden. Opmerkelijk was dat *pabB* van *Lactococcus lactis* groter is dan *pabB* in andere organismen. Door middel van BLAST-analyse bleek dat *pabB* in *Lactococcus lactis* gefuseerd is met *pabC* (Fig. 1, pagina 151). Hierdoor nemen wij aan dat *Lactococcus lactis* alle genen heeft om *p*ABA te maken. Vervolgens hebben wij de aanwezigheid, activiteit en fysiologische rol van dit *p*ABA-gencluster geëvalueerd. Dit hebben wij gedaan door het gencluster uit te schakelen en tot overexpressie te brengen. Het uitschakelen van dit gencluster leidt er toe dat de foliumzuurproductie gestopt wordt. In afwezigheid van *p*ABA, nucleobasen en nucleosiden is de groei van deze deletiestam gereduceerd, wat laat zien dat onder deze conditie de foliumzuurproductie essentieel is voor groei. Groei van deze deletiestam is wel mogelijk wanneer nucleobasen en nucleosiden worden toegevoegd aan het groeimedium. Dit toont aan dat op dit medium de productie van foliumzuur niet essentieel is.

Het tot overexpressie brengen van het *p*ABA-gencluster in *Lactococcus lactis* resulteert in een ophoping van *p*ABA. Dit experiment laat zien dat alle drie genen die nodig zijn voor het maken van *p*ABA aanwezig zijn in het *p*ABA-gencluster. Echter, het over-produceren van *p*ABA resulteert niet in een verhoogde foliumzuurproductie. Dit suggereert dat de expressie van de foliumzuurbiosynthesegenen beperkend zijn voor verhoogde foliumzuurproductie. In voorgaand onderzoek is een *Lactococcus lactis*-stam gemaakt waarbij het foliumzuurgencluster tot overexpressie is gebracht. Deze stam is alleen in staat veel foliumzuur te maken als *p*ABA wordt toegevoegd aan het groeimedium. Vervolgens hebben wij het foliumzuurgencluster en het *p*ABA-gencluster tegelijk tot overexpressie gebracht in *Lactococcus lactis*. Deze stam was in staat om 100 keer meer foliumzuur aan te maken dan de controle-stam. Bovendien was deze stam niet afhankelijk van toevoeging van *p*ABA aan het groeimedium. Hieruit concluderen wij dat wanneer het foliumzuur en het *p*ABA-gencluster afzonderlijk tot overexpressie gebracht wordt, dit niet leidt tot verhoogde foliumzuur-productie. Alleen als beide genclusters tegelijk tot overexpressie gebracht worden leidt dit tot verhoogde foliumzuurproductie. Op basis van dit gegeven kan gesuggereerd worden dat in wildtype-stammen beide biosyntheseroutes gebalanceerd tot expressie worden gebracht. Wij geloven dat de *p*ABA-opname geschiedt via passief transport over het membraan in de ongedissocieerde vorm. Het genoom van *L. plantarum* lijkt alle *p*ABA-biosynthesegenen te ontberen. Wij hebben getracht de *p*ABA-genen van *Lactococcus lactis* over te brengen naar *L.*

plantarum, wat er toe heeft geleid dat er iets meer foliumzuur gemaakt wordt; wij hebben echter geen verhoogde *p*ABA pools gevonden. Hieruit hebben wij afgeleid dat de precursoren van *p*ABA niet voldoende gemaakt worden in *L. plantarum* om de *p*ABA-productie te vergroten. In dit hoofdstuk tonen wij het belang aan van *p*ABA op de productie van foliumzuur..

In **hoofdstuk 5** beschrijven wij de transformatie van een stam die foliumzuur consumeert in een stam die foliumzuur produceert. De vijf genen in het foliumzuurgencluster van *Lactococcus lactis* zijn gekloneerd op een hoge-kopie-plasmide. Vervolgens is deze plasmide overgebracht naar de foliumzuurconsumerende bacterie *Lactobacillus gasseri*. Deze getransformeerde stam was in staat om te groeien in afwezigheid van foliumzuur, dit in tegenstelling tot het wildtype. Wij geloven dat dit principe gebruikt kan worden om de foliumzuurniveaus van gefermenteerde voedselproducten te vergroten. Daarnaast zou de consumptie van een dergelijke probiotische, foliumzuurproducerende stam kunnen helpen om de foliumzuurniveaus van mensen te kunnen vergroten. Dit is belangrijk omdat een gebrekkige inname van foliumzuur kan leiden tot bepaalde gezondheidsaandoeningen. Echter het foliumzuur die door *L. gasseri* geproduceerd wordt zou wel moeten worden vrijgelaten gedurende de passage door de darmen. Hier hebben wij momenteel echter geen gegevens over.

In **hoofdstuk 6** beschrijven wij het effect van overproductie van foliumzuur op het plasmidebehoud in *L. plantarum*. Eerst vergelijken wij het foliumzuurgencluster van verschillend lactobacilli met het bekende foliumzuurgencluster van *Lactococcus lactis*. Uit dit vergelijk blijkt dat het foliumzuurgencluster van *L. plantarum* geen gen bezit die homoloog is aan *folQ* (hoofdstuk 3). Wel bevat het gencluster van *L. plantarum* een gen (*xtp2*) met een vermeende vergelijkbare functie, nucleotide triphosphate pyrophosphatase (vergelijkbaar aan *folQ*). Vervolgens is het gencluster van *L. plantarum* gekloneerd op een hoge-kopie-plasmide en getransformeerd naar *L. plantarum*. Deze stam is in staat om veel foliumzuur aan te maken (3.29 mg/L per OD_{600} eenheid), in vergelijking tot de controle-stam (lege vector) (9.6 µg/L per OD_{600} eenheid). Opmerkelijk is wel dat *L. plantarum* niet in staat is om foliumzuur aan te maken in afwezigheid van *p*ABA; dit is in overeenstemming met het ontbreken van de *p*ABA-genen op het chromosoom van *L. plantarum*. Opvallend is wel dat het tot overexpressie brengen van het foliumzuurgencluster in *L. plantarum* leidt tot een 25% reductie in groeisnelheid. Het kweken van de foliumzuur-overproducerende stam in afwezigheid van de selectiemarker (chloramphenicol) leidt tot een snelle reductie van het plasmide-kopie-nummer en de foliumzuurproductie. Het toevoegen van chloramphenicol aan het kweekmedium verhindert dat het plasmide-kopie-nummer en de foliumzuurproductie sterk daalt. Echter de aan- of afwezigheid van chloramphenicol heeft geen invloed op het kopie-nummer van *L. plantarum* met de controle-plasmide. Dit geeft aan dat de oorzaak voor de kopie-nummer-reductie ligt in het tot overexpressie brengen van het foliumzuurgencluster. Als laatste is de relatie tussen het plasmide-kopie-nummer, de foliumzuurproductie en de

groeisnelheid bepaald. Uit dit experiment is gevonden dat isolaten (van een kolonie) die weinig foliumzuur produceren een lager plasmide-kopie-nummer hebben maar daardoor ook een hogere groeisnelheid. Dit staat in contrast met isolaten die veel foliumzuur produceren; genoemde isolaten hebben een hoog plasmide-kopie-nummer en hierdoor een gereduceerde groeisnelheid. In competitieve groei-experimenten zullen hoog-foliumzuurproducerende cellen snel worden overgroeid door laag-foliumzuurproducerende cellen. Sequentie-analyse van een isolaat die weinig foliumzuur aanmaakt laat zien dat een groot gedeelte van de plasmide verloren gaat, waarschijnlijk doordat de plasmide herrangschikt.

In een industriële toepassing is het essentieel om de hoeveelheid generaties laag te houden, vooral als de productie van metabolieten of enzymen tot een reductie in groeisnelheid leiden. Daarom kan het gebruik van selectiemarkers (die geschikt zijn voor voedsel-toepassingen) helpen om het plasmide-kopie-nummer stabiel te houden. Als alternatief zou ook het gen of de genen geïntegreerd kunnen worden op het genoom. Dit heeft echter als nadeel dat de expressie lager zal uitvallen door de reductie in kopie-nummer.

De fysiologische respons van foliumzuur-overproductie in *L. plantarum* is onderzocht in **hoofdstuk 7**. In hoofdstuk 6 hebben wij beschreven dat de homologe overexpressie van het foliumzuurgencluster in *L. plantarum* tot een grote stijging van de foliumzuur productieniveaus leidt. Met behulp van een functionele genomics-benadering hebben wij de impact van overproductie van foliumzuur op het organisme beoordeeld. De differentiële metabolome van *L. plantarum* laat zien dat 18 metabolieten significant verschilden in relatieve hoeveelheid tussen de foliumzuur-overproducerende stam en de controle-stam. Vijf van deze metabolieten hebben wij kunnen correleren aan het overproduceren van foliumzuur. De metaboliet met het hoogste relatieve verschil is 10-formyl-foliumzuur. Daarnaast hebben wij door middel van metabolomics gevonden dat in afwezigheid van *p*ABA de biosyntheseroute van foliumzuur geblokkeerd wordt wat leidt tot ophoping van 6-hydroxymethylpterin. DNA-microarrays analyse laat zien dat geen van de genen specifiek reageerde op hoge foliumzuurniveaus. Wij hebben echter wel aangetoond dat respectievelijk 8 en 11 genen reageerden in continue en batch fermentatie op het tot overexpressie brengen van de foliumzuurgenen (wat anders is dan hoge foliumzuurniveaus). Wij hebben dit onderscheid kunnen maken, omdat het tot overexpressie brengen van deze genen heeft geleid tot een groeivertraging. We geloven er daarom in dat deze genen daarbij betrokken zijn.

Het feit dat maar enkele genen specifiek reageerden op deze groeivertraging is opmerkelijk. We hebben vervolgens enkele mogelijke verklaringen weten te vinden voor de groeisnelheids-reductie. Door gebruik te maken van data die gebruikt is voor het valideren van het metabole-model van *L. plantarum* hebben wij uitgerekend dat ongeveer 30% van de totale hoeveelheid GTP (precursor van foliumzuur) is opgeslagen in foliumzuur. De GTP is een zeer belangrijk molecuul voor de bacterie en men kan zich voorstellen dat als zo'n grote hoeveelheid GTP wordt opgeslagen in foliumzuur dit een

effect kan hebben op de groeisnelheid. Een andere verklaring van de reductie in groeisnelheid zou de grote hoeveelheid foliumzuurspecifieke mRNA's en enzymen kunnen zijn die zich in de cel ophopen na het tot overexpressie brengen van deze genen. Wij hebben gevonden dat de foliumzuur-specifieke mRNA ongeveer 8 tot 30% van de totale mRNA-hoeveelheid uitmaakt. Voor de enzymen varieert dit tussen 4 en 8%. Wij geloven dat verhoogde invoer van GTP in foliumzuur, al dan niet gecombineerd met de ophoping van foliumzuurspecifieke mRNAs en enzymen, kan leiden tot de groeivertraging.

Het gebruik van een functionele genomics-benadering met behulp van metabolomics en transcriptomics heeft ons inzicht van hoge foliumzuurniveaus op de fysiologie van de cel vergroot. Bovendien geeft deze benadering ons inzicht in mogelijke factoren die de reductie in groeisnelheid kunnen verklaren. Deze kennis kan gebruikt worden voor het maken van stammen die hoge foliumzuurniveaus kunnen maken zonder reductie in groeisnelheid. Deze stammen moeten een verhoogde productie van GTP hebben, dit in combinatie met een verhoogde aanwezigheid van alle componenten die nodig zijn in de transcriptie/translatiemachinerie.

In **hoofdstuk 8** beschrijven wij de ontwikkeling van een minimaal groeimedium voor *L. plantarum*. In bijna alle andere eerder genoemde hoofdstukken hebben wij gebruik gemaakt van CDM (chemische gedefinieerd medium) voor het laten groeien van *L. plantarum*. Het CDM bevat een hoeveelheid componenten die niet essentieel zijn voor de groei van *L. plantarum*. In een stapsgewijze benadering worden alle niet-essentiele componenten verwijderd uit dit medium, wat resulteerde in een minimaal medium (PMM5). Elke component die vervolgens uit dit medium verwijderd wordt voorkomt groei van *L. plantarum*. Een nadeel van dit minimale medium is dat de hoeveel biomassa (OD_{600} 0.1) die behaald wordt op dit medium niet veelbelovend is voor reproduceerbare experimenten. Om deze reden hebben wij drie componenten teruggevoegd aan dit medium: pyridoxamine, cysteine en ammonium citrate. Het medium wat hierbij ontstond (PMM7) had een tien keer zo hoge biomassa (OD_{600} 1.0). Het metabole-model voor *L. plantarum* voorspelde dat groei op dit medium door *L. plantarum* niet mogelijk was. Dit is (onder andere) omdat foliumzuur niet gemaakt kan worden op dit medium, hetgeen als essentieel wordt geacht door het model. Er kan geen foliumzuur aangemaakt worden omdat de genen voor *p*ABA-productie ontbreken in *L. plantarum*. Om deze onvolkomenheid in voorspelde groei en gemeten groei te verklaren hebben wij de foliumzuurproductie getest op PMM7 in aan- en afwezigheid van *p*ABA. De productieniveaus van het foliumzuur verschillenden bijna 12.000-voudig na groei op PMM7 in aan- en afwezigheid van *p*ABA. Tegelijkertijd hebben wij gevonden er geen verschil in groeisnelheid en biomass-formatie was voor *L. plantarum* op PMM7 met en zonder *p*ABA, wat aangeeft dat een 12.000-voudige reductie van foliumzuurproductie niet leidt tot vertraging in de groei. Wat vervolgens suggereert dat *L. plantarum* veel minder foliumzuur nodig heeft voor groei dan dat wat hij maakt, mogelijk zal een

alternatieve C1 donor kunnen bestaan in *L. plantarum* die betrokken is bij de productie van purines, pyrimidines en aminozuren.

In **hoofdstuk 9** is onderzocht of overproductie van foliumzuur in *L. plantarum* tot resistentie tegen de foliumzuur analoog methotrexate leidt. Er zijn verschillende manieren waarop een organisme resistentie tegen de foliumzuur analoog methotrexate (MTX) kan verkrijgen. Echter, overproductie van foliumzuur is nooit geopperd als modus van resistentie. In de hoofdstukken 6 en 7 hebben wij een foliumzuur-overproducerende *L. plantarum*-stam beschreven. De foliumzuur-overproducerende stam en de controle-stam hebben wij gebruikt voor het bepalen van de groeisnelheid in aanwezigheid van MTX. Opmerkelijk genoeg is de foliumzuur-overproducerende stam ongevoelig voor MTX, terwijl de controle-stam erg gevoelig is voor deze stof. Bij een concentratie van 2,5 mg/L MTX is de controle-stam niet in staat binnen 60 uur te groeien. Het uitplaten van deze twee stammen op CDM verrijkt met MTX laat vergelijkbare resultaten zien. Alle foliumzuur-overproducerende cellen zijn in staat te groeien op deze platen, terwijl maar één van elke 500.000 controle cellen kan groeien op deze platen. Wij hebben 576 van deze natuurlijk MTX-resistente kolonies geïsoleerd en gescreend of deze in staat waren om verhoogde foliumzuurniveaus te produceren. Eén van deze isolaten produceerde 70% meer foliumzuur in vergelijking tot wildtype-cellen. Dit toont aan dat deze screeningmethode een goede methode is om natuurlijke foliumzuur-overproducerende stammen te selecteren. Vervolgens hebben wij 1920 gemutageniceerde isolaten gescreend voor verhoogde foliumzuurproductie, en twee van deze cellen waren in staat om 30% meer foliumzuur aan te maken. Opmerkelijk genoeg leidt het doorkweken van deze geïsoleerde kolonies tot een verder reductie van foliumzuurproductieniveaus, wat suggereert dat specifieke mutaties geïnduceerd worden met reductie van foliumzuurproducties als resultante.

Samenvattend: uit het bovenstaande experiment kan geconcludeerd worden dat overproductie van foliumzuur als potentieel mechanisme gebruikt kan worden voor MTX-resistentie. Door deze kennis kunnen natuurlijke foliumzuur-overproducerende isolaten geselecteerd worden om voedselproducten te verrijken met foliumzuur door deze geselecteerde stammen.

De gecombineerde productie van foliumzuur en vitamine B12 door *L. reuteri* wordt beschreven in **hoofdstuk 10**. In Europa wordt fortificatie van voedselproducten met foliumzuur niet geadviseerd, omdat een hoge inname van foliumzuur de deficiëntie van vitamine B12 kan maskeren. In dit onderzoek hebben wij de mogelijkheid onderzocht of *L. reuteri* beide vitaminen kan aanmaken. De biosyntheseroute van foliumzuur en B12 hebben weinig overlap (Fig. 2, pagina 156); de enige precursor die gebruikt wordt in beide biosyntheseroutes is glutamaat. Op het chemisch gedefinieerd medium (CDM) is *L. reuteri* in staat om foliumzuur en B12 in gelijke hoeveelheden aan te maken (w/w). Deze ratio wordt echter niet geadviseerd in voedselproducten. Voor de aanbevolen dagelijkse hoeveelheid van foliumzuur en B12 behoort deze ratio 170 op 1 te zijn. Daarom hebben

wij getest of door middel van metabole engineering de natuurlijke foliumzuur/B12-ratio geschikt gemaakt kan worden voor voedingsproducten. Het foliumzuurgencluster van *L. plantarum* is daarom op een hoge-kopie-plasmide geplaatst en getransformeerd naar *L. reuteri*. Deze stam blijkt nu in staat om foliumzuur en B12 aan te maken met een 100 tot 1 ratio. Deze ratio lijkt zeer geschikt voor voedselproducten. Nu de optimale foliumzuur/B12-ratio gemodelleerd kan worden, hebben wij getest of deze tevens op voedselproducten behaald kan worden. Op meloensap is de geëngineerde *L. reuteri*-stam in staat om een hoeveelheid foliumzuur en B12 aan te maken in een verhouding van 220 tot 1. Meloensap is dus een ideaal medium voor het aanmaken van beide vitaminen en dit tevens in een ideale verhouding.

Het is opmerkelijk dat het *L. reuter*-wildtype in staat is om meer foliumzuur te produceren op het meloensap dan op het synthetisch medium (CDM). Bovendien laat *L. plantarum* vergelijkbare resultaten zien. Deze stam is in staat om ongeveer 10 keer meer foliumzuur aan te maken op meloensap dan op het CDM-medium. Deze groeiproeven zijn ook getest op komkommersap. Op dit medium werden echter geen verhoogde foliumzuurproductieniveaus gevonden, wat aangeeft dat meloensap specifieke componenten bevat die foliumzuurproductie door Lactobacilli kunnen vergroten.

Deze vindingen die hier vermeld staan kunnen leiden tot de ontwikkeling van gefermenteerde voedselproducten, zoals meloen, met een verlengde houdbaarheidsdatum en bovendien een verhoogde nutritionele waarde. Wat belangrijk is, is dat gefermenteerd meloensap met een verhoogde foliumzuurinhoud meteen gebruikt kan worden in de voedselindustrie omdat de gebruikte stammen wildtype-stammen zijn.

Toekomst visie

Kennis van de biosynthesegenen van foliumzuur en de metaboleroutes heeft ons in staat gesteld om de foliumzuurniveaus meer dan één miljoenvoudig te moduleren. Deze foliumzuurniveaus varieerden van 1 ng/L tot 3,3 mg/L foliumzuur voor *L. plantarum* (Tabel 1, pagina 157).

Andere vitaminen zoals riboflavine en B12 zijn via random-mutagenese of middels overexpressie plasmiden in verschillende bacteriën eveneens tot verhoogde expressie gebracht. De productieniveaus van riboflavine ligt in dezelfde orde grootte als foliumzuur. Echter, *Propionibacterium freudenreichii* is in staat om B12 te produceren dat twee ordegroottes hoger ligt dan dat wat bereikt is met foliumzuur of riboflavine. Vitamine B12 is een erg complex molecuul. Dit maakt het erg moeilijk om het chemisch te synthetiseren. Vitaminen zoals foliumzuur en riboflavine zijn makkelijk te synthetiseren wat deze componenten erg nuttig maakt voor voedselfortificatie. De laatste tijd is er echter enige twijfel ontstaan over de gezondheidseffecten van chemisch foliumzuur bij mensen. Onderzoekers hebben gevonden dat hoge inname van chemisch foliumzuur kan leiden tot gezondheidsproblemen bij ratten. Deze onderzoekers hebben geopperd dat het type foliumzuur dat gemaakt wordt in natuurlijke voedselproducten niet deze gezondheids problemen opleveren.

Essentieel voor de mogelijkheid om de foliumzuurproductie te modeleren in bacteriën is de aan- of afwezigheid van *p*ABA, maar ook de expressie van de foliumzuurgenen. Voor fermentatiefortificatie doeleinden is het noodzakelijk dat *p*ABA in het voedsel aanwezig is, specifiek als dit voedsel wordt geïnoculeerd met een bacterie die niet in staat is om *p*ABA aan te maken. *L. plantarum* is een dergelijke stam. Opmerkelijk genoeg is deze stam in staat om te groeien in afwezigheid van *p*ABA. Het blijft derhalve onduidelijk of deze stam nog foliumzuur gebruikt om purines, pyrimidines en aminozuren te maken. Het zou kunnen dat deze stam een alternatieve C1-donor maakt. Genoemde alternatieve C1-donor is nog niet ontdekt. Een geschikte manier om deze te ontdekken is door gebruik te maken van functionele genomicsmethoden, zoals metabolomics en transcriptomics (vergelijkbaar zoals uitgevoerd in hoofdstuk 7). Men zou *L. plantarum* op PMM7 in aan- en afwezigheid van *p*ABA kunnen laten groeien. Vervolgens zou door middel van metabolomics and transcriptomics alternatieve C1-donoren kunnen worden geïdentificeerd.

De verhoogde productie van foliumzuur door lactobacilli op meloensap is een goed voorbeeld van fermentatiefortificatie. Het is nog niet helemaal duidelijk waarom deze stammen in staat zijn om veel foliumzuur aan te maken op meloensap. Ook hier zou het gebruik van functionele genomicsmethoden gebruikt kunnen worden om een verklaring te vinden waarom op dit medium zoveel meer foliumzuur gemaakt wordt dan op andere media.

In dit proefschrift hebben wij laten zien dat wij in staat zijn om productieniveaus van foliumzuur behoorlijk goed te kunnen modeleren in verschillende bacteriën. De data die wij hier verkregen hebben kunnen helpen om voedingsproducten te maken die verhoogde foliumzuurniveaus hebben, wat kan helpen om de foliumzuurinnameniveaus bij dieren en mensen te verhogen.

Dankwoord

Na een paar jaar hard werken is het eind in zicht en met nog een paar maanden op de teller maak ik de balans op. Het was een fantastische periode. Ik had op voorhand verwacht dat ik veel zou leren over bacteriën en experimenten. En toegegeven, ik heb over deze zaken inderdaad veel opgestoken. Maar ik had niet verwacht dat ik een expert zou worden in John Travolta-dansen, een witte broek zou kopen, olijven zou gaan eten, mijn collega's zou gaan missen, nog steeds uren kan praten over niets, 35 uur non-stop kan werken, het saaie/zware werken in de kassen zou gaan missen, ben gaan duiken en het werk als AIO straks ga missen waarbij er lekker gevoetbald werd in de zaal en op het lab. Ik wil de mensen toch even persoonlijk bedanken. Het liefst zou ik hieraan vele bladzijden willen wijden om iedereen te bedanken middels super-anekdotes, maar dat kan helaas niet. Maar huivert niet lees verder en zoekt uw naam.

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Curriculum Vitae

Arno Wegkamp werd geboren op 15 december 1975 te Weiteveen. In 1992 behaalde hij het MAVO-diploma aan het St. Michael te Klazienaveen. In hetzelfde jaar besloot hij om naar het Middelbaar Laboratorium Onderwijs (MLO) te Emmen te gaan waar hij de BACO-richting volgde: Biochemisch Analytisch Chemisch Onderwijs. In 1996 is Arno verder gaan studeren aan het Hoger Laboratorium Onderwijs te Groningen waar hij zich specialiseerde in biotechnologie. De stage en het afstudeerproject voerde hij uit bij het biologisch centrum van de universiteit van Groningen te Haren. Tijdens zijn afstudeer-project deed hij onderzoek naar de populatiegenetica van het zeewier '*Ascophyllum nodosum*'. Hij keek binnen de populatie naar de verdeling van DNA-microsatellieten waarbij de genetische verwantschap bepaald kan worden. Na het behalen van zijn diploma in 1999 is Arno als analist gaan werken voor de afdeling proceskunde aan de universiteit van Wageningen waar hij onderzocht hoe hybridoma-cellen op plantaardige hydrolisaten kunnen worden gekweekt in perfusieculturen. In 2001 is Arno een klein jaar gaan werken aan een project binnen het Wageningen Centre for Food Sciences, waar hij hielp bij het opstellen van een metabole flux-model voor de melkzuurbacterie '*Lactocuccus Lactis*'. Vervolgens werkte Arno één jaar aan het metabole-engineeren van foliumzuurbiosynthese in *L.Lactis*. Na dit project is Arno op 1 juli 2003 begonnen aan zijn promotieonderzoek bij NIZO food research BV in Ede voor de vakgroep microbiologie van Wageningen Universiteit dat in opdracht van het Wageningen Centre for Food Sciences (later, TI Food and Nutrition) plaatsvond. Het promotieonderzoek, dat in dit proefschrift wordt beschreven, stond onder begeleiding van Prof. dr. Willem M. de Vos en dr. Eddy J. Smid. Arno zal zijn promotieonderzoek verdedigen op 8 februari 2008 in de Aula van de Wageningen Universiteit.

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Activities in the Frame of VLAG Research School

Courses:
- Working with isotopes, and radioactive components, 2005
- Food summit (2004 and 2007, WCFS course)
- Simpheny course (2004, Computer program course)

Conferences:
- Conference on folate and pterines (Egmond aan Zee, 2004 oral presentation)
- Lactic Acid Bacteria 8 (Egmond aan Zee, 2005, oral presentation)
- Folate conference (Warschau, 2004, poster presentation)
- Kluyver symposium (Noordwijkerhout, 2004, 2005, 2006 and 2007, poster and oral presentations)
- NVvM/NVMM voorjaarsmeeting (Arnhem, 2006, oral presentation)
- FEMS conference (2006, Madrid, poster presentation)

General courses
- Techniques for writing and presenting a scientific paper, 2003
- Oral presentations, 2005
- WCFS intranet course, part of maintenance intranet WCFS, 2003
- Rhetoric course, by University of Groningen, 2005