Metabolic Engineering of Folate Production in Lactic Acid Bacteria

Wilbert F. H. Sybesma

Promotor

Prof. Dr. W.M. de Vos
Hoogleraar in de microbiologie

Co-promotor

Dr. J. Hugenholtz
Projectleider Wageningen Centre for Food Sciences

Promotie-commissie

Prof. Dr. M.B. Katan
Wageningen Universiteit

Prof. Dr. A.J.J. Van Ooyen
Wageningen Universiteit

Prof. Dr. K. Hammer
Technical University of Denmark, Lyngby, Denmark

Dr. D. Van Sinderen
University College Cork, Cork, Ireland

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Proefschrift

Ter verkrijging van de graad van doctor op gezag van de rector magnificus van Wageningen Universiteit, prof. Dr. Ir. L. Speelman, in het openbaar te verdedigen op vrijdag 3 oktober 2003 des namiddags te 16.00 uur in de Aula.

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Voor SR,
Beslissingen waar je niet over hoeft na te denken zijn de krachtigste beslissingen

Contents

Voorwoord

De afgelopen vier jaren stonden voor mij in het teken van het onderzoek naar het verhogen van de foliumzuurproductiecapaciteit van melkzuurbacteriën. Door gebruik te maken van eerder ontwikkelde genetische technieken, door nieuwe technieken te ontwikkelen, en door samen te werken met experts op verwante gebieden is het onder meer gelukt om een melkzuurbacterie te creëren die meer dan 50 keer zoveel foliumzuur produceert als de zogenaamde wild-type stam. De bijdrage die op deze wijze is geleverd aan de maatschappij en wetenschap is mede tot stand gekomen dankzij de expertise en bereidheid tot efficiënte samenwerking van Jeroen Hugenholtz, Marjo Starrenburg, Willem de Vos, Michiel Kleerebezem, Igor Mierau en Arno Wegkamp. Bovendien was de infrastructuur op NIZO Food Research zeer geschikt om goed moleculair biologisch onderzoek uit te voeren. Een speciaal woord van dank voor Jan van Riel, Kees Olieman en Roelie Holleman voor de technisch ondersteuning bij het opzetten van een HPLC methode voor scheiding en detectie van polyglutamylfolaten. Verder dank voor het secretariaat en de staf van WCFS voor de prettige ondersteuning.

Een aantal onderzoeksresultaten zijn behaald dankzij de nuttige samenwerking met University College Cork, Department of Food Sciences, Cork, Ireland; The Institute of Technology for Chemistry and Biology (ITQB), Oeiras, Portugal; Universiteit Groningen, department of Molecular Genetics; CERELA (Lactobacillus Reference Center), Tucuman, Argentina; WCFS Programme I, Nutrition and Health; en het EU research project NutraCells. Verder ben ik blij met de creatieve inspanningen van Brigitte Schellens die de omslag van dit proefschrift heeft ontworpen.

Tenslotte kijk ik met plezier terug op de wetenschappelijke discussies en leuke manier van samenwerken met naaste collega's binnen WCFS en de afdeling Flavour Nutrition and Ingredients.

Wilbert Sybesma, Utrecht, September 2003

Logic brings you from A to B, imagination and cooperation bring you everywhere.
(afgeleid van een uitspraak van Albert Einstein)

Abstract

Folate is an essential compound in the human diet. Folate deficiency occurs frequently among certain population groups even in highly developed countries and may increase the risks for several diseases like neural tube defects, cardiovascular diseases and certain forms of cancer. The dairy starter bacterium *Lactococcus lactis* is able to synthesize this vitamin. The use of metabolic engineering has enabled the generation of a *L. lactis* strain with a more than 50-fold increased folate production. The synthesis of polyglutamyl folates and the associated release or retention of folate from or in the bacterial cell could be controlled by the expression levels of genes involved in folate biosynthesis and by the culture conditions. Moreover, the formation of monoglutamyl folate from polyglutamyl folate could enhance the bioavailability of folate, because folate is mainly consumed in the monoglutamyl form. In this thesis, we present the first animal trial on bioavailability of folates produced by genetically modified lactic acid bacteria. The outcome of this experiment forms the basis for a human clinical trial with these bacteria. Transformation of the entire folate gene cluster to a folate auxotroph *Lactobacillus gasseri* led to the production of folate in this bacterium and opens new avenues for the food industry to increase folate levels in fermented products. Further research on the production of vitamins in lactic acid bacteria has resulted in the development of a multivitamin producing *L. lactis* strain with increased production of folate and riboflavin. Such multivitamin producing strains could contribute to the development of nutrigenomics, facilitating a tailor made diet for individuals that have specific requirements for these two vitamins due to a genetic polymorphism.

This research provides a basis for the development of functional foods with increased levels of vitamins that could be beneficial for health of the general population.

Chapter 1

Folate: Functionality, Deficiency, Bioavailability and Biosynthesis.

Wilbert Sybesma

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

Introduction

Folate, vitamin B11, is a vitamin that can not be synthesized by humans. Human life could not exist without folate that is involved in various essential functions in cell metabolism, such as the synthesis of DNA and RNA. Folate is present in various foods that can be found in an ordinary diet, like orange juice, dark green leafy vegetables, asparagus, strawberries, legumes, meat (liver) and fermented (dairy) products. Still, there are people that suffer from folate deficiency and that may have increased risks for the occurrence of diseases associated with shortage of folate, as discussed below. As a solution, it is possible to fortify foods with chemically synthesized folate, or to increase the *in situ* folate levels in fermented foods by using folate-synthesizing food-grade bacteria.

The main objective of the research presented in this thesis, is to increase the folate production capacity of lactic acid bacteria by using metabolic engineering and optimization of growth conditions. The application of food grade lactic acid bacteria with increased production levels of folate could lead to the production fermented dairy and other food products with increased levels of natural folate, which may contribute in increasing the number of individuals that comply with the daily recommended intake for folate.

In the following paragraphs, we will briefly describe the functionality of folate, and provide an overview of diseases that are associated with folate deficiency. Moreover, folate bioavailability and folate absorption levels of humans will be discussed. Our work rationalizes that an increased intake of folate is desirable for many individuals, but we will also mention some potential harmful effects that may occur because of excess of folate consumption. Finally, this chapter will describe the folate biosynthesis pathway that exist in the lactic acid bacterium *Lactococcus lactis* and an outline of this thesis will be given.

Folate functionality

Folate is the generic term for all compounds that have a similar function as folic acid (pteroyl monoglutamic acid). In natural folates the pteridine ring is reduced to 7,8-dihydrofolate, or 5,6,7,8-tetrahydrofolate. The tetrahydrofolates are unsubstituted or substituted with various one-carbon groups, such as formyl, methenyl, methylene, methyl, and others (Fig. 1), that have an active function in C1-metabolism. For instance, 10-formyl tetrahydrofolate is used as C1-donor by various transferases and other enzymes involved in purine biosynthesis. 5,10-Methylenetetrahydrofolate is used in the synthesis of dTMP from dUMP by thymidylate synthase. Methionine synthase uses 5-methyl

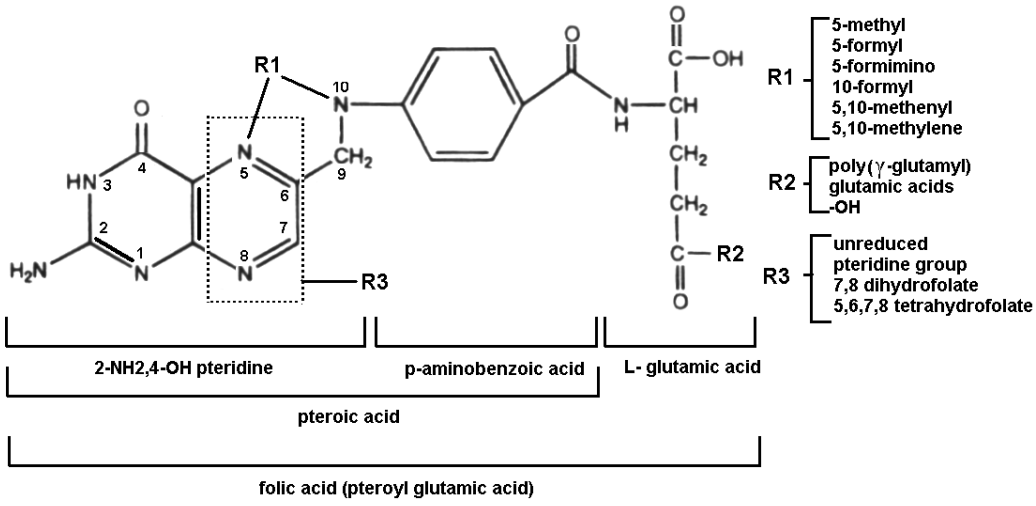

Fig. 1.
Structure of folic acid and folate derivatives.

tetrahydrofolate in the conversion of L-homocysteine to L-methionine. This last reaction is catalyzed by a B12-containing methyltransferase. Much of the methionine which is formed is converted to S-adenosylmethionine (SAM), a universal donor of methyl groups for DNA, RNA, hormones, neurotransmitters, membrane lipids, proteins and others. The different folate derivatives can be synthesized from tetrahydrofolate by various enzymes (e.g. 10-formyltetrahydrofolate synthetase, serine hydroxymethyltransferase, methenyltetrahydrofolate cyclohydrolase, 5.10-methylenetetrahydrofolate reductase, and others). Various comprehensive and recent overviews of C1-metabolism have been reported (Selhub, 2002; Stover and Schirch, 1993).

One of the oldest observed diseases related to the functional role of folate is megaloblastic anemia as a result of ineffective erythropoiesis. Later, independent clinical trials showed that supplementing a woman's diet with folic acid before and during early pregnancy reduced the occurrence of neural tube defects (NTDs) by more than 70% (reviewed by van der Put *et al.,* 2001). More recently, the influence of folate on DNA methylation, both genome-wide and gene-specific, has raised particular interest in the area

of aging and certain diseases that affect the aging population, such as loss of cognitive function and Alzheimer's disease, but also diseases that may affect the whole population, such as cardiovascular disease, cancer and others. The occurrence of these diseases may be in part explained by inadequate intake or inadequate status of B vitamins. Reduced folate levels in the blood are directly correlated to elevated homocysteine levels. Homocysteine, both a product of methionine metabolism and a precursor of methionine synthesis, was recently shown to be a partial risk factor for cardiovascular disease, stroke, and thrombosis, when its concentration in plasma is slightly elevated. Moreover, there is growing evidence about a possible association between elevated plasma homocysteine levels and loss of neurocognitive function and Alzheimer's disease. These associations could be due to a neurotoxic effect of homocysteine or to decreased availability of SAM which results in hypomethylation in the brain tissue. Hypomethylation is also thought to intensify depressive tendency in people and to be the link between inadequate folate status and (colorectal) cancer (Selhub, 2002).

Folate deficiency - Anemia

Anemia is clinically defined as low haemaglobuline or low haematocrit concentrations in the blood. Anemia has been associated with numerous poor health-related factors such as impaired cognition, reduced work capacity, increased maternal mortality during delivery, low birth weight, and increased fetal and neonatal death. The main factor that is responsible for development of anemia is iron deficiency. However, the maintenance of normal haemotopoietic functions also requires adequate levels of many other nutrients acting in concert, like vitamin A, cobalamine (vitamin B12), riboflavin (vitamin B2), and pyridoxine (vitamin B6) (Fishman *et al.,* 2000).

Folate is an essential cofactor during human erythropoiesis. Low folate levels lead to prolongation of the synthesis phase of cell division and retardation of germ cell maturation. This could result in abnormal red cell precursors, megaloblasts. These megaloblasts undergo grossly disturbed cell proliferation and those that mature are often ingested and degraded by bone marrow macrophages. As a result, erythropoiesis is ineffective, the rate of delivery of new erythrocytes into circulation is depressed, and a macrocytic anemia gradually develops. Haematologically, this may be reflected in a high mean corpuscular cell volume and low haemoglobuline concentration. Most, but not all, megaloblastic anemia is produced by ineffective erythropoiesis in the bone marrow due to either folate or vitamin B12 deficiency. The cause of folate deficiency is frequently

inadequate dietary intake, whereas vitamin B12 deficiency is almost always conditioned by some specific type of malabsorption (reviewed by Castle, 1978, and Fishman *et al.,* 2000)

Folate deficiency - Neural tube defects

Based on animal studies, epidemiologic studies and intervention trials, folic acid in pregnant women is known to be protective for neural tube defects (NTD), primarily spina bifida and anencephaly. A remarkable aspect was that folic acid supplementation was not correcting a clinical deficiency in most of these women. It was later shown that the risk of having an NTD-affected birth was negatively associated with maternal red cell folate status, and the level of risk varied throughout the normal range, suggesting an interaction of genetic factors with folate nutritional status (reviewed by Molloy, 2002). To reduce the risk of NTD, the U.S. Food and Drug Administration mandated to enrich all cereal grain products with folic acid as of January 1998. Recent data demonstrate that this public health action is associated with increased folate blood levels among U.S. women of childbearing age and that the national rate of spina bifida has decreased by 20%. Epidemiologic data on use of folate and folate antagonists have also implicated folic acid in prevention of other birth defects such as facial clefts and cardiac and limb defects. Rates of anencephaly appear not to have declined (Green NS, 2002). The C677T and A1298C mutations in the gene encoding the folate requiring methylenetetrahydrofolate reductase enzyme (MTHFR), are also associated with an increased risk of NTD. Although the MTHFR polymorphisms are only moderate risk factors, population-wide they may account for an important part of the observed NTD prevalence (van der Put *et al.,* 2001).

Folate deficiency - Cardiovascular diseases

It is commonly recognized that folates may play a role in the prevention of cardiovascular disease by reducing the homocysteine concentrations in the blood. Adequate intake of folate reduces homocysteine levels in the blood (reviewed by Verhoef *et al.,* 1998). Over the last few years, several studies have reported beneficial effects of folates on endothelial function, a surrogate end point for cardiovascular risk. Consistently, epidemiological studies have demonstrated an association between folate levels and (fatal) cardiovascular disease. The exact mechanisms underlying the healing effects of folates on the endothelium remain to be elucidated, but may involve direct interactions with the enzyme endothelial NO synthase. Recently, more benefits of folates, independent of homocysteine lowering, have also been reported. Potential mechanisms include antioxidant actions, or effects on cofactor availability. Obviously, beneficial effects of folates on cardiovascular risk would have important clinical and dietary consequences.

However, for definite conclusions, the completion of ongoing randomized controlled trials will have to be awaited (reviewed by Verhaar *et al.,* 2002, and Ashfield-Watt *et al.,* 2001).

Folate deficiency - Carcinogenesis

Many clinical studies have suggested that the folate status may modulate carcinogenesis. Although these observations have been made in a number of tissues, the data show a potential association with colon rectum diseases. The mechanism(s) by which this modulation is mediated remains unknown, but alterations in either genome-wide or gene-specific DNA methylation and/or alterations in DNA stability, resulting from DNA strand breaks or uracil misincorporation, may be involved. Folate has, as discussed above, a central role in biological methylation and nucleotide synthesis, and therefore it is not surprising that folate depletion has been observed to alter DNA methylation and diminish DNA stability. The hypothesis that these two pathways are the means by which folate modulates cancer risk is also supported by the epidemiological observation that a common polymorphism in the methylenetetrahydrofolate reductase gene differentially affects the relative risk of colon cancer depending on folate status, because MTHFR catalyzes the reaction that determines whether cellular folate is diverted into biological methylation or nucleotide synthesis. This phenomenon suggests that an imbalance between biological methylation and nucleotide synthesis may be responsible for folate-related carcinogenesis. The control of cell proliferation, which also is related to DNA methylation, is another possible mechanism by which folate status may modulate carcinogenesis. In cell culture studies, folate supplementation has been observed to suppress excessive cell proliferation (reviewed by Choi and Mason, 2002). High alcohol consumption, probably in combination with a diet low in some micronutrients such as folate and methionine, and smoking early in life, are likely to further increase risk of colon cancer (Giovannucci, 2002).

Folate deficiency - Cognitive diseases

The study of different neurological problems, including stroke, Alzheimer's disease, and depression, has propelled a greater interest in interrelationships among folate, homocysteine, and neurological function. Specifically, low folate status is a suspected risk factor for depression, that also results in an increase in circulating levels of homocysteine. Homocysteine has emerged as an independent risk factor for stroke, and recent studies suggest that vascular disease affecting the brain and Alzheimer's disease may result together in senile dementia. The relationship between stroke and Alzheimer’s disease is now hypothesized to reflect some common pathogenic factors involving folate, homocysteine, or both. It remains unclear whether there is a causal relationship between neurological dysfunction in either condition with folate or homocysteine, and whether the

neurotoxicity of increased homocysteine and/or reduced folate is derived from direct detrimental effects on neurons themselves, or is instead derived indirectly following perturbation of nervous system vasculature. However, recent reports from several laboratories provide growing evidence that homocysteine not only induces direct neurotoxicity, but also potentiates both amyloid-beta and glutamate neurotoxicity. These latter studies leave open the possibility that even mild elevations in homocysteine may place neurons at risk for additional trauma (reviewed by Morris *et al.,* 2002, and Shea *et al.,* 2002). Nevertheless, since improvement of folate status lowers homocysteine levels, the hypothesis that folate supplementation may lower the risk of several important health consequences of aging, including various forms of neuropsychiatric dysfunction, warrants the current intensive exploration.

Folate bioavailability

Folate nutritional status depends on intake from food and supplements as well as on the bioavailability of the various ingested forms of this vitamin. In most biological material folate is present in a conjugated form, containing a polyglutamyl tail. Folate absorption only occurs in the monoglutamyl form. Folate auxotrophic organisms, such as humans, contain a polyglutamyl folate hydrolase activity to remove the glutamyl tail and make the folate available for uptake by the tissue cells. This conversion is catalyzed by intestinal γ-glutamyl hydrolases. The effective intake level of dietary folate is therefore strongly influenced by the degree of bioavailability of folate, characterized by the level of polyglutamylation and the level of glutamyl hydrolase enzyme activity. Several authors have suggested that the bioavailability of monoglutamyl folate is higher than that of polyglutamyl folate (Clifford *et al.,* 1991, Gregory, 1989, Melse-Boonstra *et al.,* 2003). Although significant advances in the understanding of folate bioavailability have occurred in recent years, many areas of uncertainty remain, especially with respect to naturally occurring dietary folate. It is hypothesized that with increased levels of monoglutamyl folate the need for intestinal hydrolase activity to deconjugate polyglutamyl folate is relieved and may be advantageous under the conditions that hydrolase activity is reduced by certain food components present in the diet or by genetic polymorphism. Moreover, monoglutamyl folate will have reduced intracellular retention and consequently be better released from the food matrix compared to polyglutamyl folates, leading to a larger bioaccessibility. Another folate characteristic dealing with bioavailability is the difference between natural folate, naturally present in the foods (5-formyl tetrahydrofolate, 5-methyl tetrahydrofolate, etc.) and chemically synthesized folate (folic acid), that is added to fortified foods and present in vitamin supplements. It is hypothesized that folic acid is more

stable than most natural folates (except 5-formyl tetrahydrofolate) and that most natural folates may be degraded before absorption in the digestive system occurs. Moreover, it may occur that natural inhibitors for efficient folate uptake are present in food leading to decreased bioavailability. Folate bioavailability may also be affected by physiological and pharmacological factors, such as alterations in intestinal pH because of achlorhydria, pancreatic insufficiency or administration of certain drugs (Gregory, 2001). An overview of folate bioavailability studies till 1999/2000 is presented by Brouwer (Brouwer, 1999).

Folate absorption levels in humans and folate production levels in foods

The daily recommended intake (DRI) of folate for an adult is 200/400 µg (EU/USA). For pregnant women 400/600 µg is recommended. Recent reports have indicated that folate intake levels are inadequate among various population groups in developed countries including women of childbearing age (studies done in The Netherlands and Ireland) (Brussaard *et al.,* 1997, Konings *et al.,* 2001, O'Brien *et al.,* 2001). There are many factors that may contribute to the fact that the status of folate (and other vitamins) among several population groups is inadequate, of which the most important is the reduced dietary folate intake. Other factors that may be involved could be on a genetic level, such as the occurrence of thermolabile tetrahydrofolate reductase, physiological, such as the achlorhydria which affects vitamin B12 absorption, socioeconomic, resulting in monotonous diets, and habitual, for example interactions with various drugs. Under certain conditions it could be considered to increase the DRI for people, for instance with impaired C1-metabolism, due to genetic polymorphisms like H475Y in the glutamate carboxypeptidase II gene, or the C677T and A1298C mutations in the methylene-tetrahydrofolate reductase gene.

A recent study has listed the most important foods contributing to folate intake in The Netherlands. The list is headed by bread, boiled potatoes, low fat milk, orange juice, and fresh cauliflower. Due to the ability of lactic acid bacteria to produce folate (Lin, 2000, Sybesma *et al.,* 2003), folate levels in fermented (dairy) products could be further increased compared to the corresponding non-fermented dairy products (Crittenden *et al.,* 2003, Alm, 1980). Recent studies have shown that fermented foods are among the 15 most important food items contributing to the folate intake (Konings *et al.,* 2001). The natural diversity amongst dairy starter cultures with respect to their capacity to produce folate can be further exploited to design new complex starter cultures which yield fermented (dairy) products with elevated folate levels (Smid *et al.,* 2001), which will have a much higher contribution to the human daily folate intake. Currently the contribution to the

DRI for folate by fermented dairy products varies in average between 15-20% (Konings *et al.,* 2001). In some countries, like the USA, other important sources of folate are synthetic folic acid supplements. The differences between folate bioavailability of synthetic forms of folate and natural forms of folate have not been unambiguously determined (see above). However, folate-fortified foods are not widely available all over the world, either because of legislation, or limited industrial development. Within the European Union consumers have limited access to fortified foods, because different regulations exist in the Member States related to (i) the permission of food fortification; (ii) the allowance of specific nutrients; (iii) the levels at which they can be added. When foods fortified with folic acid are not allowed, as in The Netherlands, the availability of (fermented) foods containing naturally sources of folate may give a significant contribution to general health status.

Excess of folate consumption

In the USA, folic acid is now added to grain products and continues to be included in the majority of ready-to-eat breakfast cereals. Recent data indicate that the folate status in the USA population has improved significantly, presumably due to the effects of fortification (Rampersaud *et al.,* 2003). Moreover, there have been reports stating that excessive folic acid intake occurs due to the over consumption of fortified foods. The effects of potentially too high intake of folic acid must be carefully assessed, especially before calls for higher levels of fortification are considered (Quinlivan and Gregory, 2003). One of the concerns is that folic acid (not food folate) intake in excess of the tolerable upper intake level, 1000 μg per day, may mask the diagnosis of a vitamin B12 deficiency, which is more prevalent in the elderly than in younger individuals. Shortage of vitamin B12 impairs methylation in C1-metabolism resulting in accumulation of 5-methyl tetrahydrofolate. A severe B12 deficiency could lead to the occurrence of pernicious anemia, that may result in neuropathy and a megaloblastic anemia, morphological identical to that seen in folate deficiency. However, the addition of excess of folic acid, via fortified foods, may initially reactivate that part of C1-metabolism that could redress the anemia, but not the neuropathy. The restart of the complete methylation cycle, that may be potentially blocked because of vitamin B12 deficiency, could not occur. Consequently, the neuropathy could continue, and would probably not be detected in an early stage (Scott, 1999). Thus, when folic acid supplements are provided, it is recommended to provide a multivitamin that includes vitamin B12. The Dutch health council has set the maximal tolerable upper intake level of folic acid at 1000 μg per day. Moreover, to enable a safe and effective increase of folate intake in the elderly, naturally occurring folate-rich food sources could be promoted. Other studies, although controversial, suggest that exposure

to excess folate, perhaps at levels that occur at the upper end of the intake distribution curve, may have unintended consequences in promoting embryo viability. In conclusion, it could be suggested that the individualizing of folic acid dietary recommendations necessitates a detailed understanding of all genetic and physiological variables that influence the interaction of folate with the genome and their relationship to the disease process (Stover and Garza, 2002).

Folate biosynthesis

As described above, the capacity of some food grade micro-organisms to produce folate could be used to increase folate levels in fermented foods. The folate biosynthesis pathway in micro-organisms can be divided in several parts. The pteridine proportion of folate is made from GTP, that is synthesized in the purine biosynthesis pathway. p-Aminobenzoic acid originates from chorismate and can be synthesized via the same biosynthesis pathways required for the aromatic amino acids, involving glycolysis, pentose phosphate pathway and shikimate pathway. The third component of a folate molecule is glutamate, that is normally taken up from the medium. In a series of reactions that require six enzymes, these three components are modified and coupled to synthesize folate (Fig. 2). GTP cyclohydrolase I (*folE*, EC 3.5.4.16), catalyses the reaction from GTP to dihydroneopterin triphosphate through an intermediate and release of formate. A phosphate residue is then removed presumably by the action of a phosphatase. Dihydroneopterin aldolase (*folB*, EC 4.1.2.25) then acts on the product to give glycolaldehyde and 6-hydroxymethyl-7,8-dihydropterin, which is converted to 6-hydroxymethyl-7,8-dihydropterin pyrophosphate by 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (*folK*, EC 2.5.1.15). Dihydropteroate synthase (*folP*, EC 2.7.6.3) couples p-aminobenzoate to produce 7,8-dihydropteroate. Addition of glutamate to the carboxy part of p-aminobenzoate by the bifunctional protein folate synthetase/polyglutamyl folate synthetase (*folC*, EC 6.3.2.12/17) produces dihydrofolate. After further reduction to tetrahydrofolate, by dihydrofolate reductase (*folA*, EC 1.5.1.3), polyglutamyl folate is produced by subsequent addition of glutamate molecules to the glutamyl residue of folate by the activity of polyglutamyl folate synthetase (*folC*, EC 6.3.2.17) (See Fig. 2). Different substituted folate derivatives are synthesized in a number of enzymatic steps involved in C1-metabolism and used to perform their specific metabolic activities.

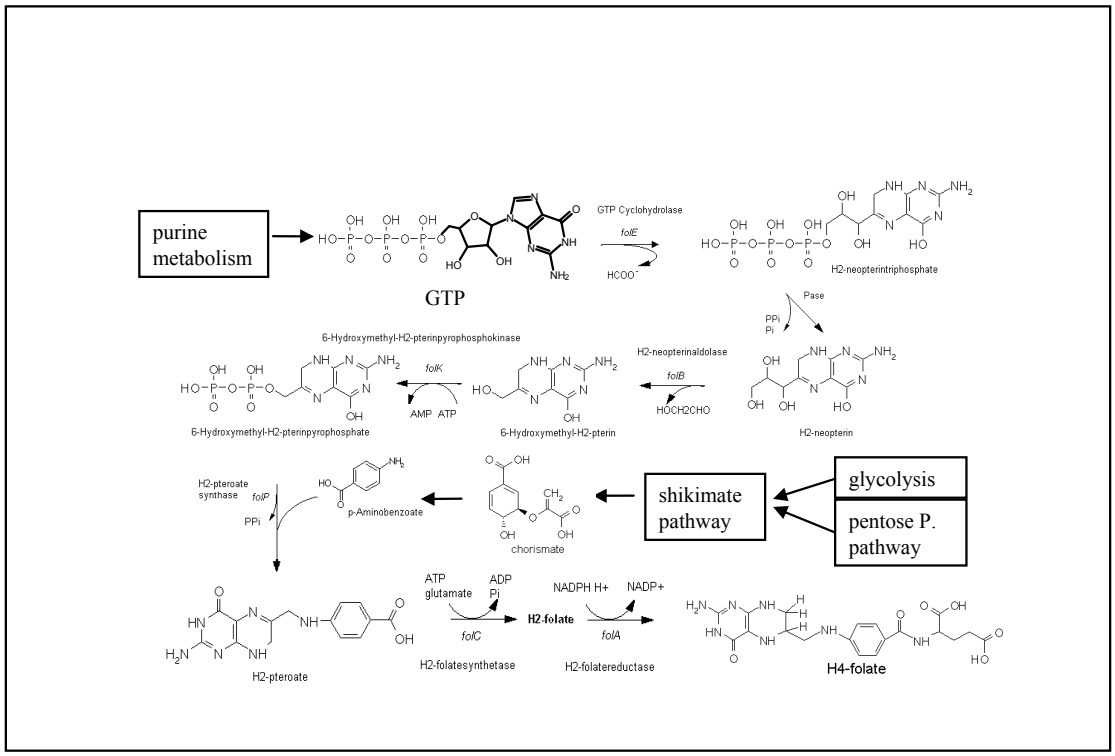

Fig. 2.
Chemical structure of tetrahydrofolate and folate precursors in the folate biosynthesis pathway of *L. lactis*. Relevant enzymes and their gene designation are indicated.

Outline of the thesis

The general aim of the research described in this thesis is to increase the folate production capacity of lactic acid bacteria. As a model organism the dairy starter bacterium *Lactococcus lactis* is used. Metabolic engineering strategies and optimization of growth conditions are used to increase folate production levels in *L. lactis*. A second objective of the research described in this thesis is to control the production of bioavailable folate in fermenting lactic acid bacteria. Finally, the results of the research can be a reference guide for the increased production of other B-vitamins in fermenting micro-organisms. Application of this research in the food industry is foreseen to contribute to the development of fermented foods with increased levels of natural folates.

Chapter 1 provides an overview of general aspects of folate related to its functionality, deficiency, bioavailability, consumption and biosynthesis. Chapter 2 analyses the debate about the use of genetically modified organisms in foods and recommends a

distinction in risk assessment between genetically modified organisms derived from plant, animal or microbial origin. Chapter 2 considers also the current safety assessment procedure for novel foods. A profound food safety analysis using genome-based approaches is proposed, that may result in the conclusion that novel foods derived from genetically modified lactic acid bacteria could be considered as safe as traditionally fermented foods. The reason for writing this chapter is that the majority of the research described in this thesis will discuss the successful application of metabolic engineering to increase the production of bioavailable folate in fermenting lactic acid bacteria. However, in our today's society, the use of genetically modified organisms is not widely accepted due to assumed risks for human safety and the environment. Chapter 3 will present the results of a screening of a variety of lactic acid bacteria for their ability to produce folate, both intra- and extracellularly. Furthermore, several physiological and environmental conditions are analyzed for their influence on folate production in *Lactococcus lactis* and *Streptococcus thermophilus*, such as growth rate, presence of inhibitory concentrations of antibiotics or salt, pH, and addition of the medium compounds p-aminobenzoic acid, tyrosine and hemin. Chapter 3 also describes the setup of a methodology that enables the identification of different folate derivatives with different polyglutamyl tail length by using liquid chromatography. In Chapter 4 a folate gene cluster in the genome of *L. lactis* is identified and analyzed by DNA sequence analysis. This knowledge is subsequently used to overexpress several genes involved in folate biosynthesis in *L. lactis* and, as a result, the intra- and extracellular folate production and distribution could be controlled. Chapter 5 focuses on the nature of the folate derivatives produced in *L. lactis* and studies the control of the polyglutamyl tail length by increasing the flux through the folate biosynthesis pathway and by overproduction of the enzyme polyglutamyl folate synthetase, which is responsible for the elongation of the glutamyl tail of folate. Moreover, the potential application of engineered food microbes producing folates with different tail length is discussed. Chapter 6 describes the functional expression of human or rat γ-glutamyl hydrolase in *L. lactis*. The production of this intestinal enzyme in the lactic acid bacterium results in deconjugation of intracellular polyglutamyl folates and subsequent release of bioavailable monoglutamyl folate. Chapter 7 summarizes the research that was done to select a *L. lactis* strain with a deregulated riboflavin biosynthesis. In combination with the knowledge about increasing folate production levels by using metabolic engineering, a new *L. lactis* strain could be obtained with increased production levels of both folate and riboflavin. The potential application of this strain in the diet of individuals with increased requirement for these vitamins is discussed. Chapter 8 gives an example of metabolic engineering aimed at the transformation of a folate-consuming lactic acid bacteria into a

folate-producing lactic acid bacteria. Chapter 9 describes the generation of the ultimate folate producing *L. lactis* that has more than 50 times increased folate production levels. The biosynthesis of the folate precursor p-aminobenzoic acid and its controlling role on folate production levels is discussed as well. Moreover, the effects of folate overproduction on the transcriptome of *L. lactis* are shown. In Chapter 10 the presence of a new open reading frame within the folate gene cluster is described. In addition, the potential involvement of an unknown gene, *ylgG*, in the folate biosynthesis in *L. lactis* is analyzed. Chapter 11 provides the preliminary results of a folate rat bioassay that has the objective to study the variation in folate bioavailability of folates with a different polyglutamyl tail length. A summary of the work and relevant concluding remarks are given in Chapter 12. Finally, an appendix describes a new tool to perform improved, efficient and reliable RT-PCR using tag-extended RT primers and temperature gradient PCR. This technique has been applied to show that the folate gene cluster is transcribed as a poly-cistronic operon in *L. lactis*.

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Chapter 2

Safe Use of Genetically Modified Lactic Acid Bacteria in Food.
- Bridging the Gap between the Consumer, the Green Society, and the Industry -

Wilbert Sybesma, Jeroen Hugenholtz, and Willem M. de Vos

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

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Contents

Introduction

Ever since human domestication took place, approximately 10,000 years ago, parts of nature have been cultured in order to support survival of the human race. In our times the main objective of agriculture is still the same, the assurance of efficient food supply in the first place, and the increase of quality of the supplied foods for the consumer in the second place. Modern biotechnology using recombinant DNA technology offers ample opportunities to further improve food production in the expanding world of today.

Within the European Union the production of novel foods obtained by using genetically modified organisms, GMOs (see box 1), is subject to strong debate, in which the scientific community states that there is sufficient legislation to guarantee that such novel foods are safe for the consumer and the environment, while the green society in many cases states an opposite opinion. The support for the use of GM crops and foods among the European population has increased in 2002 in some countries. Yet at the same time a majority of the Europeans do not support agri-food biotechnologies (Eurobarometer 58.0, 2003). In the current debate about the application and safety of GMOs in food industry no clear distinction is made between GMOs derived from, animal, plant, or microbial origin. Moreover, ethical aspects could play a role in the debate about GMOs from animal or plant origin, but these are considered less relevant in the discussion about genetically modified micro-organisms. Apart from this, the current European legislation on novel foods and the use of GMOs in food production processes as laid down in the regulation EC No 258/97, directive 2001/18/EC (revised directive 90/220/EC), directive 90/219/EC, and Commission Recommendation 97/618/EC, suffers from several scientific inconsistencies. 1. The legislation is exclusively focusing on the methodology rather than on the end product and hence, holds too strong to the definition of GMO. Consequently, organisms in which the genetic material has been altered by recombinant DNA techniques in a way that does occur naturally, for instance by point mutations or small deletions, are considered to be GMOs. 2. Foods with a new structure that date from before 1997 are not considered as novel foods (see box 2). 3. Self cloning (see box 3) of non-pathogenic micro-organisms is not considered to lead to a GMO, as long as it is only used for contained use, but the same self-cloned micro-organism when used in products (deliberate release) is considered again to be a GMO. Moreover, the market introduction of foods with organisms that have been improved via classical breeding or random mutagenesis is currently acceptable without profound safety analysis, but it could be questioned whether these foods are more safe than GMO-derived novel foods.

Box 1: Definition of GMO according to Council Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (repealing Council Directive 90/220/EEC) and Council Directive 90/219/EEC of 23 April 1990 on the contained use of genetically modified micro-organisms.

A GMO is described as an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. This means that all organisms which are produced by means of recombinant DNA techniques using vector systems, or techniques involving the direct introduction of genetic material into an organism, or cell fusion techniques, are in principle GMOs. Organisms that have been subjected to processes involving modification of DNA by means of transduction, conjugation, polyploidy induction, cell fusion, or mutagenesis involved by exposure to certain environmental conditions, are not considered to be GMOs. (Self-cloning is not considered as a GMO under Directive 90/219/EEC).

The rational for food safety of any food produced (including, but not especially, GMO derived foods) is the assurance that there will not be any adverse effect on human health, or on the environment. The starting point for a food safety approach is the comparison between the new foods and the conventional counterparts that have a history of safe use, both for the food produced and for the micro-organism that is modified. Following this approach, identification of new or altered hazards relative to the conventional counterpart (substantial equivalence, see box 4) is essential (OECD, 1993).

Micro-organisms applied in food fermentation include bacteria, yeast and filamentous fungi. This paper mainly discusses the use of genetically modified lactic acid bacteria (GM-LAB) in food industry. The application and safety assessment of genetically modified yeast and fungi is not discussed, neither is the use of GMOs derived from plants (reviewed by Kuiper *et al.,* 2001) or animals. The use of genetically modified microbial inoculants for use in agriculture, such as biological control agents, biofertilizers, or phytostimulators is reviewed elsewhere (Amarge, 2002, Morrissey *et al.,* 2002). An overview will be given of the specific genetic techniques that generate GM-LAB and the advantages that the novel foods fermented with GM-LAB may bring to the consumer. Moreover, the current safety assessment procedures are considered and further suggestions are made for an adequate risk analysis when GM-LAB are applied in food production. Careful analysis of the nature of GM-LAB combined with a profound safety assessment procedure for foods that are produced through the actions of GM-LAB, are essential for comparing the safety of such products with traditional foods. Finally, based upon the principle of assuring food safety for the consumer, it can be argued to make no distinction between foods produced by LAB that have been altered in an uncontrolled way,

for instance by random mutagenesis, or controlled way, such as GM-LAB. This could imply that all foods fermented by LAB should be subjected to a safety assessment procedure, before commercialization can occur. Recently, such issues have been considered for non-GM-LAB in a draft document introducing the concept of Qualified Presumption of Safety (QPS), which will be discussed below. Furthermore, we will examine the value of the principle of a long and safe history of use of traditionally used LAB and discuss whether this could be a starting point for the acceptance of GM-LAB.

Lactic acid bacteria and genetic engineering

LAB have a long history of use by man for food production and food preservation. LAB are gram-positive, non-spore forming bacteria and naturally present in raw food material and in the human gastro-intestinal tract. The heterogenous group of LAB include the rod-shaped bacteria like lactobacilli and cocci, such as streptococci, lactococci, pediococci and leuconostocs. LAB are widely used as starter cultures for fermentation in the dairy, meat and other food industries. Their properties have been used to manufacture products like cheese, yogurts, fermented milk products, beverages, sausage, olives, etc. These food-grade bacteria can also improve the safety, shelf life, nutritional value, flavor and quality of the product. Moreover, LAB can be used as cell factories for the production of food additives and aroma compounds. It is further assumed that LAB may function as probiotics and contribute to the general health of the consumer upon consumption. Among these probiotics are popular dairy products such as yogurts and drinks. The use of probiotics falls currently within a gray area between food and medicine and many health claims assigned to probiotics are not yet scientifically proven. An application less related to food, but more related to pharmaceutics encompasses the use of LAB in the production of proteins for application in health care or for development of new vaccines (Mercenier *et al.,* 2000). For the future it is foreseen that knowledge about the interaction between LAB and the human host will open new avenues for improving LAB to benefit human health.

The uncontrolled genetic alteration of LAB that may occur by random mutagenesis and is followed by selection, may result in the development or isolation of strains with improved traits. These may be either attractive for the manufacturer of fermented foods, or have benefits for the consumer. Both controlled and uncontrolled genetic alterations result in a change of the genetic code of the micro-organism that may affect the transcription and translation processes and, consequently, may influence metabolic processes in the cell. In the current legislation about the use of GMOs in food industry, the nature of the DNA

modification determines partially the acceptability of application of GMOs in food fermentation based upon the definition of GMO (Table 1).

Uncontrolled genetically altered LAB

Spontaneous mutations may occur in LAB by natural events such as insertion sequence elements (Visser *et al.,* 2003), radiation, erroneous DNA replication or transcription, and other factors. The level of such mutations depends on the growth conditions. By screening of natural isolates of LAB, strains with improved fermentation characteristics can be selected. The frequency of mutations can be further increased by exposing LAB to mutagenic conditions such as UV light or chemicals like N-methyl-N'-nitro-N-nitrosoguanidine (NNG), or ethyl methyl sulfonate. Specific screening on desired traits may result in the identification of the new strains with improved capacities for application in food industry.

Foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances.

Box 2: Definition of novel foods according to regulation EC No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel food and food ingredients.

Foods and food ingredients that were not used for human consumption to a significant degree within the Community before 15 May 1997. In addition, these food and food ingredients must fall into particular ingredients, namely they must be:
Foods and food ingredients containing or consisting of genetically modified organisms (GMOs) within the meaning of Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms.
Foods and food ingredients with a new or intentionally modified primary molecular structure.
Foods and food ingredients consisting of or isolated from micro-organisms, fungi or algae.
Foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for food and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use.

Table 1: Types of DNA modification methods and the acceptability to be used in food production

Modification of DNA	Controlled genetic alteration	Un-controlled genetic alteration	Acceptance of contained use, 90/2219/EEC	Acceptance of deliberate release, 2001/18/EC
Spontaneous mutations	-	+	+ non-GMO	+ non-GMO
Induced mutations	-	+	+ non-GMO	+ non-GMO
Mutations via insertion elements	-	+	+ non-GMO	+ non-GMO
Conjugation	+	-	+ non-GMO	+ non-GMO
Transduction	+	-	+ non-GMO	+ non-GMO
Self-cloning	+	-	+ non-GMO	- GMO
Non-self-cloning	+	-	- GMO	- GMO

Examples of GM-LAB selected after uncontrolled genetic alterations.

Selection of strains that have been subjected to uncontrolled genetic alterations is used in the dairy industry to improve certain intrinsic characteristics of the fermented end product, like flavor, structure, nutritional value, or phage resistance. Spontaneous mutations in single genes can lead to altered lactose metabolism, citrate uptake, and increased proteolytic activity. In *Lactobacillus bulgaricus* a spontaneous IS element-mediated deletion of the *lacZ* gene altered lactose metabolism resulting in limited fermentation capacities of lactose. As a consequence, yogurt, made by these altered *L. bulgaricus*, is not suffering from further acidification after fermentation (Mollet *et al.,* 1990). A gene inactivation method for several lactic acid bacteria has been described that is mediated via insertion sequences (IS elements) which may integrate in a gene, resulting in inhibition of function (Maguin *et al.,* 1996). According to current legislation, however, strains with inactivated genes obtained by this strategy are considered to be GMOs.

Box 3: Definition of self-cloning according to Council Directive 98/81/EC amending Directive 90/219/EEC.

"self-cloning" means the removal of nucleic acid from a cell or organism, followed by the re-insertion of all or part of that nucleic acid — with or without further enzymic, chemical or mechanical steps — into the same cell type (or cell-line) or into a phylogenetically closely related species which can naturally exchange genetic material with the donor species. (Accordingly, the temporary introduction of plasmids, the deletion of specific DNA sequences, or introduction of DNA from another micro-organism belonging to the same species fall within the definition of self-cloning).

L. lactis strains, that are involved in the fermentation of Roquefort cheeses were randomly mutagenized and selection was done on carbon dioxide production that improves the quality of the cheese (Attar 2000). In a similar way LAB defective in lactate production, but with increased production levels of the butter flavor compound diacetyl were selected after random mutagenesis with the mutagen NNG (Boumerdassi, 1997). The selection of a spontaneously mutated *L. lactis* strain that overproduced diacetyl, responsible for the butter flavor in many fresh dairy products, is described by Monnet (Monnet *et al.,* 1997). A stronger and more direct selection procedure was applied by the temporarily introduction of a plasmid introducing valine prototrophy in the bacterium (Curic *et al.,* 1999). After selection of the desired mutation in the aldB gene, encoding α-acetolactate decarboxylase, the plasmid was removed, leading to strain that did not contain any foreign DNA. Nevertheless, under the actual EU legislation this strain is still considered as a GMO (Henriksen *et al.,* 1999). This illustrates one of the inconsistencies of the current legislation that is mentioned above.

The nutritional value of fermented foods can also be improved by (over)production of healthy components, like vitamins, in the fermenting bacteria. To increase vitamin B2, riboflavin, production levels in the dairy starter bacterium *L. lactis*, bacterial cells were exposed to increasing concentrations of roseoflavin. This resulted in the selection of a roseoflavin-resistant strain with a deregulated riboflavin biosynthesis and increased production levels of riboflavin, up to 1 mg/L. Food products obtained after fermentation with this mutant *L. lactis* could give a strong contribution to the daily recommended intake (DRI) of 1.3 mg for riboflavin. Partial analysis of the genome of this mutant revealed a mutation in a supposed regulatory sequence just upstream of the first gene of the riboflavin gene cluster (Burgess *et al.,* 2003).

Box 4: The concept of substantial equivalence according to OECD 1993 (Safety evaluation of foods produced by modern biotechnology – concepts and principles, OECD Paris).

The concept of substantial equivalence is part of a safety evaluation framework based on the idea that existing foods or traditionally used micro-organisms in food production can serve as a basis for comparing the properties of a genetically modified food or GMM with the appropriate counterpart. Careful risk analysis should investigate the nature and effects of new metabolites and proteins that could be produced by the GMM. The concept of substantial equivalence is not a safety assessment in itself; rather it represents the starting point that is used to structure the safety assessment of both a GMM relative to its conventional counterpart. This concept aims to identify similarities and differences between the old and new micro-organism. The safety assessment carried out in this way does not imply absolute safety of the new product, but it focuses on assessing the safety of any identified differences, so that the safety of the GMM and the GMM derived food can be considered relative to their conventional counterparts.

LAB can also be selected for the removal of undesirable compounds from raw food material. Traditionally used LAB can degrade the undesirable sugar lactose from milk during yogurt fermentation. However, despite the decrease of lactose, one of the degradation products is the harmful galactose that may cause cateract problems (Liu *et al.,* 1998). Vaughan describes the selection of spontaneous galactose-fermenting mutants of *Streptococcus thermophilus*, that contain up-mutations in the *gal* operon and may assist in removal of undesired galactose from the food matrix (Vaughan *et al.,* 2001). The selection of a double mutant strain of *L. lactis* with an inactivated glucose uptake system and deficient in glucokinase, could also reduce the concentrations of lactose and galactose during fermentation. Moreover, this strain could also be used as natural sweetener due to the excretion of the undegradable lactose moiety glucose (Thompson *et al.,* 1985). An overview of LAB selected after uncontrolled modification of DNA is given in Table 2.

Controlled genetic alteration of LAB

Opposite to the random mutagenesis, we place the controlled modification of the DNA. These directed modifications vary from single base pair substitutions, mutations, insertions of genes into the chromosomes, or removal of DNA from the chromosome (deletions) resulting in the inactivation of specific enzymes. A summary of the practices of controlled genetic alteration of LAB is given in Table 3. According to the current legislation, all such controlled genetic alterations lead to strains that are considered as GMOs, except for strains obtained by self-cloning, transduction and conjugation (Table 1).

A variety of techniques has been developed to generate GM-LAB, such as cloning systems, chromosome modification systems and expression systems (de Vos, 1999). The most popular transformation system for generating controlled genetic alterations in LAB is electroporation with self-replicating vectors. Alternative systems are conjugation and transduction (Gasson, 1990). Besides efficient cloning systems, adequate expression systems have been developed allowing the controlled expression of homologous and heterologous genes. Controlled constitutive expression is possible by using a system of synthetic promoters (Solem and Jensen 2002), while a nisin induced controlled expression system (NICE) allows the gradual overexpression of genes (Kleerebezem, 1997). Other systems are controlled by promoters based on sugars, e.g. the lactose operon promoter (Payne 1996), by salt, e.g. *gadC* promoter (Sanders 1997), by pH decrease (Madsen 1999), or by temperature up-shift (Nauta 1996) (reviewed by De Vos, 1999). Targeted gene replacement or removal and inactivation of genes can also be applied via (non-replicative) vectors using the natural event of crossing-over during cell division and DNA

replication. (Leenhouts *et al.,* 1996). Compared to the use of replicative vectors in GM-LAB that results in new or enhanced cellular behavior, the deletion of genes after double crossing-over by using a non replicative plasmid does not result in the addition of any DNA to the genetic content of the cell.

Table 3: Practices of controlled genetic alterations of LAB.

- Improved LAB made by introduction of plasmids via the natural event of conjugation or transduction.
- Improved LAB made by passage (introduction and subsequent elimination) of a recombinant plasmid.
- Improved LAB constructed by deletions of DNA as a consequence of double cross-over recombination.
- Improved LAB constructed by self cloning that does not change the total genetic make-up of a LAB cell.
- Insertion of DNA from micro-organisms with a long tradition of safe use in the food industry.
- Insertion of DNA from micro-organisms without a long tradition of safe use in the food industry
- Insertion of DNA from other organisms

A specific aspect related to the application of vectors in strain engineering is the use of selection markers. The use of antibiotic resistance markers has been compromised by their application in transgenic plants. Hence, food-grade resistance markers are preferred. Currently there are many food-grade selection markers for vector cloning in LAB. For instance, transfer of the α-galactosidase gene (*aga*) and a gene coding for a putative transcriptional regulator from the LacI/GalR family (*galR*) of *Lactococcus raffinolactis* ATCC 43920 into *L. lactis* and *Pediococcus acidilactici* strains modifies the sugar fermentation profile from melibiose negative (Mel(-)) to melibiose positive (Mel(+)) (Boucher *et al.,* 2003). A similar food-grade vector is based on complementation of the lactose operon in *L. lactis* NZ3600 or *L. casei* by introduction of *lacF*, or *lacG,* respectively, enabling growth on lactose (Platteeuw *et al.,* 1996, Takala *et al.,* 2003). An alternative system is based on a suppressor tRNA allowing growth in milk of a purine auxotrophic strain (Dickely, 1995). A new developed food-grade marker is characterized by the requirement of D-alanine in the medium to enable growth of the micro-organisms (Bron *et al.,* 2002). An overview of general strategies for constructing food-grade markers has previously been reported (de Vos, 1999).

Table 2: Overview of lactic acid bacteria with controlled or uncontrolled genetic alterations

host strain	donor strain	gene involved	intended effect	modification technique	Reference
L. bulgaricus	Not applicable	*lacZ*	limited lactose fermentation	IS mediated deletion	Mollet et al., 1990
L. lactis	Not applicable	*ldh and others*	increased carbon dioxide production	spontaneous and induced random mutagenesis	Attar *et al.,* 2000
L. lactis	Not applicable	*ldh and others*	increased acetoin and diacetyl production	NNG induced random mutagenesis	Boumerdassi *et al.*, 1997
L. lactis	Not applicable	*aldB*	increased diacetyl production	NNG induced random mutagenesis,	Monnet *et al.,* 2000
L. lactis	Not applicable	*aldB*	increased diacetyl production	Spontaneous random mutagenesis	Goupil *et al.,* 1996
L. lactis	Not applicable	*ribC*	increased riboflavin production	induced random mutagenesis	Burgess *et al.,* 2003
S. thermophilus	Not applicable	gal operon	fermentation of galactose	spontaneous random mutagenesis	Vaughan *et al.,* 2001
L. lactis	Not applicable	*glk, eII*$^{man/glc}$	no glucose fermenting capacity	spontaneous random mutagenesis	Thompson *et al.,* 1985.
L. lactis	*L. lactis*	*aldB*	increased diacetyl production	double crossover homologous recombination	Swindell *et al.,* 1996
L. lactis	*Lb. helveticus*	*pepN, pepX, pepC, pepI*	modulation of proteolytic system for enhancement cheese ripening	food grade vector cloning	Joutsjoki *et al.,* 2002
L. lactis	*L. delbrueckii*	*pepI, pepL, pepW, pepG*	modulation of proteolytic system for enhancement cheese ripening	NICE (Nisin Induced Controlled Expression)System	Wegmann *et al.,* 1999
L. lactis	*Peptostreptococcus*	*gdh*	increased production	vector cloning	Rijnen *et al.,* 2000

	asaccharolyticus		of alpha-ketoglutarate		
L. lactis	lytic phage *phi31*	phage inducible promoter	expression of lethal three-gene restriction cassette LlaIR+,	vector cloning	Djordjevic *et al.,* 1997
L. lactis	lytic phage *phi31*	anti sense phage RNA	silencing of phage genes	vector cloning	Walker *et al.,* 1998
L. lactis	lytic phage	anti sense phage RNA	silencing of phage genes encoding structural genes	vector cloning	Kim *et al.,* 1991
L. lactis	Not applicable	*pip*	inactivation of phage infection protein	double crossover homologous recombination	Monteville *et al.,* 1994
L. lactis strains	*L. lactis strains*	lacticin encoding genes	lacticin production	Conjugation	O'Sullivan *et al.,* 2003
L. lactis strains	*L. lactis strains*	lacticin encoding gene	lacticin production	conjugation (plasmid stacking)	Mills *et al.,* 2002
L. lactis	*S. thermophilus*	abiA, abiG	abortion of cells upon phage induction	vector cloning	Tangney *et al.,* 2002
L. lactis and others	*Pediococcus acidilactici* and others	*lcnC, lcnD*	lantibiotic production	vector cloning	Horn *et al.,* 1999
L. lactis	phage	*lytA, lytH*	production of lysin and holin	NICE	de Ruyter, *et al.,* 1997
L. lactis	*S. thermophilus Sfi6*	EPS gene cluster	altered EPS production	vector cloning	Stingele *et al.,* 1999
L. lactis	*S. thermophilus Sfi39*	EPS gene cluster	altered EPS production	vector cloning	Germond *et al.,* 2001
L. gasseri	*L. lactis*	folate gene cluster	introduction folate biosynthesis pathway	vector cloning	Wegkamp *et al.,* 2003
S. thermophilus	*Not applicable*	*pgmA, gal U*	inactivation of phosphoglucomutase	double crossover homologous recombination and vector cloning	Levander *et al.,* 2002
L. lactis	*B. sphaericus*	*ldh, alaD, alr*	rerouting of pyruvate	double crossover	Hols *et al.,* 1999

			to L-alanine	homologous recombination, vector cloning	
L. lactis	*L. lactis*	riboflavin gene cluster	overexpression riboflavin biosynthesis pathway	vector cloning	Burgess *et al.,* 2003
L. lactis	*L. lactis*	folate gene cluster	overexpression folate biosynthesis pathway	vector cloning	Sybesma *et al.,* 2003bc
L. lactis	*L. lactis*	*glk, pfnABCD, pfcBA, genes* lactose-PTS and tagatose-6P	inactivation of glucose fermenting system and introduction of lactose fermenting system	double crossover homologous recombination, vector cloning	Pool *et al.,* 2003
L. lactis	*L. lactis*	*galA, aga*	introduction α-galactosidase activity	food grade vector cloning	Boucher *et al.,* 2002
L. plantarum	*B. subtilis*	*phyC*	introduction phytase activity	vector cloning	Kerovuo *et al.,* 2000
L. plantarum	*L. amylovorus*	*amyA*	introduction α-amylase activity	chromosomal integration	Fitzsimons *et al.,* 1994

Other controlled modifications of the genetic content of DNA may occur via conjugation and transduction (Gasson, 1990), that are considered as naturally events. Following the current legislation, bacteria that are changed by using these transfer systems are not considered as GMOs (Table 1).

Examples of GM-LAB engineered via controlled genetic alterations

Directed mutagenesis is widely applied in research to improve fermented food products. The following paragraphs will provide an overview of successful examples of metabolic engineering aimed at the improvement of certain characteristics of food products, like flavor, structure, shelf life, nutritional value, or product performance in general. An overview of GM-LAB is given in Table 2.

The flavor and flavor stability of buttermilk was improved by inactivation of the *aldB* gene encoding α-acetolactate decarboxylase (Swindell *et al.,* 1996). The overexpression of peptidase genes in LAB via self-replicating plasmids could also enhance flavor formation and cheese ripening. Peptidolytic activity can be increased by overproduction of peptide degrading enzymes originating from the same species or from other species. An example of the self-cloning strategy is the overproduction of *PepN* by *L. lactis* (Gasson and De Vos, 1994). GM-LAB with increased proteolytic propertied are generated by the overexpression of the genes encoding PepN, PepX, PepC and PepI peptidases from a highly proteolytic *L. helveticus* strain (Joutsjoki *et al.,* 2002) or PepI, PepL, PepW, and PepG from *L. delbrueckii* (Wegmann *et al.,* 1999) into *L. lactis*. Moreover, the expression of *gdh* from *Peptostreptococcus asaccharolyticus*, encoding glutamate dehydrogenase, into *L. lactis* increases the production of alpha-ketoglutarate (Rijnen *et al.,* 2000). This enhances the degradation of amino acids, which also benefits the cheese ripening process.

Food fermentation processes that, because of size, cannot be operated under strict axenic conditions, may suffer from bacteriophage infections resulting in lysis of starter bacteria. The transformation of industrially important strains with phage resistant genes from other LAB could generate new phage resistant strains. Engineering of starter strains aimed at inhibiting the phage development is possible by creating a system of lethal gene induction upon phage infection (Djordjevic *et al.,* 1997) or by production of antisense RNA inhibiting translation of phage RNA (Kim *et al.,* 1991, Walker *et al.,* 1998). The inactivation of a phage infection protein, *pip*, involved in phage adsorption and DNA injection resulted also in a phage resistant *L. lactis* strain (Monteville *et al.,* 1994). The lactococcal abortive

infection mechanisms AbiA and AbiG were introduced into *Streptococcus thermophilus*, and a range of phages capable of infecting this host were sensitive to these mechanisms (Tangney *et al.,* 2002). The quality of food fermentation processes could be further improved when they are protected from spoilage by other bacteria. The expression of the food-grade lantibiotics in dairy starter strains could prevent spoilage from gram positive bacteria during the process of cheese ripening (Horn, 1999). The production of lantibiotics to prevent growth of *Listeria monocytogenes* and *Lactobacillus fermentum,* during the production of cheese was also achieved via the non-GMO approach of conjugation (Hugenholtz *et al.,*1991, O'Sullivan *et al.,* 2003). Bacteriophage-resistant dairy starter cultures were also selected after the natural event of conjugation (Gasson, 1990, Hill *et al.,* 1989) or after conjugation (stacking) of plasmids with phage resistant genes (Mills *et al.,* 2002).

Contrarily to the unwanted event of cell lysis during early stage of fermentation in the process of cheese making, as could performed by phages, cell lysis could also decrease ripening time and enhance flavor due to the release of many enzymes. The controlled production of lysin and holin bacteria has shown the possibility to induce cell lysis at desired states of food fermentation (De Ruyter *et al.,* 1997).

Another important feature of food products is texture. Complete gene clusters, encoding exopolysaccharide producing enzymes have been transformed from one LAB strain to another one. The new generated strains could influence viscosity and texture of the fermented product (Germond *et al.,* 2001). In *S. thermophilus* the phosphoglucomutase gene was inactivated resulting in improved exopolysaccharide production enhancing the viscosity of the fermented food product (Levander *et al.,* 2002). Engineering of exopolysaccharide productio in *L. lactis* was also achieved by using a self-cloning strategy (Boels, 2002).

Genetic engineering using genes from non related micro-organisms could also be used to produce high added value products, such as L-alanine. By introduction of a *Bacillus* alanine dehydrogenase gene into a *L. lactis* strain deficient in lactate production, pyruvate conversion was pushed in the direction of alanine. The subsequent inactivation of the host gene encoding alanine racemase leads to the production of the stereo specific and thus high valuable L- alanine (Hols *et al.,* 1999).

The nutritional value of fermented foods can be improved via use of GM-LAB. It was shown that by overexpressing the complete riboflavin gene cluster (four genes) via a multi copy plasmid and strong promoter, the riboflavin production reached levels as high as 8 mg/L (Burgess *et al.,* 2003). Application of this strain in a fermented dairy product would lead to enormous decrease of intake levels in terms of volume to acquire the daily recommended intake of 1.3 mg of riboflavin. Another important vitamin in the human diet is folate. This B-vitamin decreases the risks for neural tube defects, cardiovascular diseases, some forms of cancer, and Alzheimer disease. Nowadays, still a big part of the population in both developed and lesser developed countries does not meet the DRI of folate (200-400 μg). The overexpression of all the genes encoding for the folate biosynthesis pathway in *L. lactis* has shown that under optimal environmental conditions, folate levels ranging from 5 - 8 mg/L can be achieved (Sybesma 2003a). This could result in fermented food products that require an intake of less than 100 ml to acquire the DRI for folate. More than 1.5 liter of the fermented food product should be consumed to administer the same level of folate by using the traditional natural strains. It should be stated here that both riboflavin and folate overproducing strains, were developed as proof of principles and in the current status contain plasmids with chloramphenicol resistant markers. These strains could only be applied in food fermentations, after the substitution of the antibiotic resistance marker with a food-grade marker. The research aimed at the generation of starter cultures that would increase the vitamin levels in fermented food, also focused upon control of the bioavailability of folate produced in the fermented foods. In nature folate predominantly exists as polyglutamyl folate, however, folate is absorbed as monoglutamyl folate. Several studies show that the bioavailability of monoglutamyl folate, that does not need enzymatic deconjugation, is higher than the bioavailability of polyglutamyl folate. The overexpression of the first enzyme in the folate biosynthesis pathway increases the production of monoglutamyl folate (Sybesma *et al.,* 2003ab).

Recently, the engineering of a *L. lactis* strain was described, that will contribute to the formation of lactose-free and naturally sweetened foods. By the deletion of 3 genes involved in glucose uptake and degradation and introduction of the genes for lactose-PTS and tagatose-6P pathway the strain can grow on lactose. The galactose-moiety of lactose is used as C-source, while the glucose moiety is completely excreted (Pool *et al.,* 2003). This GM-LAB obtained by self-cloning has identical properties as the earlier selected non-GM-LAB of Thompson (Thompson *et al.,* 1985). However, the genetic background of the latter strain was spontaneously altered and, except for the desired mutations, it is unknown to what extent more mutations have occurred.

An example of the use of recombinant DNA technology in the development of probiotic strains is removal of undesirable sugars from the gastro-intestinal tract. This project is promising for improvement of human health. Undegradable sugars such as raffinose and stachyose, that may be present in raw agricultural products like milk and soy, are causing health problems in a lot of people. Because of the lack of digestive enzymes for these sugars in the human GI tract, gas producing bacteria degrade these sugars resulting in intestinal pains and flatulence. The expression of genes encoding enzymes that are able to degrade raffinose, stachyose, etc, such as α-galactosidases (Boucher *et al.,* 2003; Silvestroni *et al.,* 2002), in certain LAB, results in the (extracellular) production of sugar degrading enzymes. Consequently, upon passage of these sugars through the GI tract, they will be degraded by the probiotic LAB and not by the resident gas-forming bacteria (Piard, 2003).

Also in feed industry strain improvement can be successfully applied. *Lactobacillus plantarum* is used as an inoculum in grass silage and vegetable products due to its pH lowering effect, which allows preservation of the products (Seale 1986; Daeschel *et al.,* 1987). Kerovuo describes the cloning of the phytase gene, *phyC*, from *Bacillus subtilis* in *L. plantarum* (Kerovuo *et al.,* 2000). Phytases are enzymes which hydrolyse phytate. These enzymes have a significant value as feed additives. The application of a phytase-producing silage strain in feed preparation may improve the nutritive value of feed and, at the same time, reduce the environmental phosphate pollution following consumption by monogastric production animals. Another engineered *L. plantarum* strain designed to improve the nutritional value of feed is an amylolytic *L. plantarum* silage strain with improved starch degrading capacity (Fitzsimons *et al.,* 1994).

Risk factors for use of genetically modified lactic acid bacteria

The process of introducing a novel trait into a micro-organism by the addition, substitution, removal, or rearrangement of defined DNA sequences, including the DNA sequences used for the maintenance or transfer of the new DNA into the recipient strain could lead to the generation of micro-organisms with novel genetic properties and a modified cellular behavior. It is anticipated that this will result in the intended effects. However, unintended effects, that could be a consequence of the modification, could also occur in the newly generated micro-organisms. A distinction can be made into the primary and secondary effects that differ in their origin. Primary effects are caused by the intended genetic alterations and secondary effects are caused by the unintended genetic alterations or they occur as a consequence of the primary effects.

The primary unintended effects

The primary unintended effects could occur during controlled genetic alterations. For example, by insertion or modification of genes polar effects, which affect adjacent genes, may occur. Another kind of primary unintended effects may arise as a result of the synthesis of a chimeric protein (fusion protein), due to the insertion of foreign DNA. These new proteins may provoke a different cellular behavior, but they might also lead to allergic responses of the consumer because of their different chemical structure. Careful analysis of the genomic organization prior to and following the controlled genetic alterations could be used to predict the occurrence of such primary unintended effects. Similarly, the use of a well-characterized locus for the insertion of the new DNA may prevent the occurrence of fusion proteins.

It should be noted that despite the fact that strains obtained via random mutagenesis are not GMOs, the occurrence of such primary unintended effects is very likely to occur within such mutant strain. In addition, it is possible that DNA elsewhere in the genome will also be affected by random mutagenesis that could result in synthesis of modified enzymes and altered metabolite production (secondary unintended effects), which might affect the health of the consumer. Therefore, it could be assumed that certain GM-LAB may have a lower risk profile than LAB isolated after random mutagenesis.

The secondary unintended effects

The occurrence of secondary unintended effects is much less predictable and could be a consequence of unintended genetic alterations, but also of the direct intended and primary unintended effects of the alterations. The overproduction of certain desired metabolites in the cell may result in unexpected and undesired cellular responses. For instance, a LAB defective in lactate dehydrogenase capacity may produce other acids and alcohol under certain environmental conditions. Increased concentrations of desired metabolites may also result in feed back inhibition of parallel biosynthetic pathways in the cell. Hence, a careful analysis of alterations in metabolism is desirable. Recently, it was shown that in *Lactobacillus johnsonii* by the inactivation of *ldhD*, responsible for the formation of D-lactate, primary and secondary end products were not easily redirected in high amounts to other routes (Lapierre *et al.,* 1999). Another secondary unintended effect may occur when by the overproduction of one product, the production of another compound is decreased. For instance, the synthesis of the vitamins folate and riboflavin starts with GTP as a substrate. An increased flux through the riboflavin biosynthesis pathway, as obtained in *L. lactis* producing increased riboflavin levels (Burgess *et al.,*

2003), may reduce the concentration of GTP available for folate biosynthesis. As a consequence, folate production levels may be decreased. Furthermore, the insertion of new DNA sequences into a LAB, may result in genetic reorganization causing unintended effects such as changed production levels of certain enzymes and, consequently, changed production levels of metabolites.

Another class of secondary unintended effects is the change of the microbial population when the GM-LAB is applied in mixed cultures. Several starter strains may grow simultaneously or in succession. For instance, during yoghurt fermentation *S. thermophilus* grows prior to *L. bulgaricus*, that starts growing after acidification of the milk by the *S. thermophilus*. During the fermentation of Sauerkraut, heterofermentative *Leuconostoc mesenteroides* initiates the fermentation and after 3 and 7 days it is usually succeeded by the more acid-tolerant homofermentative *Lactobacillus* species, while *Lactobacillus plantarum* completes the fermentation (Lu *et al.,* 2003). Hence, the synthesis of new metabolites, specifically those with antimicrobial activity may disturb the balance in a mixed culture, resulting in unintended changes in the final product.

The phenomenon of gene transfer can be seen also as a secondary unintended effect. In the previous section many examples are given about self-replicating vectors to introduce additional DNA in the LAB cell. The production and consumption of GM-LAB will result in their contact with micro-organisms in the environment, including the intestinal microbial flora of the consumer. Consequently, transfer of DNA between these micro-organisms may occur (horizontal gene transfer) (Doolittle *et al.,* 2003, Jain *et al.,* 2002). Transfer between the GM-LAB and the consumer has not been demonstrated (Genereux *et al.,* 2003). The horizontal gene transfer is not restricted to plasmid-based DNA as other modes of gene transfer, such as those mediated by transposons and phages, can also take place (Droge *et al.,* 1998). In case a selective advantage is conferred by transferred DNA, the likelihood of stable gene transfer may be higher. For this reason the use of transferable antibiotic resistance markers in fermenting micro-organisms is not allowed, because these could provide a selective advantage following transfer to intestinal micro-organisms as soon as consumers are treated with antibiotics. This is a realistic scenario since there is evidence indicating that gene transfer of antibiotic resistance genes has occurred to bacteria that are present in traditionally fermented foods (Teuber *et al.,* 1999). In contrast, the inactivation of genes by deletion does not result in the addition, but in the omission of DNA. Consequently, any discussion about potential gene transfer in GM-LAB with inactivated genes is void.

Considerations for restrictions in development GM-LAB

Partially following earlier recommendation (LABIP, 1998), and to ensure the safety of humans and the environment, it is recommended to make some restrictions regarding the uncontained use of GM-LAB. 1. Only LAB with a qualified presumption of safety (see below) should be used as a recipient. 2. The use of transferable antibiotic resistance markers should not be permitted. 3. The expression of far distantly related genes in LAB is acceptable under contained use only, unless the inserted gene will not induce growth or survival advantages of other micro-organisms after potential horizontal gene transfer. 4. The strain engineering may never result in production of toxic compounds or generation of a pathogenic strain that may affect human health.

Food-grade micro-organisms

Use of micro-organisms in food production is accepted when they have a long history of safe use, however it is not scientifically defined what is a long history and what is a safe use. Moreover, a recent draft document (SANCO, 2003) suggests that only micro-organisms that have a long and safe history of use in food and that also have a qualified presumption of safety status (see below) should be applied in food industry. These strains could be used as recipients for the genetic modification. The use of pathogenic micro-organisms should not be allowed. However, the history of safe use in food should not be an ever lasting grant that the strain could be always be applied in food fermentation. It goes without saying that, in case new research shows that strains with a long history of safe use are producing toxic components in levels that may harm human health, these strains should not be accepted anymore in food fermentations. For instance, certain LAB have an unblemished history of safety in food fermentation, but, as was discovered later, may produce unfavorable amines (Ten Brink *et al.,* 1990, Cantoni *et al.,* 1994, Faeth and Radler, 1994). Evidently, these strains should not be used anymore in preparation of foods without a profound safety assessment, that, especially in this case, also investigates the actual concentrations of the harmful compound to which the consumer will be exposed.

Transferable antibiotic resistance markers

The use of transferable antibiotic resistance markers in LAB used in food production may increase the risk of their transfer to human intestinal flora and cannot be allowed. The use of safe and sustainable food-grade selection markers is recommended (de Vos, 1999). However, contrarily to current legislation, the use of antibiotic resistance markers in intermediate strains that are used for the generation of new bacterial strains with improved characteristic and that are completely removed before the final production strain is

developed, should not be rejected on basis of reasons related to antibiotic resistance. For instance, the selection of the earlier described GM-LAB with inactivated α-acetolactate decarboxylase (Curic *et al.,* 1999), should be allowed, because the vector containing the antibiotic resistance gene was removed from the selected strain. It is noteworthy that antibiotic resistance could also occur spontaneously. Well known examples include resistance to streptomycin and rifampicin that are a consequence of point mutations in rRNA or RNA polymerase genes, respectively. A specific case is resistance to vancomycin, one of the last clinically used antibiotics. While strains with vancomycin resistance genes have been described that were transferable and that should not be used for food fermentations, also LAB can spontaneously acquire vancomycin resistance. In such vancomycin resistant strains spontaneous mutations in genes encoding cell wall synthesizing enzymes may result in altered peptidoglycan intermediates bearing D-alanine–D-lactate termini, that confer vancomycin resistance (Baptista *et al.,* 1997, Arthur *et al.,* 1996). Similarly, LAB that produce D-lactic acid show enhanced resistance to vancomycin. These naturally occurring phenomena however, are not a reason to exclude such strains from consideration as recipients in constructing GM-LAB, since they are not transmissable. Specific consideration needs to be given to tolerance to antibiotics that is caused by certain classes of transporters. Many of these are naturally present in LAB and may be essential for their survival in certain environments where they could be involved in export of toxic compounds. It is conceivable that these transporters also export structurally related compounds, such as some antibiotics. In most cases the level of antibiotic resistance is far from that found in clinically relevant antibiotic-resistant pathogens. Hence, there appears to be no need for specific precautions when such antibiotic tolerant LAB strains are used as recipients.

Expression of homologous or heterologous genes

The potential of gene transfer following expression of foreign genes in LAB used in food fermentations is one of the major concerns that affect the safety assessment for consumer and environment. When genetic elements that are used for the modification originate from LAB that have a long and safe history of use in food, it is often suggested that no new risks are introduced. Although this argumentation is very logical, it does not make the fermented food safe on forehand, and adequate safety assessment remains necessary. However, it could eliminat the risk for negative consequences for the environment. The net effect of isolating a gene from a strain, duplicate or copy it to the same or to another LAB, and release it, does not change the gene frequency in the environment. For LAB (and other microbes) it is said that all genetic information is already

omni-present, but that nature selects. Thus, fear for negative consequences after the release of such engineered strains in the environment is unfounded. Evidently this is also valid for strains that have been subjected to spontaneous DNA modifications, or deletions of DNA.

After millions of years of evolution guided by natural selection, it is unlikely that genetically modified micro-organisms can be developed that have improved fitness in the environment. However, the introduction of more distantly related genes in micro-organisms is a less natural event. The cloning of such genes on a plasmid would not be acceptable due to the possibility of horizontal gene transfer in case a selective advantage could be envisaged. Therefore, some precautions are suggested for the expression of heterologous genes in LAB from non-microbial origin. As long as heterologous genes are only inserted in the chromosome via double cross over, and the inserted heterologous gene will not lead to evolutionary advantages and survival of such strains, there are no reasons to fear the consequences of potential gene transfer. Containment of such engineered strains may prevent interactions with the environment. This may be realized by limiting their survival in the environment because of dependence on the presence of certain medium components, pH, or temperature.

Genes that may cause pathogenicity (e.g. generation of virulence factors) or that encode for enzymes that are known to be involved in synthesis of toxic compounds or precursors of toxic compounds, should *a priori* be excluded as target for genetic engineering. Finally, the use of sequences that mediate integration into other genomes should be avoided in development of genetic constructs.

Considerations of the safety assessment procedures for foods derived from traditional LAB and GM-LAB

Introduction

With the exception of those LAB not previously used to a significant degree in the preparation of a human food within the EU, LAB for food use are not subject to EU regulation. Implicit in this absence of any formal requirement for a safety assessment is the recognition that there has been a long history of presumed safe use. The long and safe history of use status of LAB is in itself an arbitrary criterion and may need further consideration. If the long history of safe use status is based on the absence of reports of the occurrence of adverse effects on consumers of fermented foods, it could be questioned whether also GM-LAB could be accepted until negative reports appear. This

would imply the immediate acceptance of all non-pathogenic GM-LAB for food production. However, is this whole debate not made for the assurance of safety for the society? Before regulation about food safety was an issue, the society decided autonomously that it wanted to consume certain fermented foods, probably based on positive experience. These facts could be a convincing social, but less scientific, argument that LAB with a long history of safe use may be accepted in food fermentations until adverse effects will be reported. The European Food and Feed Culture Association (EFFCA) has produced a list of starter species for which a documented history for use in food manufacture exists. Documented history of use is defined by EFFCA as cultures sold for human consumption in quantities exceeding 100 kg of freeze dried culture. Although for many species belonging to LAB safety documentation is ample, the list also contains species that are potentially pathogenic species.

Qualified Presumption of Safety (QPS)

Within the EU it is proposed to introduce a system similar in concept and purpose to the GRAS (Generally Recommended As Safe) definition used in the USA, which could be applied to micro-organisms and their products. Such a system should lead to a listing of qualified micro-organisms that will not have properties that may adversely affect human health or the environment (SANCO, 2003). For this purpose, Qualified Presumption of Safety (QPS) is being introduced as an assumption based on reasonable evidence. It aims to provide a qualified generic approval for non-genetically modified micro-organisms, without requiring all organisms used in food production with a long history of safe use to be subjected to a full and unnecessary safety review. A case-by-case safety assessment then could be limited to only those aspects that are relevant for the organism in question. A pre-requisite for QPS would be identity that is unambiguously established at the taxonomic level claimed. Appropriate and state of the art biochemical and molecular biological methods must be applied for this. For organisms not commonly used in food production or without a long history of safe use, this implies a need for experimental data on the genetics of the taxonomic unit and the growth and biochemical characteristics of the component strain under a variety of relevant environmental conditions. For some groups of organisms, such as those used as plant protection products, a consideration of impact on the wider environment may be appropriate. However, this could exclude organisms considered either to be of uncompromised origin and regularly introduced into the wider environment or originating from soil or water. In both cases the organisms are naturally occurring and therefore free of any need for an environmental impact assessment. The QPS could only be valid for bacteria that enter the food chain and that are free of any acquired resistance

to antibiotics of importance in clinical and veterinary medicine. The presence of antibiotic resistance determinants would not exclude their safe use for production purposes provided that only the fermentation products are retained in the final product. A decision scheme relating to the concept of QPS has been proposed and is presently subject to discussion (Fig. 1) (SANCO, 2003). It is worthwhile mentioning that this QPS decision protocol might be subjected to modifications before its implementation may occur. Especially the decision step questioning if any other concerns related to safe use of micro-organisms exist, which are not involved with pathogenicity or toxicity, is currently not well defined.

Many industrial strains will be the product of mutagenesis and selection programs designed to improve their phenotype for a particular purpose. These cryptic mutations will not affect taxonomic status. As a consequence, following the QPS decision tree, strains that are not produced via genetic modification, but contain altered DNA sequences that may cause primary or secondary unintended effects, will be accepted for use in food preparation without a further safety assessment. The next paragraph considers whether such strains of LAB should be subject to similar scrutiny as GM-LAB.

Should traditionally fermented foods be subjected to similar safety assessment procedures as novel foods?

Due to the long history of use of many LAB, their products are not systematically assessed for safety. Moreover, improved strains obtained after random mutagenesis are not assessed either. However, the omission of adequate safety assessment in the past does not render the fermented foods safe for consumer and environment in the future. The safety assessments that have been conducted thus far, are primarily intended to apply to discrete chemical entities such as food additives, or specific chemical or microbial contaminants that pose identifiable hazards and risks.

It is likely that unintended effects could always occur in the development of strains using traditional (non-recombinant DNA) techniques, or from exposure of micro-organisms to selective pressure or certain environmental conditions, or just by natural events. This justifies that each new natural LAB strain should undergo the same safety assessment procedure as engineered strains. Whether this is a viable approach is currently being investigated by analyzing differences at the global transcriptional level in strains isolated by random mutagenesis and by directed mutagenesis (Renault, 2001). The development of GM-LAB for use in food should be focused on minimizing the occurrence of unintended effects with adverse consequences on human health. Risk assessment studies using new

technologies should focus on global response in cellular metabolism as a tool to identify unintended effects.

Table 4: Essential information for safety assessment of novel foods (as laid down in Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council).

I.	Specification of the novel foods.
II.	Effect of the production process applied to the novel foods.
III.	History of the organism used as the source of the novel foods.
IV.	Effect of the genetic modification on the properties of the host organism.
V.	Genetic stability of the GMO used as novel foods source.
VI.	Specificity of expression of novel genetic material.
VII.	Transfer of genetic material from GMO.
VIII.	Ability of the GMM to survive in and colonize the human gut.
IX.	Anticipated intake/extent of use of the novel foods.
X.	Information from previous human exposure to the novel foods or its source.
XI.	Nutritional information on the novel foods.
XII.	Microbiological information on the novel foods.
XIII.	Toxicological information on the novel foods.

It could be considered that the decision to grant a QPS status to strains that are isolated after spontaneous or induced random mutagenesis, or to assess those strains to a profound safety assessment procedure as obliged for novel foods, would be taken after the performance of representative comparison profiling studies, like genomics, transcriptomics, proteomics, and metabolomics, between the mentioned strains and their naturally conventional counterparts. In case that the outcome of such studies would not indicate relevant differences between the strains that could potentially have adverse effects on the health of the consumer or that are unknown, the QPS protocol could be applied. In analogy with the outcome of these studies, the profiling of engineered strains obtained by directed genetic alterations may result in a more scientific food safety assessment procedure for novel foods derived from these GM-LAB, in which not the technical aspects but the final products are assessed.

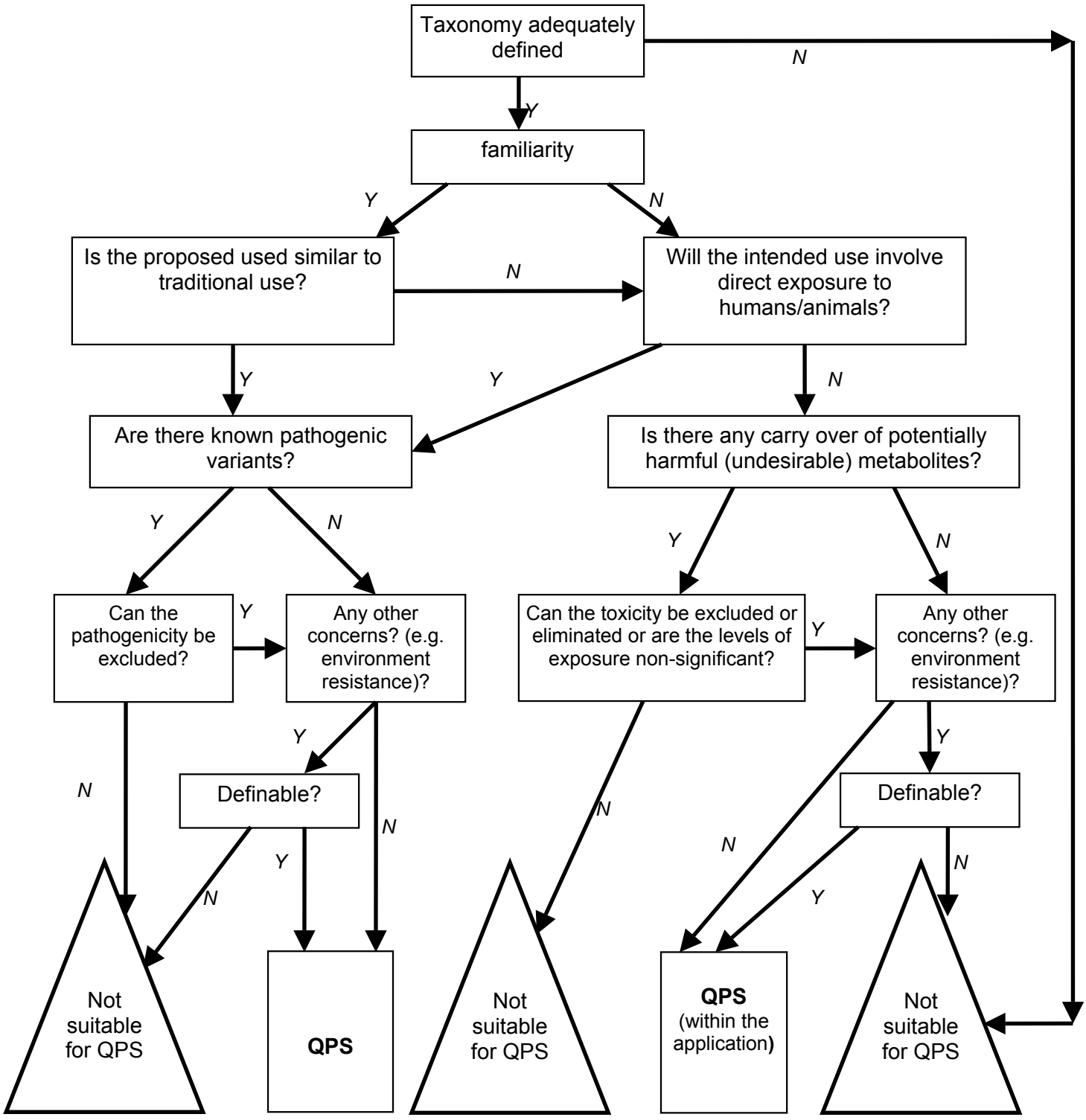

Figure 1:
A general scheme for the assessment of suitability for QPS status of micro-organisms (as proposed by SANCO, 2003).

Considerations of safety assessment procedures of GM-LAB and novel foods

The safety assessment of food produced by GM-LAB should comprise analysis of the intended effect, the nature of the modification, and detectable unintended effects that may occur in the micro-organism or in the food that is produced by the fermenting micro-organism. The development of modern genetic techniques could enhance the safety assessment, and the follow up by post monitoring surveillance may contribute to the safety assessment and increase the consumers trust. Furthermore, the safety assessment procedure should encompass the obligations stated in directive 2001/18/EC and Commission Recommendation 97/618/EC (summarized in Table 4).

Modern DNA techniques, genomics, transcriptomics, proteomics, and metabolomics

The rapid development of genetic and biochemical techniques can be applied to increase reliable, fast and profound safety assessment of the fermenting micro-organisms and the derived foods, in accordance with the concept of substantial equivalence. It is desired that the occurrence of unintended effects could be foreseen in an early stage and that potential harmful effects could be analyzed on forehand, because the presence of new proteins or the change in concentrations of certain proteins could lead to the information of altered activity of biochemical pathways that may result in changed metabolite content. Moreover, allergenic potential could be investigated for new detected proteins. The analysis of the complete set of metabolites in the cell could also provide information about potential toxicity and about the general nutritional value.

Genome wide analysis of modified strains and of their natural counterparts, for instance by using fingerprints techniques, or by complete sequencing of the genome, can reveal all loci in the genome where mutations have occurred that could possibly result in unintended effects. Transcriptome analysis, by using DNA array or DNA chip technology, could provide information about altered expression patterns of the new micro-organisms compared to the wild-type strain. The observation of altered expression levels of genes not involved in the targeted effect of the genetic engineering, could be a starting point for the analysis of the reaction products of the enzymes that the concerning genes are coding for. This might be done by studying of the literature and application of bioinformatics, and, if a harmful compound may be produced, subsequent biochemical analysis could be performed to measure the presence of these chemicals. More relevant for food safety assessment is an analysis of the proteome and metabolome of the modified micro-organisms. Proteome analysis, by using 2D-gel electrophoresis or 2D-HPLC, provides information on altered protein concentration and content produced by the modified micro-

organisms. Likewise the outcome of the transcriptome analysis, the reaction products of the new proteins or increased protein concentrations themselves could be predicted and analyzed. Moreover, suspected proteins, that have partially a similar amino acid sequence as known allergens, could be assessed for potential allergenicity. The value of proteomics has already been proven in genetically modified rice that was engineered to produce more storage proteins. 2 D-gel analysis revealed an unexpected increased level of prolamins (FAO/WHO, 2000). Metabolome analysis, by using a wide range of chromatographic and spectroscopic techniques, could provide information about the presence of new metabolites or altered concentrations of existing metabolites. Increased concentrations of known metabolites could be compared with current recommended maximum intake levels to assure the consumer's safety. Likewise, decreased concentrations of vital components could reduce the nutritional value of the new product. The identification of new proteins or new metabolites could be a starting point for further toxicological studies. To strengthen the safety assessment it could be considered to perform transcriptome, proteome and metabolome analyses under food processing conditions and under GI-tract conditions in case the new engineered strains have potential for survival in the human gut. The outcome of these data will also provide information on the occurrence of the intended and, if present, of the unintended effects. The conclusion of the analysis should be the assurance that neither the intended effects, nor the unintended effects, have adverse effects on human health, because no harmful components or proteins with allergenic characteristics are being produced.

For the near future it is possible to develop a universal proteome and metabolome reference frame, consisting of all proteins and compounds produced by fermenting micro-organisms. The proteome and metabolome reference frame should contain information about potentials for allergenicity, based upon amino acid sequence comparison with known allergens, and information about acceptable maximum and minimum levels of all compounds present in food. The changed protein and metabolome content of any new engineered strain can be easily compared with the reference frame. Comparison of the proteome and the metabolome of the new engineered strains with the reference frame, could be a starting point for the further food safety assessment procedure and could ensure that further safety assessment remains proportional to the perceived risks. For instance, if it would be verified by studying the transcriptome, proteome, and metabolome, that proteins or metabolites are not produced in harmful concentrations and that the nutritional value is not adversely affected, no further safety assessment would be required for the fermenting micro-organisms and the fermenting food.

Post market surveillance

A post market surveillance (PMS) on novel foods, involves the observation of (health) effects on the consumer over a longer period of time after release of the novel food on the market. It could be questioned whether after approved pre-market safety assessments of genetically modified foods, the use of post market surveillance would still be necessary. However, it is known that foods may contain anti-nutrients and toxicants which may lead to (delayed) adverse effects for health of humans and animals. Moreover, various other factors may also be involved in determining the safety of food on a longer term, like the consumption conditions, and the genetic profile of the consumer. Therefore, to further protect the general society including specific sub-populations, and to provide additional information on long term effects, post surveillance monitoring could be applied in the form of a recording and investigation system by national health services, perhaps similar to the adverse drug effects reporting systems used by doctors. Up to now, only a few food additives have been subjected to post surveillance monitoring, including the artificial sweetner aspartame (Butchko *et al*., 1994) and the fat replacer olestra (Slough *et al*., 2001). Recently within the EU a PMS has started on a novel food related to the use of phytosterol-esters in Yellow Fat Spreads that is assumed to have cholesterol lowering effects in the consumer (Lea, 2002). However, it may be difficult to introduce a PMS over a longer time period, if the food consumption pattern of individuals is not consistent over a long time. It should be taken into account that some consumers might be exposed to newly introduced components from different sources during their daily consumption pattern and consequently might be at risk because of overexposure to certain compounds. The post market surveillance may be applicable to confirm the actual intake or to further support the absence of well defined safety concerns such as toxicity and allergenicity

Although the application of post market surveillance to food products raises significant technical issues, besides the active surveillance, also passive surveillance could be envisaged, which is based on spontaneous reports. Apart from this, it could be considered that for novel foods derived from GM-LAB that are substantially equivalent to LAB with a QPS status, a PMS would not be necessary.

The Dutch Council of Health has recommended the introduction of a PMS characterized by 1. Setting up a government supported complaints line for all consumer complaints associated with health and foods. This would enable any side effects produced by a product to be traced, provided that they arise soon after consumption and that they attract attention. One such example is food allergy. This monitoring system will not reveal

any causal relationships. However, it can be used to generate hypothesis. In this way it would alert people to health problems, leading on to a focused follow-up study, 2. Continual monitoring of consumption of foodstuffs, carried out jointly by government and industry. A precondition here is that this must facilitate a detailed breakdown of data on individual products into information on their ingredients (down to the molecular biology level, in the case of genetically modified foods), 3. Long term epidemiological, prospective, cohort studies into the relationship between chronic diseases and diet, and 4. Active market monitoring program, carried out by companies for novel foods, that contain bio-active ingredients. The aim here is to check the accuracy of the presumed (safe) intake of the target group (Dutch Council of Health, 2002).

It may be concluded that the PMS could only prove its value by signaling hazardous events resulting in immediate withdrawal of the concerned food. The PMS could not be seen as a safety assessment, but as an early warning system that operates better as the adverse effects are stronger. Moreover, it could investigated if an increased confidence of the consumer to use novel foods could evolve, when the principle and reasoning for setting up a PMS are well communicated to the consumer.

Suggestions for cost accountability of safety assessments for novel foods

The development, approval and subsequent market release of novel fermented foods involves many economical issues for industry, consumers and the society in general. Investments in research and development by the food industry will provide opportunities for employment and the development of new scientific technologies. The consumption of novel foods with added value will benefit the consumer. Especially the introduction of health claim proven products, may contribute to the generation of a healthier community, which is expected to decrease costs for public health.

The costs that are made for the development of novel foods, can only be worth the investment in a society that accepts and buys these novel foods. The introduction of traits that benefit the production costs of the manufacturer, should lead to a price reduction, as a way to generate an advantage for the consumer too. The introduction of traits that benefit the consumer, will lead to added value of the product and may justify a price increase for the consumer. Moreover, novel traits could also be developed that have a lower environmental load and that bring advantages to the society as a whole. The obligation of performing a profound safety assessment, before the launching of any novel food, could enormously increase the initial costs to be made by industry. The question is who should

cover these costs: the industry, the consumer, national authorities, or supra national authorities?

Public money from the European Union, the European Food Safety Authority, and national food safety or research funds, could be used to set up the complete infra structure required for food safety assessment as stated in the paragraphs above, including development of genomics, transcriptomics, proteomics, and metabolomics. It will also be the task of the regulatory authorities to control and conclude the whole safety assessment protocol. The industry pays its own research and development and could pay also all the costs required to go through a complete and profound safety assessment. The pre-market assessment costs could be financed by the industry too. After approval of the novel food, post market surveillance remains a combined matter for the society and the industry and should be funded by both.

The high costs for the development and assessment of novel foods may be a limiting factor for the industry to develop novel foods, especially those with interesting traits, benefiting for instance the health of the consumers. Therefore, it should be allowed for the industry to promote novel foods with scientifically proven advantages over traditional foods by labeling and other forms of publicity. Currently, the possibilities for promoting scientifically proven functional foods are limited and not harmonized within various EU countries. It is evident that the consumer should be protected by prohibiting the labeling of untrue claims. However, not permitting the labeling of true health claims is equally wrong (Katan and de Roos, 2003). The future EU regulation, as proposed in its working paper of 2002 about nutritional and functional claims (SANCO, 2002), is promising for consumer and industry by allowing and regulating the labeling of health claims.

The consumer's perception

Genetic alteration of especially plants and animals is seen by many Europeans as an interference with nature that has unknown and potentially disastrous interactions with human genetics and natural ecosystems. Many consumers are extremely aware of health threats caused by unknown risks in food supply. The green society has raised several questions about the environmental risks of genetically altered crops and animals. Environmental risks for plants include the evolution of GM-plants into "super weeds," cross-pollination introducing herbicide resistance into existing weeds or introducing undesirable genetic traits into neighboring crops, and harm to non-target populations caused by toxins introduced to create insect resistance.

In the present work we have shown that the consequences of development and use of GM-LAB are different compared to GMO from plant or animal origin. The potential intended and unintended effects and related risks can be better predicted. In combination with profound safety assessments, safety for consumers and environment can be assured. The occurrence of unintended effects is not unique for GM-LAB, but also occurs in LAB used in conventional food fermentations. Logically, this is no reason to ban GM-LAB, but it could be a reason to analyze the unintended effects of traditionally used strains in food fermentation by genomics-based profiling methods. A further response to the public concern is to strengthen the pre-marketing data requirements and to introduce post-launch surveillance program to confirm safety as described above.

Besides an approval of the safety assessment, the GM-LAB designed for use in food production should have a clear benefit for the consumer. Not only for the sake of the industry, but also for the consumer, labeling on food products should emphasize the benefits (eventually health claims) of the novel foods. Evidently, the product labels should inform the consumer also about nature of the (genetically modified) bacteria, the risk assessment procedure, and the conventional counterpart. Moreover, denying the existence of certain scientifically proven claims is in fact a misleading policy to the society. The addition of fluor to drinking water, iodium to salt or bread are already functional foods *avant la lettre.* These measures were taken by the national regulating authorities of some EU countries. Recent work provides an overview of health claims used or proposed in the USA, Japan, and Europe (Katan and de Roos, 2003). In the USA, the FDA has approved 14 health claims such as the role of calcium in osteoporosis and folate in prevention of neural tube defects (FDA, 2000 Food Labeling Guide).

To increase trust among consumers, industry needs to accept liability in case of damage caused by novel foods. Liability for common and unpredicted adverse effects directly related to the consumption of the novel food should be for the manufacturer. Liability for predicted adverse effects for sub-populations, as is known already to occur by the consumption of traditional foods, is for the consumer only when labeling included warnings for the specified adverse effects that could occur in sub-populations. Moreover, the free choice of the consumer should be respected. Thus, alternative similar products, although they will not posses the introduced advantageous traits, should be widely available on the market.

A complex set of legislation and regulation seems at present times the only correct response to consumer demand for protection from the perceived harms that could be

caused by GMOs. It will be worth the investment to strongly rely on this precautionary principle, which directs that actions should be taken to prevent potential harms even if there is a lack of scientific certainty about the harm in the eyes of industry or scientists. However, it remains to be awaited if the consumers will accept this as a guarantee for the safe consumption of novel foods. For the future it is recommended to overcome the lack of knowledge concerning the basic science of genetics and biotechnology. Governmental sponsored research programs could be set up with the ultimate goal of further reducing the uncertainty and mistrust within the society. Educating the public on these matters may help to overcome the negative emotions related to the use of certain GMOs.

Conclusions

The low public trust in industry, regulatory authorities and sciences is a major element contributing in the EU to the general negative perception of GMOs. However, it should be questioned if this negative attitude towards GMO originates from a majority of the population, or might be induced by just a small minority that represents itself efficiently. The set up of an infrastructure by the regulatory authorities to subject all fermenting micro-organisms and fermented foods to a new and profound risk assessment procedure, might be the extra step that is currently needed to convince all parties involved that novel foods are as safe as traditional foods. A distinction within the discussion and legislation between food safety assessment procedures for fermented foods derived from micro-organisms, especially LAB, or from GMO derived from other origin, especially plant and animal origin, could bring nuances in the current controversy. As a starting point, the QPS protocol (SANCO, 2003), based on taxonomy and on the history of safe use of LAB applied in food, could in the near future be applied for any kind of LAB or GM-LAB provided that a series of modern profiling methods have verified the absence of unintended effects of altered LAB that may cause harm to the health of the consumer.

Objective safety assessments of GM-LAB could lead to correction of inconsistencies in current legislation that directly affect the use of GM-LAB in food. It is expected that study of genome evolution and profiling studies will reveal that in many organisms the genetic material, that is naturally or spontaneously changed, has been modified more than can be established by controlled alterations, including genetic modification. Direct genetic engineering, including self-cloning that does not introduce new DNA in a strain, generates targeted modifications that can be much better controlled and evaluated than strains that are exposed to random mutagenesis approaches. If such strains, not defined as GMO, are accepted in food fermentations via QPS status, the

acceptance by the society of GM-LAB obtained via controlled genetic alterations, could be improved. Apart from this, strains that were selected after the occurrence of spontaneous mutations and via a GM-LAB as intermediate strain, which is rendered a non-GM-LAB however in the final strain, should not be considered as GMO and should easily receive the QPS status.

For the moment, in line with current legislation, safety issues should dominate the acceptability of GM-LAB in food industry. A targeted approach should be based upon analysis of key compounds in the new food, like proteins, carbohydrates, fats, vitamins, minerals as well as on analysis of toxicity. A non targeted approach should use profiling methods to detect global responses in the new food. Global expression profiles will reveal whether changes additional to those expected occurred. The expression of any new or altered gene in LAB, that is either randomly mutated or self cloned, may affect the transcriptome, proteome, and metabolome profiling and could tell if the expression of the new gene results in any (undesirable) side effects. For the category of GM-LAB that have genes introduced originating from distantly related organisms, it can be added that apart from assessment by using global profiling methods horizontal gene transfer may not lead to improved growth or survival.

For the consumer's sake, novel foods should be well labeled, communicating to the consumer the evidence of health claims, toxicological and beneficial dose ranges of selected compounds, the impact on overall dietary intake and associated effects on consumers, and the possibilities for effective post market surveillance.

In conclusion, broad evaluation of the nature of novel foods derived from GM-LAB, which could be considered as a specific group of GMOs, could be a starting point to bridge the gap between industry, consumer and green society. This may lead to acceptance of GM-LAB derived novel foods to serve a better quality of life in our today's society.

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Chapter 3

Effects of Cultivation Conditions on Folate Production by Lactic Acid Bacteria

Wilbert Sybesma[1], Marjo Starrenburg[1], Linda Tijsseling[2], Marcel H.N. Hoefnagel[1,3], and Jeroen Hugenholtz[1].

1. Wageningen Centre for Food Sciences, Wageningen, The Netherlands
2. Dairy Technology Group, Department of Agrotechnology and Food Sciences, Wageningen University, Wageningen, The Netherlands
3. Process Engineering Group, Department of Agrotechnology and Food Sciences, Wageningen University, Wageningen, The Netherlands

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Abstract

A variety of lactic acid bacteria was screened for their ability to produce folate, intra- and/or extracellularly. *Lactococcus lactis*, *Streptococcus thermophilus* and *Leuconostoc sp.* all produced folate while most *Lactobacillus sp*, with the exception of *Lactobacillus plantarum* were not able to produce folate. Folate production was further investigated in *L. lactis* as a model organism for metabolic engineering and in *S. thermophilus* for direct translation to (dairy) application. For both these two lactic acid bacteria, an inverse relationship was observed between growth rate and folate production. When cultures were grown at inhibitory concentrations of antibiotics or salt, or when the bacteria were subjected to low growth rates in chemostat cultures, folate levels in the cultures were increased relative to cell mass and (lactic) acid production. *S. thermophilus* excreted more folate than *L. lactis*, presumably as a result of differences in the number of glutamyl residues of the produced folate. In *S. thermophilus* 5,10-methenyl and 5-formyl tetrahydrofolate were detected as the major folate-derivatives, both containing 3 glutamyl residues, while in *L. lactis* 5,10-methenyl and 10-formyl tetrahydrofolate were found, both with either 4, 5 or 6 glutamyl residues. Excretion of folate was stimulated at lower pH in *S. thermophilus*, but the pH had no effect on folate excretion by *L. lactis*. Finally, several environmental parameters were observed which influence folate production in these lactic acid bacteria; high external pH increased the relative folate production, addition of *p*-aminobenzoic acid stimulated folate production, while high tyrosine concentrations led to decreased folate biosynthesis.

Introduction

Folate is an essential component in the human diet. It is involved, as a cofactor, in many metabolic reactions, including the biosynthesis of the building blocks of DNA and RNA, the ribonucleotides. The daily recommended intake for an adult varies from 200 µg in Europe to 400 µg in the USA. For pregnant women a double dose is recommended, since folate is known to prevent neural-tube defects in newly borns (29,42). Recently, high folate diets were claimed to protect against cardiovascular diseases (7,8) and, even, some forms of cancer (2). Several recent reports have indicated that folate deficiency is common among various population groups including women of childbearing age (18,26).

Folate is produced by various green plants and by some micro-organisms. Vegetables and dairy products are the main source of folate for humans. Milk is a well-known source of folate. It contains between 20 and 50 µg/l folate and thus contributes significantly to the daily requirement of the average human. Some fermented milk products, especially yogurt, are reported to contain even higher amounts of folate (1). Up to 110 µg/l folate has been found in yogurt. This high level is a direct result of the production of additional folate by the lactic acid bacteria in the yogurt. Of the two lactic acid bacterial species in yogurt, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, only the latter is reported to produce folate (30). Recently, also some other lactic acid bacteria were observed to produce folate during milk fermentation (19).

Folate is synthesized from the precursors GTP, *p*-aminobenzoate (pABA) and glutamate. In a number of enzymatic steps these three building blocks are modified and used for the further production of various folate derivatives that are necessary for C1-metabolism, such as tetrahydrofolate, 5-formyl tetrahydrofolate, 5,10-methenyl tetrahydrofolate,10-fomyl tetrahydrofolate, 5,10-methylene tetrahydrofolate and some others (13,36).

In earlier work we reported the influence on folate production of metabolic engineering of the folate biosynthesis pathway (39). In the present work we study the influence of growth conditions and medium composition, especially the role of pH and *p*-aminobenzoic acid (pABA), on folate biosynthesis and folate distribution in lactic acid bacteria. We concentrate on *Lactococcus lactis* MG1363 (12) as best-studied model lactic acid bacterium for metabolic engineering and starter bacterium for cheese, butter and buttermilk production, and on *Streptococcus thermophilus* NIZO strain B119, as a starter bacterium for the production of yogurt, probiotic dairy products and several cheese varieties. Large differences in folate production were observed between different strains and different growth conditions.

Materials and methods

Bacterial strains and growth conditions. 15 *Lactococcus lactis* strains, 2 *Leuconostoc* strains, 7 *Lactobacillus* strains, 3 *Streptococcus thermophilus* strains and 1 *Enterococcus* strain were tested for their ability to produce folate. The strains are listed in Table 1 and included is the NIZO strain no., the original code as found in official strain collection, literature references in case of previous publications, or the source of isolation.

Lactococcus strains were cultivated in M17-medium (41) or chemically defined medium (CDM, 28) supplemented with 0.5% glucose at 30°C. *Lactobacillus* and *Leuconostoc* strains were cultivated in MRS medium (9) at 37°C and 30°C respectively. *Streptococcus thermophilus* strains were cultivated at 37°C in M17-medium. All strains were routinely stored in litmus milk with 0.1% yeast extract at - 40°C.

For the study of cultivation effects on folate production, *Lactococcus lactis* strain MG1363 was grown in chemically defined medium (28) without folate, *p*-aminobenzoic acid and the aromatic amino acids, phenylalanine, tryptophane and tyrosine. *Streptococcus thermophilus* strain B119 was grown in Difco Folic Acid Casei Medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA). The growth medium was supplemented with erythromycin (0 - 0.1 µg/ml), chloramphenicol (0 - 2.5 µg/ml), NaCl (0 - 0.8 % w/v), or hemin (10 µg/ml), as indicated in the figures and tables. Growth was determined by the increase in optical density at 600 nm. Aerobic growth conditions were obtained by growing 5 ml cell culture in a 100 ml tube under continuous shaking, 210 rpm, in a waterbath at 30°C (Gyrotory waterbath G76, New Brunswick Scientific, Edison, N.J. USA).

Continuous fermentation. For continuous cultivation a 1 L glass vessel (Applikon Dependable Instruments, Schiedam, The Netherlands), filled with 0.5 L growth medium, was used, as described previously (35). Temperature was controlled at 30°C or 37°C and pH controlled by titration with 5N NaOH using the Biocontroller ADI 1020 (Applikon dependable Instruments, Schiedam, The Netherlands). Anaerobic conditions were maintained by sparging the headspace of the culture with N_2-gas. For cultivation of *Streptococcus thermophilus* NIZO strain B119, modified M17-medium was used with potassium phosphate (0.6 g/l) replacing ß-glycerophosphate and 0.5% lactose as the growth-limiting substrate. For continuous cultivation of *Lactococcus lactis* MG1363 chemically defined medium without *p*-aminobenzoic acid, folate and phenylalanine was used with 0.5% glucose as the growth-limiting substrate.

Analysis of intra- and extracellular folate concentration. Folate was quantified using a *Lactobacillus casei* microbiological assay (14). Cells and supernatant were

Table 1: Extra- and intracellular folate production before and after enzymatic deconjugation by different species and strains of lactic acid bacteria.

Strain	Origin or original strain code	Nizo strain no.	Extracellular folate (µg/l)	Extracellular folate$^{deconj.}$ (µg/l)	Intracellular folate (µg/l)	Intracellular folate^{deconj} (µg/l)	Total folate^{deconj} (µg/l)	Total folate^{deconj} (µg/l/unit OD 600nm)
Lactococcus lactis subsp. *cremoris*[A]	HP	B42	14	14	44	81	95	39
L. lactis subsp. *cremoris*[A]	E8	B64	10	9	47	99	92	33
L. lactis subsp. *cremoris*[A]	SK110	B697	41	46	40	69	116	50
L. lactis subsp. *lactis*[A]	MG1363 (14)		10	8	37	87	94	31
L. lactis subsp. *lactis*[A]	KB (25)	B628	9	6	39	84	90	25
L. lactis subsp. *lactis*[AC]	KB (25)	B628	15	11	165	245	256	29
L. lactis subsp. *lactis*[AC]	NZ 9000 (6)		13	9	38	106	116	43
L. lactis subsp. *lactis*[AC]	NZ 9010 (6) *Ldh* negative		20	22	184	269	291	39
L. lactis subsp. *lactis*[A]	Radish	B26	20	26	25	65	91	32
L. lactis subsp. *lactis*[A]	Frozen peas	B27	12	8	27	61	69	25
L. lactis subsp. *lactis*[A]	Silage (3)	B1171	11	14	30	47	62	22
L. lactis subsp. *lactis*[A]	Silage (3)	B1172	11	10	29	53	63	23
L. lactis subsp. *lactis*[A]	Silage (3)	B1173	4	5	25	52	57	20
L. lactis subsp. *lactis* biovar *diacetylactis*[A]	C17 (35)	B621	17	21	40	77	98	37
L. lactis ssp. *Lactis* bv *diacetylactis*[A]	Ru4 (35)	B86	17	16	42	84	100	38
L. lactis ssp. *Lactis* bv *diacetylactis*[A]	ZK (35)	B87	19	14	34	65	79	35
Enterococcus[A]	Termite-gut	B28	-8	-18	1	0	-18	-8

Streptococcus thermophilus[A]	Yoghurt	B103	6	25	3	4	29	13
S. thermophilus[A]	Yoghurt	B108	25	23	83	179	202	51
S. thermophilus[A]	Spray-dryer	B119	48	40	69	80	120	214
Leuconostoc lactis[B]	60 (35)	B911	8	37	5	7	45	26
Leuconostoc paramesenteroides[B]	7-1 (35)	B629	6	33	8	10	44	25
Lactobacillus plantarum[B]	WCFS-1		6	27	11	18	45	4
Lb. helveticus[B]	ATCC10797	B219	-9	-1	2	90	89	22
Lb. helveticus[B]	Cheese		-9	3	0	-1	2	1
Lb. acidophilus[B]	Ki (17)	B230	-8	0	0	1	1	0
Lb. casei[B]	Cheese		-9	-45	1	32	-13	-3
Lb. casei ssp. *Rhamnosus*[B]	ATCC 7469	B236	-10	-98	1	34	-63	-7
Lb. delbrueckii subsp. *bulgaricus*[B]	Rb from RR starter (10)	B194	-9	12	2	41	54	31
M17 medium			10	69				
MRS medium			9	108				

Standard deviation varied between 5% and 20%. Folate concentrations are corrected for folate present in the medium.
Positive values: folate production. Negative values: consumption of folate present in the growth medium.
A. growth medium is M17, B. growth medium is MRS, C. aerobically grown cells,
Folate ^{deconj} = measured folate concentration after enzymatic deconjugation of the polyglutamyl-tail.

recovered from a cell culture (5 ml) to measure both intra- and extracellular folate concentrations, as described in earlier work (39). The microbiological folate assay has nearly equal response to mono- di- and triglutamyl folate, while the response to longer chain polyglutamyl folate (n-glutamyl more than 3) decreases markedly in proportion to chain length (40). Consequently, total folate concentrations can only be measured after deconjugation of the polyglutamyl tails in samples containing folate derivatives with more than three glutamyl residues. The analysis of total folate concentration including polyglutamyl folate was done after enzymatic deconjugation of the folate samples during 4 hours at 37°C, pH 4.8, with human plasma (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), as a source for γ-glutamyl hydrolase activity (39). Microbiological assay measurements were replicated, standard deviation varied between 0-20%. The micro-organism used for detection, *Lactobacillus casei* subsp. *rhamnosus* strain ATCC 7469, was stored at - 80°C in MRS medium supplemented with 15% glycerol. For use in the microbiological assay, *L. casei* was pre-grown in filter-sterilized Folic Acid Casei Medium supplemented with 0.3 µg/l folate by incubation for 18 h at 37°C. Cultures were cooled-down in ice and 40%, cold and sterile, glycerol was added. 1.5 ml aliquots of this mixture were stored in sterile tubes at - 80°C until use for folate determination. The actual microbiological assay was performed in 96-well microtiter plates with each well containing 300 µl volume. The wells were filled by adding 150 µl of two-fold concentrated Folic Acid Casei Medium to 150 µl of an unknown or reference sample in 0.1 M potassium phosphate buffer, pH 6.3 containing 1% ascorbic acid. As reference samples, folic acid was dissolved in the same buffer at the concentration range of 0 – 0.25 µg/l. Control wells were not inoculated to check for sterility of the procedure. Growth in the microtiter plates was determined by measuring increase in absorbance at 580 nm using the microplate reader Emax (Molecular Devices Corporation, Sunnyvale, California)

Folate measurement by HPLC. For analysis of intracellular folate concentrations by HPLC concentrated cell-extracts were prepared as follows: *L. lactis* or *S. thermophilus* cells were recovered from a cell culture (50 ml) by centrifugation (12,000 g, 10 min, 4°C) and washed with 20 ml of 50 mM H_3PO_4 (pH 2.3), containing 1% ascorbic acid. The cells were resuspended in 1 ml of the same buffer. A cell-free extract was made by adding 1 g of silica beads to the cell suspension followed by disruption of the cells in an FP120 Fastprep™ cell disrupter (Savant Instruments inc., Holbrook, NY, USA). The cell-extract was heated at 100°C for 3 minutes to release folate from folate binding proteins and to precipitate proteins. Following centrifugation (two times at 12,000 g, 3 min, 4°C) 100 µl of concentrated cell-free extract was injected onto the chromatograph as soon as possible after extraction, although samples were stable over the working day.

Folate derivatives were purchased from Schircks (Jona, Switzerland). Small volumes of folate stock solutions were prepared at a concentration of 1 mg/ml and frozen. Working solutions were prepared by thawing microliter volumes and diluting to a concentration within the range 10-1000 ng/ml according to need. The concentrated polyglutamyl folate samples were analyzed by masspectrometry using a VG Quattro II mass spectrometer (Micromass UK Ltd., Manchester, U.K.).

The high performance liquid chromatograph consisted of a Waters 600E pump (Waters assoc., Watford, UK), Waters 767 plus autosampler injector, Shimadzu SPD-M10A photodiode array detector and a Waters 470 fluorescence detector. Different mono- and polyglutamyl folate derivatives are discriminated with a betasil phenyl column (250mm x 3mm ID, 3μm) (Keystone Scientific inc. Bellefonte, PA) protected with a betasil phenyl guard column. Freshly prepared mobile phase consisting of 9% methanol and 1.5% formic acid, pH 3.0, was filtered through a 0.45 μm millipore filter (type durapore) and degassed. Chromatography was performed at 50°C using a flow rate of 0.5 ml/min which produced a back pressure of 1200 psi. Detection was by one of the following: (a) Fluorimetric detection was achieved with an excitation wavelength of 310 nm and emission setting of 352 nm. The optimal signal to noise ratio for sensitive detection was an attenuation of 64 and a gain value of 100 with a filter value of 4 s. (b) Photodiode array detection (PAD). PAD data was collected between 220 and 500 nm at 2 nm optical resolution in order to discriminate fine structural details of the similar mono- and polyglutamyl folate spectra. Post-analysis routines were achieved using Shimadzu Class VP 5.0 software.

Results

Screening for folate production. Several species and strains from the lactic acid bacterial genera *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Leuconostoc* were screened for intra- and extracellular folate production (Table 1). The highest folate levels were detected in an aerobically grown *L. lactis* strain defective in lactate dehydrogenase, 291 μg/l. However, the highest folate production per biomass was found in *S. thermophilus* strain B119, 214 μg/l/$OD600_{nm}$. In general, *Lactobacillus* strains consumed (small amounts of) folate and did not produce folate, with the exception of *Lb. plantarum*. In most strains, e.g. *L. lactis* MG1363, folate production levels were higher after deconjugation of the polyglutamyl-tail (see Materials & Methods), indicating the (intracellular) presence of polyglutamyl folates with more than three glutamyl residues. In contrast, in some other strains, e.g. *S. thermophilus* B119, deconjugation of the folate samples had no effect on

folate analysis indicating the absence of polyglutamyl folates with more than three glutamyl residues.

The extent to which cells excreted the produced folate into the medium varied from strain to strain (Table 1). All *L. lactis* strains, except SK110, showed high intracellular accumulation (approximately 90%) of folate. The other lactic acid bacteria, including *S. thermophilus*, showed extensive excretion of folate into the medium. For *S. thermophilus* strain B119, this excretion of folate was observed to be strongly dependent on the pH of the cultivation medium. Under controlled growth conditions in a chemostat at pH 5.5, the cytoplasmic retention of folate in *S. thermophilus*, was relatively low (45%), resulting in increased excretion of folate into the medium. At higher pH (6.0 and 6.5) most folate was found inside the cells (Table 2). Interestingly, increased dilution rates, and consequently increased growth rates, also resulted in increased retention of folate by *S. thermophilus* (data not shown). At the higher pH-values and higher growth rates, a smaller fraction of the total folate was retained inside the streptococcal cells than the 90% (or more) intracellular retention usually observed for *L. lactis* under all growth conditions. The pH did not have an effect on the folate distribution over the inside and outside of the cell in *L. lactis* (Table 2).

Table 2: Folate production by *Streptococcus thermophilus* strain B119 and *Lactococcus lactis* MG1363 in continuous cultures at different pH.

	Folate (µg/l) production							
pH	*S. thermophilus*				*L. lactis*			
	Extracellular	Intracellular	Excreted %	Total	Extracellular	Intracellular	Excreted %	Total
5.0	nd	nd	nd	nd	3 ± 2	32 ± 5	8%	37
5.5	77 ± 7	65 ± 15	54%	142	4 ± 2	36 ± 3	9%	44
6	94 ± 8	292 ± 30	24%	386	6 ± 1	42 ± 9	11%	54
6.5	103 ± 8	431 ± 30	19%	534	nd	nd	nd	nd
7.0	nd	nd	nd	nd	16 ± 1	91 ± 9	8%	107
7.5	nd	nd	nd	nd	15 ± 1	88 ± 1	8%	102

nd: not determined. *S. thermophilus* strain B119 was cultivated in modified M17-medium (potassium phosphate instead of β-glycerophosphate) with 0.5% lactose as growth-limiting substrate and *L. lactis* in chemically defined medium (28) without phenylalanine, para-aminobenzoc acid, and folate, with 0.5% glucose as growth-limiting substrate.

Identification of polyglutamyl folates. The total folate concentrations and the distribution over the inside and the outside of the cell in *L. lactis* and *S. thermophilus* was determined by a microbiological assay. HPLC was used to determine the different intracellular mono- and polyglutamyl folate derivatives in these two lactic acid bacteria. The retention times and spectral characteristics of specific folate calibrators were used to

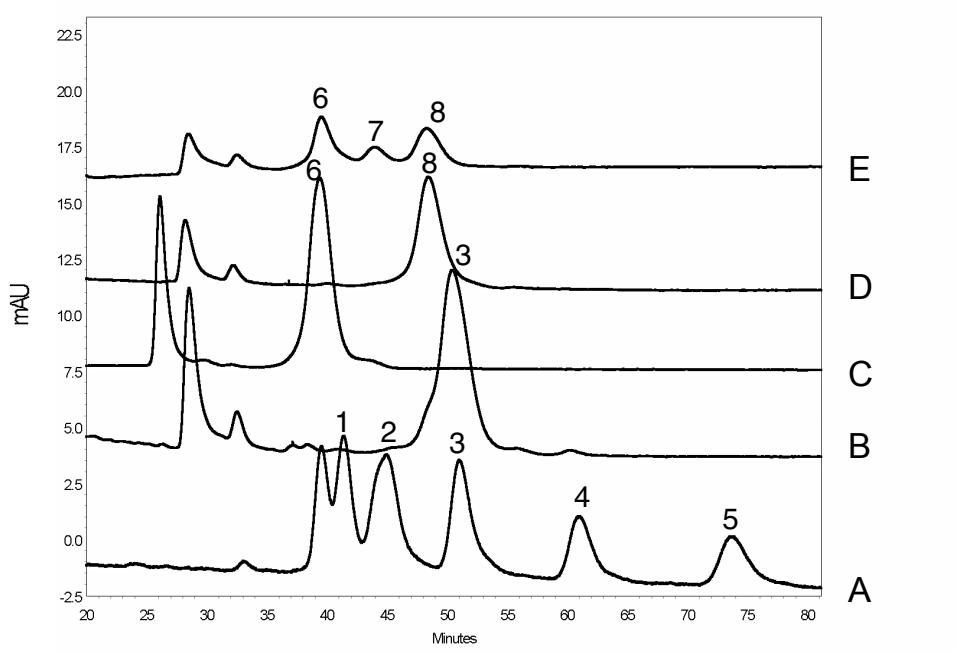

Figure 1.
Chromatograms of folate calibrators (A, C), cell free extracts of *S. thermophilus* before (B, D) and after deconjugation (E) monitored by UV absorption at 280 nm (A, B) and 360 nm (C, D, E)
Legend: **1,** 5-formyl tetrahydrofolate-glu_1 (S and R diastereoisomer); **2,** 5-formyl tetrahydrofolate-glu_2; **3,** 5-formyl tetrahydrofolate-glu_3; **4,** 5-formyl tetrahydrofolate-glu_4; **5,** 5-formyl tetrahydrofolate-glu_5, **6,** 5,10-methenyl tetrahydrofolate-glu_1; **7,** 5,10-methenyl tetrahydrofolate-glu_2;**8,** 5,10-methenyl tetrahydrofolate-glu_3.

identify the different folate derivatives in the cells of *S. thermophilus* and *L. lactis*. *S. thermophilus* produces 5-formyl tetrahydrofolate and 5,10-methenyl tetrahydrofolate, both in the triglutamyl form: Fig. 1 shows the chromatograms of several 5-formyl polyglutamyl tetrahydrofolate calibrators (at 280 nm) and 5,10-methenyl tetrahydrofolate (at 360 nm) and cell-free extracts of *S. thermophilus*. Based upon the characteristic UV absorption maximum around 360 nm of 5,10-methenyl tetrahydrofolate (20), the components detected at 360 nm can be identified as 5,10-methenyl tetrahydrofolate with different lengths of the polyglutamate tail. The elution time of 5,10-methenyl tetrahydrofolate is two minutes less than the elution time of 5-formyl tetrahydrofolate. It can be assumed that 5,10-methenyl polyglutamyl folates elute also just before the corresponding 5-formyl polyglutamyl folates.

Thus, the detected 5,10-methenyl tetrahydrofolates in *S. thermophilus* could be identified as 5,10-methenyl tetrahydrofolate with 3 glutamate residues. After enzymatic deconjugation most of the 5,10-methenyl polyglutamyl folate was transformed into 5,10-methenyl mono- and diglutamyl folate.

In the cell free extracts of *L. lactis* also 5,10-methenyl polyglutamyl folate could be detected at 360 nm, in this case with 4, 5 and 6 glutamate residues (data not shown). The fluorescence detection and UV absorption of the *L. lactis* samples show also the presence of polyglutamyl folates with a UV absorption maximum of 262 nm (data not shown). Based on the UV absorption maximum of 262 nm, we assumed that the unknown polyglutamyl folates (also with 4, 5, or 6 glutamate residues) are 10-formyl polyglutamyl folates (M.D. Lucock, personal communication). No other folate derivatives, including 5-formyl polyglutamyl folate, could be identified.

Cultivation effects on folate production. The influence of growth conditions and medium composition was further analyzed, especially the influence of pH, pABA, hemin and growth rate. Under controlled growth conditions in a chemostat the total amount of folate produced in both *L. lactis* and *S. thermophilus* increased more than three-fold by increasing the pH from 5.5 to 7.5 (Table 2).

A further increase in folate production was observed in pH controlled batch fermentations with glucose excess. The amount of biomass produced increased by more than a factor 3 in pH-controlled batch fermentations compared to non-pH-controlled batch fermentations. The increase in folate production is even more than the increase in biomass and reaches levels up to 400 ng/ml (data not shown). Folate production per cell was also increased in the *L. lactis* strain KB, naturally defective in lactate dehydrogenase (LDH) activity, and an engineered LDH-negative strain (*L. lactis* NZ9010) (Table 1). To promote maximum cell growth, these strains were grown aerobically. A further increase in biomass and folate production per cell was observed in strains that were defective in lactate dehydrogenase activity and that were grown under aerobic conditions in the presence of hemin (Fig. 2). Analysis of the fermentation characteristics of these cells showed that lactate production was decreased and the production of acetate and acetoin was increased compared to cells grown anaerobically (data not shown).

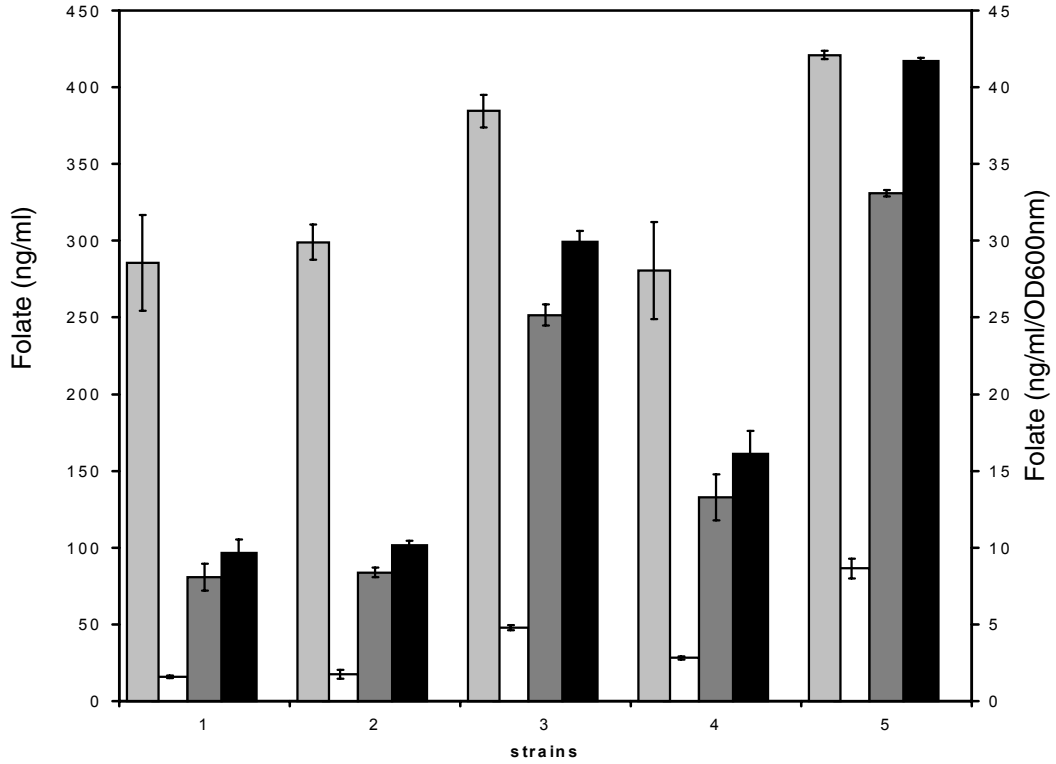

Figure 2.
Folate production and distribution in different *L. lactis* strains grown aerobically and anaerobically with and without hemin in M17 medium.
On right Y-axis: Total folate production per $OD600_{nm}$ (grey bars).
On left Y-axis: Extracellular folate (open bars), intracellular folate (hatched bars) and total folate production (closed bars)
Legend: **1,** wild type strain, anaerobic; **2,** wild type strain, aerobic; **3,** *ldh*- strain, aerobic; **4,** wild type strain, anaerobic + hemin; **5,** *ldh*- strain, aerobic + hemin.

One of the folate precursors is *p*-aminobenzoic acid, that is synthesized via glycolysis, pentose phosphate pathway and shikimate pathway. In *L. lactis* the folate production was shown to be dependent on the concentration of pABA in the medium. The addition of pABA in a concentration range between 1 and 100 μM to a minimal medium, lacking aromatic amino acids, purines and folate, resulted in a two-fold increase of folate production (Fig. 3). Concentrations of pABA above 100 μM did not result in a further increase in folate. Folate production decreased two-fold by the addition of tyrosine (1.2 mM) to the medium (Fig. 3). However, the negative effect of tyrosine on folate production could be compensated by the addition of pABA (Fig. 3). The changes measured in the folate microbiological assay upon addition of pABA or tyrosine were not a result of direct growth stimulation or inhibition of the *Lb casei* (data not shown).

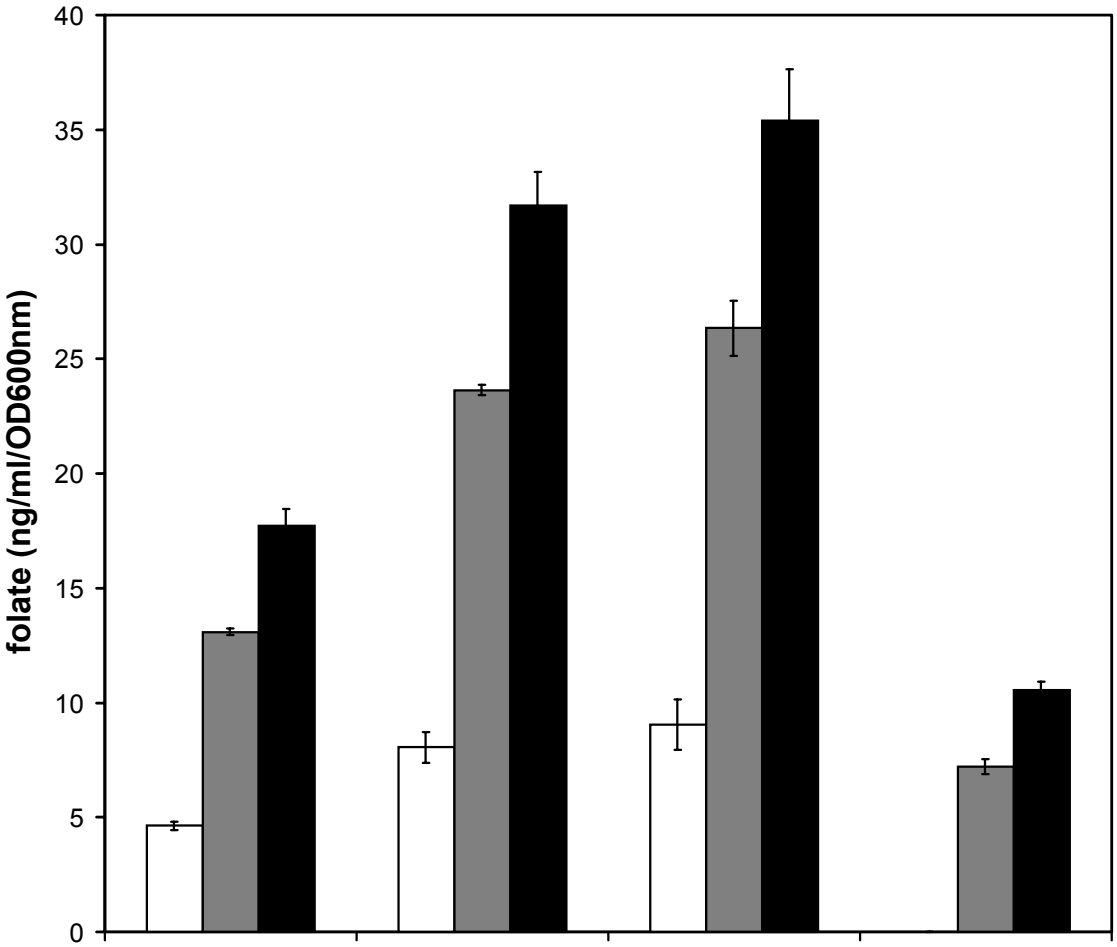

Figure 3.
Influence of *p*-aminobenzoic acid and tyrosine on folate production of *L. lactis* grown in chemically defined medium.
Extracellular folate (open bars), intracellular folate (hatched bars) and total folate production (closed bars).
Legend: 1, - pABA, - tyrosine; 2, + pABA 100 μM, + tyrosine 1.2 mM; 3, +pABA 100 μM, -tyrosine; 4, - pABA, + tyrosine 1.2 mM.

Folate production could further be improved in the two lactic acid bacteria by inhibiting growth with antibiotics or with increasing concentrations of NaCl. In *L. lactis*, addition of growth-inhibitory concentrations of 2 μg/ml chloramphenicol and 0.1 μg/ml erythromycin lead to 40% resp. 215% increase in folate production. Addition of NaCl to the growth medium increased folate production in both *L. lactis* and *S. thermophilus*. In the latter bacterium, 0.8% (w/v) NaCl was the highest salt concentration allowing growth in a semi-synthetic medium. Under these conditions the specific growth rate was strongly reduced (90%) and a ten-fold increase in folate production was observed. The relationship between growth rate and folate production was further investigated in continuous cultures. *S. thermophilus* NIZO strain B119 was grown in modified M17-medium with lactose as

growth-limiting substrate. The growth rate was controlled by the flow rate of the incoming growth medium. At low dilution rates, the yield of folate in the bioreactor was increased (Fig. 4), although the specific folate production (ng/ml/OD600nm/h) did decrease slightly with the lower dilution rates. A similar inverse relationship between growth rate and folate production was observed for *L. lactis* MG1363, using glucose as growth-limiting substrate in a chemically defined medium (data not shown).

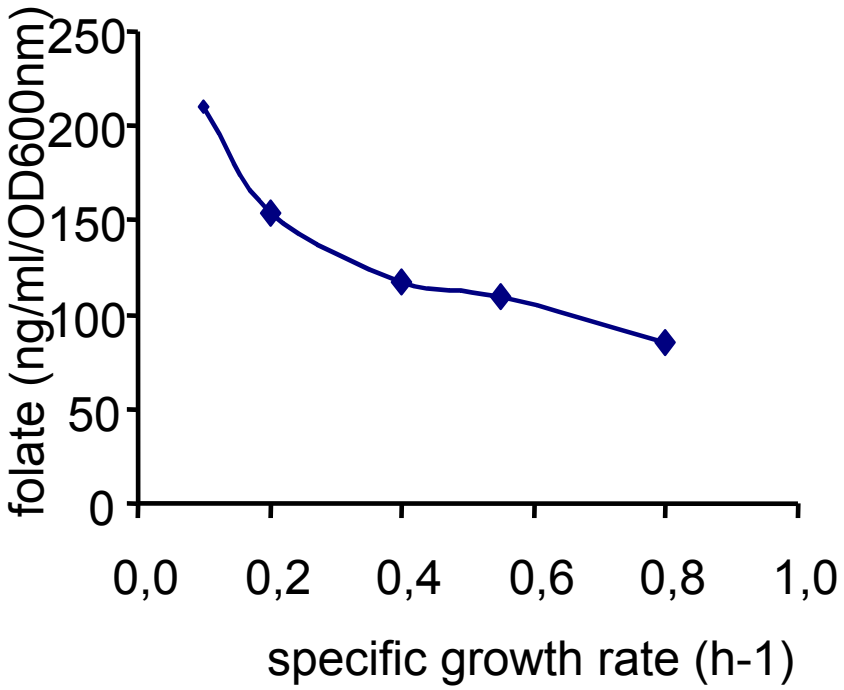

Figure 4.
Total folate production by *Streptococcus thermophilus* NIZO strain B119 grown at different dilution rates (= growth rates) in continuous culture in modified M17-medium, see Material and Methods.

Discussion

Several species and strains from the lactic acid bacterial genera *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Leuconostoc* were screened for folate production. The lactic acid bacteria *Lactococcus lactis* MG1363 and *Streptococcus thermophilus* B119 were further analyzed for folate production at different growth conditions. *Lactococcus lactis*, *Streptococcus thermophilus* and *Leuconostoc* produced folate in the range of 5-291 µg/l. Lactobacillus strains, with the exception of *Lb. plantarum*, did not produce folate. In several strains folate analysis performed after deconjugation resulted in detection of higher folate levels. This indicates that part of the folate is present as polyglutamyl folate with more than three glutamate residues.

All folate producing strains showed partial excretion of folate into the external medium. In *L. lactis* up to 90% of the total produced folate remained intracellular and was identified as 5,10-methenyl tetrahydrofolate and presumably 10-formyl tetrahydrofolate both with 4,5, and 6 glutamate residues. In *S. thermophilus* much less of the total produced folate remained intracellular and was identified as 5-formyl tetrahydrofolate and 5,10-methenyl tetrahydrofolate both with 3 glutamate residues. The difference in distribution can probably be explained by the different length of the polyglutamyl tail of the two micro-organisms. One of the functions of the polyglutamyl tail is believed to be the retention of folate within the cell (24,33). The longer polyglutamyl tail identified in *L. lactis* improves the retention of folate. It can be assumed that cell retention of folates is mainly a result of the negative charge of the carboxyl groups of (polyglutamyl) folate (pKa of 4.6). Hence, polyglutamyl folates with longer glutamyl tail length will have a more negative charge than polyglutamyl folates with short glutamyl tail length. Moreover, in *S. thermophilus* the intra- and extracellular folate distribution was influenced by the pH. Cells that were grown at low pH had a higher extracellular folate fraction than cells that were cultured at a high pH. It was observed in both *S. thermophilus* and *L. lactis* cells that by decreasing the extracellular pH, the intracellular pH (15,34) was decreased also. Consequently, at lower intracellular pH a higher concentration of the folates are protonated and electrically neutral, enhancing transport across the membrane. In *L. lactis* no influence of pH on intra- and extracellular folate distribution was observed. We assume that the polyglutamyl folates with 4, 5 and 6 glutamate residues are not protonated at lower intracellular pH to the same extent as the polyglutamyl folates with 3 glutamate residues as detected in *S. thermophilus*.

In continuous cultures it was observed that the production of folate was also increased with increasing pH. This is in agreement with literature data that report on the

high pH optima between 7.3 and 9.3 of all the enzymes involved in folate biosynthesis as was analyzed in several (micro)-organisms (4,22,23,27,31,32,37,44). Therefore, we assume that the activity of folate synthesizing enzymes is increased under conditions with a high external pH, that lead to a more alkaline cytosol compared to conditions with low external pH (15,34). The former hypothesis is supported by the observations that more folate per cell is produced in batch fermentations with controlled pH. Under such conditions acidification of the medium is delayed and, as a consequence, growth of the micro-organisms is prolonged and the intracellular pH may be maintained at a more optimum level for efficient folate biosynthesis. Slow acidification of the fermentation broth and increased folate production is also achieved during the growth of a *L. lactis* strain that is defective in lactate production because of the deletion of a lactate dehydrogenase encoding gene. The total folate levels were increased in this strain because of increased biomass and, probably, more efficient folate biosynthesis at higher intracellular pH. The addition of hemin to the growth medium resulted in a further increase of folate produced per cell biomass. We assume that hemin stimulates the direct oxidation of NADH by oxygen. As a consequence, NADH levels decrease and pyruvate is consumed via alternative non reducing pathways resulting in decreased acidification (less lactate, more acetoin) (21). Recently it was reported that during aerobic growth the addition of hemin extends the growth period (11) and that hemin may reconstitute proton extrusion (5). This may indicate that the proton gradient in aerobically grown cells in the presence of hemin is higher and that, consequently, a higher intracellular pH is maintained. As was already described for pH controlled fermentations, the folate biosynthesis is assumed to increase at higher intracellular pH.

Folate production was further stimulated when growth was inhibited. This was observed in batch cultures in the presence of growth inhibiting concentrations of several antibiotics and in the presence of high salt concentrations. In continuous cultures folate concentration in the reactor increased at lower growth rate (Fig. 4). However, the folate production per biomass per hour decreased at lower growth rates. The reason for increased folate production when growth was inhibited is not yet known. It could be that under conditions of low growth rate GTP, one of the folate precursors, is accumulated, because of decreased DNA and RNA synthesis. Interestingly, in earlier work we reported that overproduction of GTP cyclohydrolase I (EC 3.5.4.16), the first enzyme involved in folate biosynthesis, leads to increased production of folates by *Lactococcus lactis* (39). Despite uncertainties about the mechanism, our results show that food fermentations aimed at increasing in situ folate levels could be best performed at low growth rates and a high pH.

Folate can be synthesized in *L. lactis* in the absence of *p*-aminobenzoic acid indicating that *L. lactis* has the capacity to synthesize pABA. However, addition of pABA to the medium leads to an increase of folate production. This suggest that the synthesis of pABA is a rate-determining step in the production of folate by *L. lactis*. The biosynthesis pathway of pABA and aromatic amino acids may proceed via a common pathway to chorismate. Studies related to the synthesis of aromatic amino acids in *Corynebacterium glutamicum* have shown that this shikimate pathway is under tight control of tyrosine (16). The addition of tyrosine to the medium of *L. lactis* resulted in a decrease in folate production. It can be assumed that in *L. lactis* also tyrosine leads to a feedback inhibition of phospho-2-dehydro-3-deoxyheptonate aldolase and that folate production is indirectly affected by the biosynthesis of aromatic amino acids. Currently we are working on the development of strains that are deregulated in the biosynthesis of aromatic amino acids as an approach to increase the *in situ* production of pABA and folate.

The observation that folate production levels are influenced by the selection of lactic acid bacteria, by the application of specific growth conditions, and by the use of different media, could have a large impact on the manufacture of dairy products. For instance, by specifically selecting high folate-producing strains as part of the starter culture, yogurt could be produced with elevated levels of folate (38,43). Furthermore, it is expected that in combination with specific growth conditions and metabolic engineering approaches (39) the current contribution of yogurt of 10-20% to the average daily intake for folate could be substantially increased.

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Chapter 4

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

Wilbert Sybesma, Marjo Starrenburg, Michiel Kleerebezem, Igor Mierau,
Willem M. de Vos, and Jeroen Hugenholtz

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

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Abstract

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

Introduction

Folate is an essential nutrient in the human diet. Folate deficiency leads to numerous physiological disorders, most notably anemia and neural tube defects in newborns (34), and mental disorders such as psychiatric syndromes among elderly and decreased cognitive performance (7,22). In addition, folate is assumed to have protective properties against cardiovascular diseases and several types of cancer (5,6,34). The daily recommended intake of dietary folate for an adult is 400 µg. For pregnant women 600 µg is recommended. Recent reports have indicated that folate deficiency is common even among various population groups in the developed countries (studies done in The Netherlands and Ireland) including women of childbearing age (25,38).

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

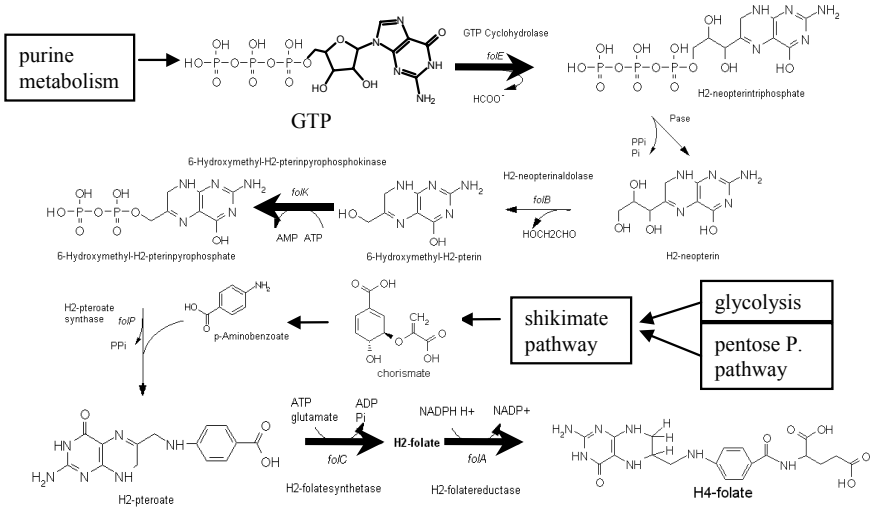
Many plants, fungi and bacteria are able to synthesize folate and can serve as a folate source for the auxotrophic vertebrates. Due to the ability of lactic acid bacteria to produce folate (32), folate levels in fermented dairy products are higher compared to the corresponding non-fermented dairy products (1). The natural diversity amongst dairy starter cultures with respect to their capacity to produce folate can be exploited to design new complex starter cultures which yield fermented dairy products with elevated folate levels.

Lactococcus lactis is by far the most extensively studied lactic acid bacterium, and over the last decades a number of elegant and efficient genetic tools have been developed for this starter bacterium. These tools are of critical importance in metabolic engineering strategies that aim at inactivation of undesired genes and/or (controlled) overexpression of existing or novel ones. In this respect, especially the nisin controlled expression (NICE) system for controlled heterologous and homologous gene expression in *Lactococcus lactis* has proven to be very valuable (9). The design of rational approaches to metabolic engineering requires a proper understanding of the pathways that are manipulated and the genes involved, preferably combined with knowledge about fluxes and control factors. Most of the metabolic engineering strategies so far applied in lactic acid bacteria are

related to primary metabolism and comprise efficient rerouting of the lactococcal pyruvate metabolism to end products other than lactic acid, including diacetyl (8,20,37,46) and alanine (18) resulting in high level production of both natural and novel endproducts. Metabolic engineering of more complicated pathways involved in secondary metabolism has only recently begun by the engineering of exopolysaccharide production in *L. lactis* (3,31,33,45). Another complicated pathway is the biosynthesis of folate (13). This biosynthesis includes parts of glycolysis, pentose phosphate pathway and shikimate pathway for the production of the folate building block *p*-aminobenzoate, while the biosynthesis of purines is required for the production of the building block GTP (Fig. 1A). In addition, a number of specific enzymatic steps are involved in the final assembly of folate and for production of the various folate derivatives. The annotated genome sequence of *L. lactis* subsp. *lactis* IL1403 (4) reveals the existence of a folate gene cluster containing all genes encoding the folate biosynthesis pathway (Fig. 1B).

In the present and previous studies we have used *L. lactis* subsp. *cremoris* MG1363 for metabolic engineering experiments (3,18,20). Although *L. lactis* IL1403 and MG1363 show a large homology on the genome level, there are considerable differences (29). For successful application of metabolic engineering in the final steps of the complicated biosynthesis pathway of folate in *L. lactis* MG1363, characterization of the folate gene cluster is necessary in this strain. The presented results are an important step in the development of fermented foods with increased bioavailable and natural folate.

1A



1B

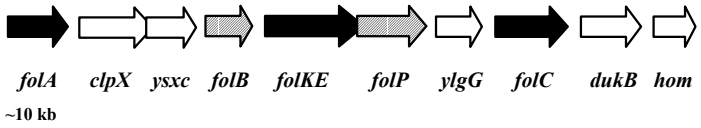


Figure 1A:

Chemical structure of tetrahydrofolate and folate biosynthesis pathway. Bold arrows indicate enzymatic reactions that are controlled in metabolic engineering experiments, see text for details.

Figure 1B:

Schematic representation of the *Lactococcus lactis* folate gene cluster as identified in *L. lactis* MG1363 and *L. lactis* IL1403. *folKE* encodes a bifunctional protein. Hatched arrows represent genes involved in folate biosynthesis, black arrows represent genes involved in folate biosynthesis that are overexpressed in metabolic engineering experiments, white arrows represent genes that are not expected to be involved in folate biosynthesis.

Materials and methods

Bacterial strains and plasmids, media and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in tryptone yeast medium at 37°C (41). *Lactococcus lactis* was grown at 30°C in M17 medium (49) supplemented with 0.5% (w/v) glucose. When appropriate the media contained chloramphenicol (10 μg/ml), or kanamycin (50 μg/ml).

DNA manipulations and transformations. Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook *et al.* (41). Large scale isolation of *E. coli* plasmid for nucleotide sequence analysis was performed with JetStar columns by following the instructions of the manufacturer (Genomed, Bad Oeynhausen, Germany). Isolation of chromosomal and plasmid DNA from *L. lactis* and transformation of plasmid DNA to *L. lactis* was performed as previously described (11). Restriction enzymes and T4 DNA ligase were purchased at Life Technologies BV, Breda, The Netherlands.

PCR amplification of DNA and nucleotide sequence analysis. Several *L. lactis* genes were amplified from chromosomal DNA by PCR with 25 ng of DNA in a final volume of 50 μl containing deoxyribonucleoside triphosphates (0.25 mM – 0.5 mM each), oligo nucleotides (50 pM) (Table 2), and 1.0 – 3.0 U of *Pfx* polymerase (Invitrogen, Paisley, Great Britain) or *Taq-Tth* polymerase mix (Clontech, Palo Alto, CA, USA). Amplification was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following regime: 30 cycles denaturation at 95°C for 30 seconds (3 minutes in first cycle), annealing at 50°C for 30 seconds and elongation at 68°C (*Pfx*) or 72°C (*Taq-Tth*) for 1-8 minutes. Sequence analysis of the genes involved in folate biosynthesis was done after amplification of a 9 kb DNA fragment flanked by the upstream region of *folA* (30) and *hom* (35) by using primers fol-F and hom-R (Table 2) and cloning of the fragment in pCR-BLUNT (Invitrogen) generating pCR-BLUNT-FOL. The generated plasmid was transformed by electroporation to *E. coli* TOP10 (Invitrogen). The nucleotide sequence of the amplified folate gene cluster was determined by automatic double stranded DNA sequence analysis using a MegaBACE™ DNA Analysis System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Primer sequences were obtained from published sequence data for *folA* (30) and *hom* (35) (Fig. 1B) and by subsequent primer walking. Amplification, cloning and sequencing were performed twice in independent experiments. Differences in both DNA sequences were re-analyzed after independent amplification and cloning of the regions flanking the ambiguous sequences.

Construction of plasmids and transformation of strains. Lactococcal plasmid pNZ8048 (26,27) is a translational fusion vector used in nisin-controlled expression systems. The vector contains a *nisA* promoter and an *Nco*I cloning site. The gene *folKE* encoding a bifunctional protein predicted to display both 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I activity was amplified from chromosomal DNA using primers *folKE*-F and *folKE*-R (Table 2). The forward primer *folKE*-F was extended at the 5' end introducing an *Nco*I restriction site resulting in a slight modification of the mature gene (nucleotides represented in italics in Table 2). The reverse primer *folKE*-R was extended at the 5' end introducing a *Kpn*I restriction site. The amplification product was digested with *Nco*I and *Kpn*I and cloned in pNZ8048 (digested with *Nco*I and *Kpn*I) thereby placing the *folKE* gene under the control of *nisA.* The new plasmid is pNZ7010. The gene *folC* encoding a bifunctional protein predicted to display both folate synthetase and polyglutamyl folate synthetase activity was amplified from chromosomal DNA using the primers folC-F and folC-R (Table 2). Both primers were extended at the 5' end introducing a *Kpn*I and an *Xba*I restriction site. The amplification product includes a ribosome binding site and was digested with *Kpn*I and *Xba*I. Next, the gene was cloned in pNZ7010 down stream of *folKE* generating pNZ7011. The gene *folP* encoding a protein predicted to display dihydropteroate synthase activity was amplified from chromosomal DNA using primers folP-F and folP-R (Table 2). Both primers were extended at the 5' end introducing a *Kpn*I and an *XbaI* restriction site. The amplification product includes a ribosome binding site and was digested with *Kpn*I and *Xba*I and cloned behind *folKE* in pNZ7010 generating pNZ7012. The gene *folA* encoding dihydrofolate reductase was amplified from chromosomal DNA using primers folA-F and folA-R (Table 2). The forward primer folA-*F* was extended at the 5' end introducing an *Nco*I restriction site resulting in a slight modification of the mature gene (nucleotides represented in italics in Table 2). The reverse primer folA-R was extended at the 5' end introducing an *Hind*III restriction site. The amplification product was digested with *Nco*I and *Hind*III and cloned in pNZ8048 (digested with *Nco*I and *Hind*III) thereby placing the *folA* gene under the control of *nisA.* The new plasmid is pNZ7013. The cloning of the antisense RNA of the gene encoding dihydrofolate reductase was achieved in a similar way as described for *folA,* except for the orientation, using primers folA-ASF and folA-ASR (Table 2).

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
L. lactis strains		
MG1363	*L. lactis* subsp. *cremoris*, plasmid free	12
NZ9000	*MG136*3 pepN:nisRK	27
IL1403	*L. lactis* subsp. *lactis*, plasmid free	4
E. coli strains		
TOP10	Cloning host	Invitrogen
Plasmids		
pCR-blunt	kan^r	Invitrogen
pCR-blunt-fol	Derivative of pCR-blunt carrying lactococcal folate gene cluster	This study
pNZ8048	Cm^r, Inducible expression vector carrying the *nisA* promoter	27
pNZ8160	Cm^r, derivative of pNZ8048 carrying terminator of *pepV*	This study
pNZ8161	Cm^r, derivative of pNZ8160 carrying constitutive promoter of *pepN*	This study
pNZ7010	Cm^r, pNZ8048 derivative containing a functional lactococcal *folKE* gene behind the *nisA* promoter	This study
pNZ7017	Cm^r, pNZ8161 derivative containing a functional lactococcal *folKE* behind the constitutive *pepN* promoter	This study
pNZ7011	Cm^r, pNZ8048 derivative containing a functional lactococcal *folKE* and *folC* gene behind the *nisA* promoter	This study
pNZ7012	Cm^r, pNZ8048 derivative containing a functional lactococcal *folKE and folP* gene behind the *nisA* promoter	This study
PNZ7013	Cm^r, pNZ8048 derivative containing a functional lactococcal *folA* gene behind the *nisA* promoter	This study
PNZ7014	Cm^r, pNZ8048 derivative containing antisense RNA of a lactococcal *folA* gene behind the *nisA* promoter	This study

Table 2: Oligonucleotides used for DNA amplification by PCR. Nucleotides represented in italics represent modifications with regard to the mature gene.

Primer	Sequence
FolF	CATACCACTTCTTTTTCGATTTGTAAAGG
Hom-R	CGATCCCGGGAAGCCCTGTGCCAACTGTCC
FolKE-F	CATGCCATG*GGG*CAAACAACTTATTTAAGCATGG
FolKE-R	GGGGTACCGATTCTTGATTAAGTTCTAAG
FolC-F	GAAGAGGTACCAGAAGAGTTTAAAAAGTATTATCG
FolC-R	TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG
FolP-F	GAATGGTACCTTTAGGAGGTCTTTTATGAAAATCTTAGAAC
FolP-R	GAGAAATCAAATCCTCATTCTAGATTAAAATTCC
FolA-F	GGAATTCCATG*GTT*ATTGGAATATGGGCAGAAG
FolA-R	GCCTCAAGCTTCATGGTTGTTTCACTTTTTC
FolA-ASF	GAGGGGTACCTATGATAATTGGAATATGG
FolA-ASR	GCCCAAAAATTGATTTTGCCATGGTTG
Pcon-F	*GAAGATCTGTCGAC CTG*CAGTAGACAGTTTTTTTAATAAG
Pcon-R	CG*GGATCCGCATGC*CTTCTCCTAAATATTCAGTATTAA
TpepV-F	CG*GGATCC*TTATGAACTTGCAAAATAAG
TpepV-R	GA*AGATCT*CACCTCTATTTCTAGAATAAAG
FolKE2-F	ATACATGCATGCAAACAACTTATTTAAGCATGGG
FolKE2-R	ATACATGCATGCGATTCTTGATTAAGTTCTAAG

The new plasmid is pNZ7014. The generation of a plasmid containing a constitutive promoter and a nisin inducible promoter separated by a terminator (pNZ8161) was created as follows: The terminator from *pepV* (18) was amplified from chromosomal DNA using primers TpepV-F and TpepV-R (Table 2). The forward primer TpepV-F was extended at the 5' end introducing a *Bgl*II restriction site. The reverse primer TpepV-R was extended at the 5' end introducing a *BamH*I restriction site. The amplification product was digested with *Bgl*II and *BamH*I and cloned in pNZ8048-•*Sph*I (digested with *Bgl*II) generating pNZ8160. The constitutive promoter from *pepN* (50) was amplified from plasmid pNZ1120 (50) using primers con-F and con-R (Table 2). The forward primer con-F was extended at the 5' end introducing a multiple cloning site including a *Bgl*II restriction site. The reverse primer con-R was extended at the 5' end introducing a *BamH*I and *Sph*I restriction site.

The amplification product was digested with *Bgl*II and *BamH*I cloned in pNZ8160 (digested with *BamH*I) generating pNZ8161. Next, the gene *folKE* was amplified from chromosomal DNA using primers *folKE*2-F and *folKE*2-R (Table 2). Both primers were extended at the 5' end introducing an *Sph*I restriction site. The amplification product was digested with *Sph*I and cloned in pNZ8161 (digested with *Sph*I) thereby placing the *folKE* gene under the control of the constitutive promoter of *pepN.* The new plasmid is pNZ7017.

L. lactis strain NZ9000 (27) was used as a host for the described plasmids (Table 1). In NZ9000 the genes for a nisin response regulator and a nisin sensor, *nisR* and *nisK* respectively*,* are stably integrated at the *pepN* locus in the chromosome and they are constitutively expressed under the control of the *nisR* promoter.

Nisin induction. An overnight culture of *L. lactis* NZ9000 harboring pNZ8048 or one of the plasmids described above was diluted (1:100) in GM17 supplemented with chloramphenicol and grown until an optical density at 600 nm (OD_{600}) of 0.5. The cells were induced with different concentrations of nisin A (referred to as nisin), ranging from 0.1 to 5 ng/ml, incubated for two hours, and harvested for further analysis. The addition of nisin and the subsequent overexpression of genes did not affect the growth characteristics of the engineered strains. Folate was analyzed in cell-extracts and fermentation broth and overproduction of proteins was monitored by SDS PAGE as described previously (3).

Analysis of intra- and extracellular folate concentration. Folate was quantified using a *Lactobacillus casei* microbiological assay (19). To measure intra- and extracellular folate concentrations, both cells and supernatant were recovered from a full grown cell culture (5 ml) after centrifugation (12,000 g, 10 min., 20°C). The supernatant was diluted 1:1 with 0.1 M NaAc buffer pH 4.8, 1% ascorbic acid. The cells were washed with 0.1 M NaAc pH 4.8, 1% ascorbic acid, and resuspended in 5 ml of the same buffer. Folate was released from the cells and from folate binding proteins by incubating the samples at 100°C for 5 minutes, which was determined to be optimal for maximum folate release. Moreover, the heating inactivates the folate producing bacteria and prevents their interference in the microbiological folate assay. The microbiological folate assay has nearly equal response to mono- di- and triglutamyl folate, while the response to longer chain polyglutamyl folate (n-glutamyl more than 3) decreases markedly in proportion to chain length (48). Consequently, total folate concentrations can only be measured after deconjugation of the polyglutamyl tails in samples containing folate derivatives with more than three glutamyl residues. The analysis of total folate concentration including polyglutamyl folate was done after enzymatic deconjugation of the folate samples during 4 hours at 37°C, pH 4.8, with human plasma (Sigma-Aldrich Chemie, Zwijndrecht,

The Netherlands), as a source for γ-glutamyl hydrolase activity: The deconjugation reaction was prepared as follows: 1 g of human plasma was diluted in 5 ml 0.1 M 2-mercaptoethanol, 0.5% sodium ascorbate and cleared from precipitates by centrifugation (10,000 g, 2 min) followed by the addition of 2.5% (v/v) of the clarified human plasma solution to the folate samples. Standard deviation of the microbiological assay varied between 0-15%. A 1% yeast extract medium solution (Difco, Becton Dickinson Microbiology Systems, Sparks, MD, USA), containing almost exclusively polyglutamyl folates, with a previously determined total folate content was used as a positive control for actual deconjugation.

Dihydrofolate reductase activity. 40 ml of a culture of *L. lactis* NZ9000 harboring pNZ8048, pNZ7013 or pNZ7014 was grown and induced with nisin as described previously. At OD_{600} of 2.5 cells were harvested, washed and resuspended in 1 ml buffer (10 mM KPO_4, 0.1 mM DTT, 0.1 mM EDTA, pH 7.0). A cell-free extract was made by adding 1 g of silica beads to the cell suspension followed by disruption of the cells in an FP120 Fastprep™ cell disrupter (Savant Instruments inc., Holbrook, NY, USA) and centrifugation (20,000 g, 10 min., 0°C). 20–100 µl of the cell free extract was used to measure dihydrofolate reductase activity as described previously (39).

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

Results

Sequencing and annotation of a folate gene cluster. Based upon the genetic organization of a folate gene cluster in *L. lactis* IL1403 (4), a 9kb DNA fragment flanked by *folA,* encoding dihydrofolate reductase and *hom,* encoding homoserine dehydrogenase, was amplified from the genome of *L. lactis* MG1363. In the latter strain the sequence of the genes involved in folate biosynthesis was not yet known, except for *folA* (30). Its nucleotide sequence was determined and revealed the presence of nine open reading frames which have all the same orientation. Sequence comparison with the genome of *L. lactis* IL1403 showed that both strains have an identical genetic organization. The nucleotide identity level of the folate gene cluster between *L. lactis* MG1363 and IL1403 is 89%. Only 5 or 6 genes in the folate gene cluster appeared to be involved in folate biosynthesis: *folA,* encoding dihydrofolate reductase (EC 1.5.1.3); *folB,* predicted to encode neopterine

aldolase (EC 4.1.2.25); *folK,* predicted to encode 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyro- phosphokinase (EC 2.7.6.3); *folE*, predicted to encode GTP cyclohydrolase I (EC 3.5.4.16); *folP,* predicted to encode dihydropteroate synthase (EC 2.5.1.15); and *folC,* predicted to encode folate synthetase / folyl polyglutamate synthetase (EC 6.3.2.12/6.3.2.17). The remaining genes that were identified in the gene cluster are *clpX*, predicted to encode an ATP binding protein for ClpP, *dukB*, predicted to encode a deoxynucleoside kinase (EC 2.7.1.113), *ysxc* and *ylgG*, both encoding an unknown protein. The genes c*lpX* and *ysxc* may be involved in stress responses (23). The overall amino acid identity levels of these nine putative proteins between the two *L. lactis* strains is 90%, ranging from 73% identity for *ylgG* to 98% for both *clpX* and *dukB*. It has been reported previously that in *L. lactis folA* contains an identified promoter region (30) and that *folKE*, *folP*, *ylgG* and *folC* are co-transcribed in a multicistronic operon (47).

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

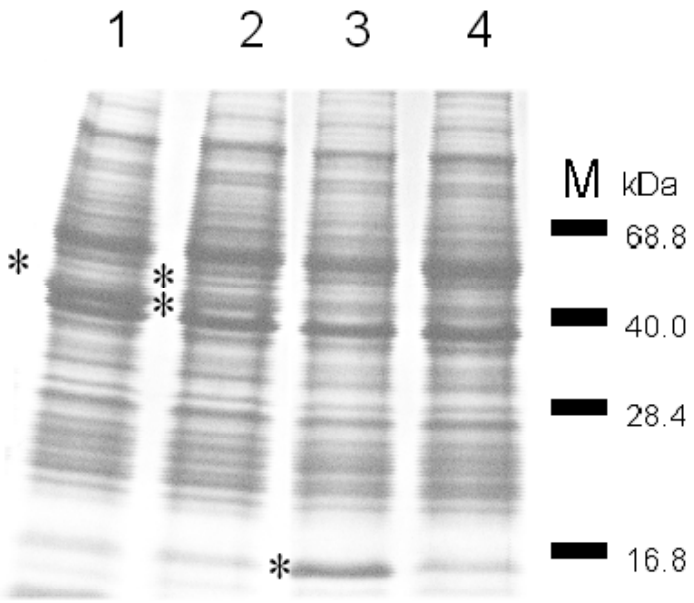


Figure 2:

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

Increased extracellular folate production by overexpression of *folKE*. GTP cyclohydrolase I, part of the biprotein encoded by *folKE*, is the first enzyme in the folate biosynthesis pathway (Fig. 1A). Compared to a non-induced strain, the overexpression of *folKE* in strain NZ9000 harboring pNZ7010 showed an increased concentration of extracellular folate from approximately 10 to 80 ng/ml. Furthermore, the extracellular folate concentration measured for the control strain NZ9000 harboring pNZ8048 was not affected by induction with nisin (Fig. 3). The folate samples were enzymatically deconjugated with human plasma in order to determine whether part of the extracellular folate was present as polyglutamyl folate with more than 3 glutamate residues that could not be measured by the microbiological assay.

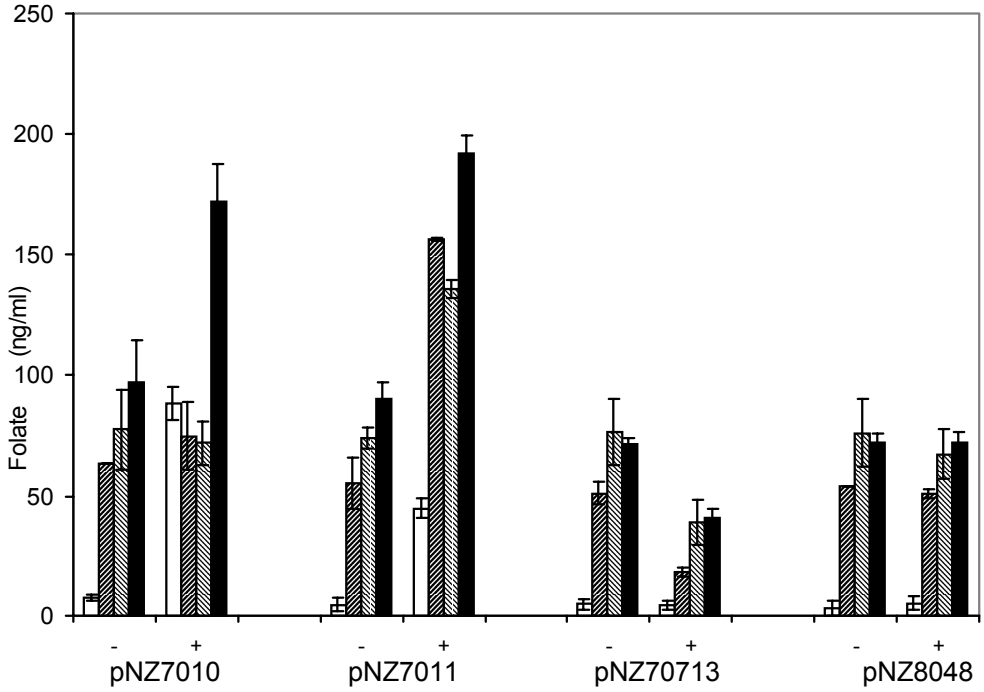


Figure 3:

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

However, no difference in folate concentration was measured with or without deconjugase treatment, indicating that the polyglutamyl folate was not excreted by the cells (Fig. 3). The intracellular folate concentration was measured by analyzing cell-extracts for the presence of folate. Under inducing conditions the *folKE* overexpressing strain displayed a minor increase in intracellular folate production compared to a control strain or a non-induced NZ9000 harboring pNZ7010. After deconjugation of the cell-extracts the intracellular folate concentrations were about 80 ng/ml in both strains (Fig. 3). The total folate production by *L. lactis* was determined by combining the extra- and intracellular folate concentration. It can be concluded that by overexpression of *folKE* the folate production is more than doubled compared to a control strain or a non-induced NZ9000 harboring pNZ7010. The majority of the extra folate produced is present as extracellular

mono, di, or tri-glutamyl folate. The constitutive expression of *folKE* behind the constitutive promoter of *pepN* that could be achieved in NZ9000 harboring pNZ7017 resulted in the same increase of folate production as observed by using the NICE system (results not shown).

Increased intracellular folate production by combined overexpression of folate genes. The extra- and intracellular folate distribution is assumed to be controlled by the ratio of mono- and polyglutamyl folates (36). The enzyme responsible for the synthesis of polyglutamyl folate is polyglutamyl folate synthetase encoded by *folC*. The simultaneous overexpression of *folKE* and *folC* (NZ9000 harboring pNZ7011) could be visualized by SDS-PAGE (Fig. 2). The overexpression of both genes resulted in a more than two-fold increase in total folate production, similar to what was observed with overexpression of only *folKE*. Differences, however, were detected in the folate distribution. Contrary to the folate produced by the overexpression of *folKE* only, the majority of the extra folate produced was present as intracellular folate in the *folKE* and *folC* overexpressing strain. After deconjugation of the intracellular folate, no increased folate concentrations were detected, indicating that the overexpression of *folKE* and *folC* had no significant effect on the amount of polyglutamyl folates with more than three glutamate residues (Fig. 3). The overexpression of *folKE* and *folP*, encoding dihydropteroate synthase, was achieved by inducing strain NZ9000 harboring pNZ7012. However, no differences in folate concentration or folate distribution were observed compared to the overexpression of only *folKE* (results not shown).

Altered folate production by overexpression of *folA* or antisense *folA*. To gain further insight in folate biosynthesis control in *L. lactis* the gene *folA* encoding dihydrofolate reductase was also overexpressed. In a similar way as described previously, the induction of strain NZ9000 harboring pNZ7013 resulted in production of the enzyme with a predicted molecular mass of 15 kDa at a level that could be visualized by SDS-PAGE (Fig. 2). The overexpression of *folA* showed a two-fold decrease in folate production compared to a control strain or a non-induced strain (Fig. 3). The intracellular folate distribution and the relative amount of polyglutamyl folates remained unchanged. In a similar experiment we studied the effect of the production of antisense RNA encoding dihydrofolate reductase. The complementary sequence of the coding strand of *folA* was cloned under the control of the nisin promoter *nisA* in pNZ8048, starting at the 5' end with the complementary sequence of the stop codon of *folA* and finishing at the 3' end with the complementary start codon of the gene. The generated plasmid, pNZ7014, was transformed to *L. lactis* NZ9000.

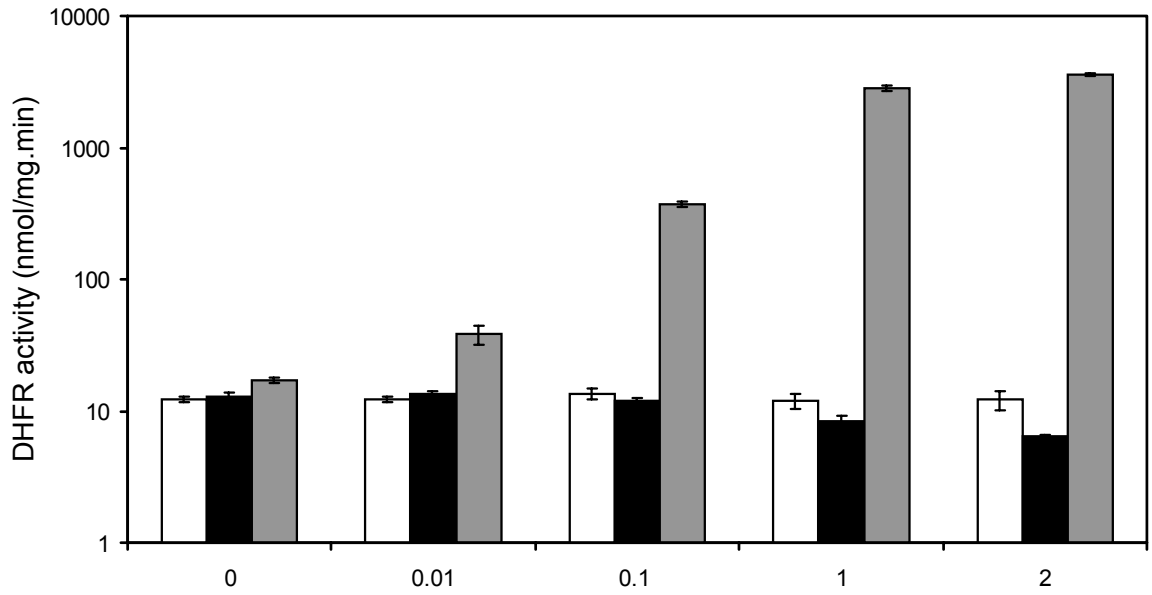


Figure 4:

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

Under inducing conditions, a small, but reproducible, increase of approximately 20% in the total folate production was observed compared to a control strain (results not shown). To confirm the effect of the transcription of antisense RNA of *folA,* the enzymatic activity of dihydrofolate reductase was determined. Cell-extracts of strain NZ9000 harboring pNZ7014, transcribing antisense RNA of *folA*, showed a two-fold decrease in dihydrofolate reductase activity (Fig. 4). In contrast, cell-extracts of *L. lactis* strains overexpressing *folA* showed a more than 1000 fold increase in dihydrofolate reductase activity compared to a control strain (Fig. 4).

Discussion

We have described successful metabolic engineering of the final part of the complicated biosynthetic pathway of folate biosynthesis and the cloning, sequencing and analysis of the folate gene cluster in *L. lactis* MG1363. Homology studies with non-redundant databases show that the folate gene cluster contains *folA,* encoding

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

In some related micro-organisms folate gene clusters have previously been identified: In *S. pneumoniae* and *Lactobacillus plantarum folP*, *folC*, *folE*, *folB* and *folK* are clustered, but in a different order (24,28). In *Lactobacillus plantarum* outside the folate gene cluster a second *folC* gene was identified. In *Bacillus subtilis folP*, *folB* and *folK* are clustered together with genes involved in p-aminobenzoate synthesis, while *folE* and *folC* are far apart in the genome (44).

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

the enzyme production levels were clearly lower. This demonstrates, not only, that functional expression of folate biosynthesis genes can also be achieved by using a constitutive promoter, but that further increase in folate production can, presumably, only be reached by combining *folKE*-overexpression with altered expression of other folate biosynthesis genes.

Most of the folate produced by *L. lactis* is intracellularly accumulated and only a minor part of the folate is secreted by the cells. More than 90% of the intracellular folate pool is present in the polyglutamyl form with 4, 5 and 6 glutamyl residues (non-published results). One of the suggested functions of polyglutamylation is retention of folate within the cell (20,36). Almost all of the extra folate produced by overexpression of *folKE* is excreted into the environment. We suggest that by the increased flux through the folate biosynthesis pathway, due to the overexpression of *folKE,* the enzymatic capacity of folate synthetase / polyglutamyl folate synthetase is not sufficient to transform all extra produced folate into the polyglutamyl form which is necessary for retention of folate within the cell. As a consequence the retention of folate in the cell is decreased. However, when *folKE* and *folC* are simultaneously overexpressed, the majority of the extra folate produced remains intracellular. This confirms that an increased capacity of folate synthetase leads to increased retention of folate in the cell due to an increased enzymatic capacity to elongate the glutamyl tail of the extra folate produced by the overexpression of *folKE*.

The decrease in folate production by overexpression of *folA* encoding dihydrofolate reductase may indicate a feed back inhibition of its reaction product, tetrahydrofolate, on one of the other enzymes involved in folate biosynthesis. Vinnicombe and Derrick (51) report an inhibiting effect of tetrahydrofolate upon dihydropteroate synthase in *S. pneumoniae*. To further analyze the observed controlling effect of *folA* we used the NICE system to produce the antisense RNA of *folA* and we measured the dihydrofolate reductase activity *in vitro*. Enzymatic activity was decreased approximately two-fold in cells expressing antisense RNA of *folA*. The same cells showed a 20% increase in folate production confirming the presumable controlling effect of the *folA* gene product. To further improve our knowledge about the suggested effect of tetrahydrofolate on total folate production we are working on the substitution of the *folA* promoter in the chromosome with the nisin inducible promoter *nisA.*

It can be assumed that the increase of extracellular folate by overexpressing *folKE* is due to an increased production of folate with a short polyglutamyl tail, such as monoglutamyl folate. It has been established that the bioavailability of monoglutamyl folate is higher than that of polyglutamyl folate (for reviews see 14,15,16). Polyglutamyl folates

are available for absorption and metabolic utilization only after enzymatic deconjugation in the small intestine by a mammalian deconjugase enzyme. Only monoglutamyl folate derivatives can directly be absorbed in the human gut. The activity of these deconjugases is susceptible to inhibition by various constituents found in some foods (2,40,43). Furthermore, the intracellular polyglutamyl folate may not be available for absorption by the gastro-intestinal tract of the consumer if the folate is not released by the, mostly dead, micro-organisms. In feeding trials, using rats as animal model, we will investigate whether besides the increase in folate production, also the folate bioavailability will increase in cells overexpressing *folKE*.

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

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Chapter 5

Controlled Modulation of Folate Polyglutamyl Tail Length by Metabolic Engineering of *Lactococcus lactis*

Wilbert Sybesma, Marjo Starrenburg, Igor Mierau, Michiel Kleerebezem, Willem M. de Vos and Jeroen Hugenholtz

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

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Abstract

The dairy starter bacterium *Lactococcus lactis* is able to synthesize folate and accumulates more than 90% of the produced folate intracellularly, predominantly in the polyglutamyl form. Approximately 10% of the produced folate is released in the environment. Overexpression of *folKE*, encoding the bifunctional protein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP-cyclohydrolase I, resulted in reduction of the average polyglutamyl tail-length leading to enhanced excretion of folate. By simultaneous overexpression of *folKE* and *folC*, encoding the enzyme folate synthetase or polyglutamyl folate synthetase, the average polyglutamyl tail length was increased again resulting in normal, wild type, distribution of folate. Overexpression of only *folC* in *L. lactis,* led to an increase in length of the polyglutamyl tail from the predominant four, five, and six glutamate residues in wild type cells, to a maximum of twelve glutamate residues in the folate synthetase overproducer and resulted in a complete retention of folate in the cells. These engineering studies clearly establish the role of polyglutamyl tail length in intracellular retention of the folate produced. Moreover, the potential application of engineered food microbes producing folates with different tail length is discussed.

Introduction

Folate is a B-vitamin and an essential nutrient in the human diet. Folate deficiency is correlated with numerous physiological disorders, like neural tube defects (20) and early spontaneous abortion (8). Moreover, altered folate homeostasis as a consequence of poor nutrition and/or genetic variability is associated with higher risks of cardiovascular diseases (2,14), several types of cancer (5,13,17) and mental disorders among elderly and decreased cognitive performance (4,11).

Folate is a general term for a large number of folic acid derivatives that differ by their state of oxidation, one-carbon substitution of the pteridine ring, and by the number of glutamate residues. The *in vivo* function of reduced folate is that of a co-factor that donates one-carbon units in a variety of reactions involved in the *de novo* biosynthesis of purines and thymidylate and for the methylation of homocysteine to methionine. In most biological systems, folate is present in a conjugated form containing a poly-γ-glutamyl tail. Polyglutamyl folates are better substrates for the enzymes of one-carbon metabolism than the corresponding monoglutamyl folates (23). Moreover, this charged tail may prevent this vitamin from leaking out of the cell (19,22,30). Therefore, the polyglutamyl tail length is assumed to be a dominant factor in the distribution of folate over the cell membrane.

In earlier work we described increased extracellular folate levels in *Lactococcus lactis* that were achieved by metabolic engineering (31). This lactic acid bacterium is widely applied in the dairy industry for the manufacture of fermented dairy products such as cheese, butter and buttermilk. A large proportion of natural folates are derived from the consumption of fermented dairy products and other (fermented) foods. The current daily recommended intake of folates for adults is 400 µg (600 µg for pregnant woman). It was also shown that the intracellular accumulation of folate was changed by the overexpression of specific genes involved in folate biosynthesis (31). Here we describe the targeted engineering of the folate polyglutamyl tail length and describe the impact of these modulations on the folate distribution between the intra- and extracellular space. These strategies could be applied to control the degree of accumulation or the release of folate during fermentation in order to modulate the bioaccessibility of folate in fermented foods.

Materials and methods

Bacterial strains and media. *L. lactis* strain NZ9000 (16) and its derivatives were grown at 30°C in M17 medium (Merck, Darmstadt, Germany) (34) supplemented with 0.5% glucose (GM17) or in chemically defined SA medium (12). When appropriate chloramphenicol was used at 10 μg/ml. Nisin induction was performed as described previously (31).

DNA techniques, construction of plasmids and transformations. The plasmids used in this study are listed in Table 1. Lactococcal plasmid pNZ8048 (16) is a vector that allows nisin-controlled expression (NICE) of genes, it contains the *nisA* promoter followed by a multiple cloning site. pNZ8048 and its derivatives and lactococcal chromosomal DNA were isolated as described previously (18,35). PCR reactions were performed with *Pwo* DNA polymerase (Boehringer, Mannheim, Germany) in a Mastercycler™ PCR apparatus (Eppendorf, Hamburg, Germany) with the following regime: denaturation at 94°C for 15 seconds (3 minutes in first cycle), annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute, for a total of 30 cycles. The gene *folC* was amplified from chromosomal DNA using primers *folC*-F (5'-GGTCCATGGTTTCTATTGAACAAGCATTAGAATGG-3') and *folC*-R (5'-TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG-3). The forward primer *folC*-F contains a *Nco*I restriction site (underlined), allowing translational fusion of the *folC* gene to the *nisA* promoter region in pNZ8048. This *Nco*I site resulted in the introduction of an additional valine residue at position 2 of the encoding FolC protein. The reverse primer *folC*-R introduces a *Xba*I restriction site (underlined). The amplification product was digested with *Nco*I and *Xba*I and cloned in similarly digested pNZ8048 thereby creating a translational fusion of the *folC* gene to the *nisA* gene. The resulting plasmid was designated pNZ7016. The construction of pNZ7010, developed for the overexpression of *folKE,* and pNZ7011, developed for the simultaneous overexpression of *folKE* and *folC,* were described before (31). *L. lactis* strain NZ9000 (16) was used as a NICE-expression host for the plasmids described. It contains the *nisRK* genes, required for nisin mediated regulation, integrated in the chromosomal *pepN* locus. Restriction enzymes and T4 DNA ligase were purchased at Life Technologies BV, (Breda, The Netherlands) and used according to the manufacturers' protocol. All other DNA manipulations were performed using established procedures (27). *L. lactis* was electroporated as described before (37).

Quantification of folate. Folate was quantified by using a *Lactobacillus casei* microbiological assay (10), including post-sampling enzymatic deconjugation, as described before (31). A 1% yeast extract medium solution (Difco, Becton Dickinson Microbiology

Systems, Sparks, MD, USA), containing almost exclusively polyglutamyl folates, with a previously determined total folate content was used as a positive control for deconjugation.

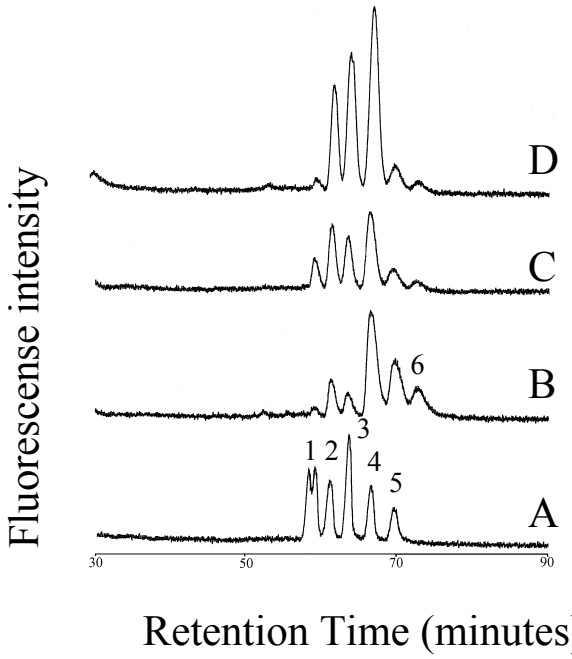
Table 1: plasmids used in this study and their characteristics

Plasmid	Relevant characteristics	Reference
pNZ8048	Cm^r, Inducible expression vector carrying the *nisA* promoter	16
pNZ7010	Cm^r, pNZ8048 derivative containing a functional lactococcal *folKE* gene under control of the *nisA* promoter	31
pNZ7011	Cm^r, pNZ8048 derivative containing a functional lactococcal *folKE* and *folC* gene under control of the *nisA* promoter	31
pNZ7016	Cm^r, pNZ8048 derivative containing a functional lactococcal *folC* gene under control of the *nisA* promoter	This study

Folate measurement by HPLC. Intracellular folate levels were measured by HPLC as described previously (32). HPLC columns, pumps and chromatographic conditions were as described previously (32). Freshly prepared mobile phase consisted of 20% methanol and 1.5% formic acid, pH 3.0 (A) and 1.5% formic acid, pH 3.0 (B). Elution conditions were 25% A and 75% B for 30 minutes, followed by 75% A and 25% B from 32 minutes till 100 minutes. Prior to analysis the column was washed with 60% acetonitril. Fluorimetric detection using a Waters 470 fluorescence detector was done at an excitation wavelength of 310 nm and emission setting of 352 nm. The optimal signal to noise ratio for sensitive detection was an attenuation of 512 and 32 for detection of intra- and extracellular folate, respectively, and a gain value of 100 with a filter value of 4 s. UV detection was performed using a Shimadzu SPD-M10A photodiode array detector (PAD). PAD data were collected between 220 and 500 nm at 2 nm optical resolution in order to discriminate fine structural details of the mono- and polyglutamyl folate spectra. Post-analysis routines were achieved using Shimadzu Class VP 5.0 software. UV absorption at 360 nm enables the discrimination of 5,10-methenyl tetrahydrofolates (21), while 5-formyl tetrahydrofolate derivatives are clearly discriminated by fluorimetric detection.

Extracellular folate levels were analyzed by HPLC after purification and concentration by solid phase extraction using C18 columns (500 μg, 3 ml, Sopachem BV, Wageningen, The Netherlands). For this purpose cells were grown in chemically defined SA medium (12) and 50 ml fermentation broth was acidified with formic acid to a pH of 2.8

1A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Retention Time (minutes)

1B

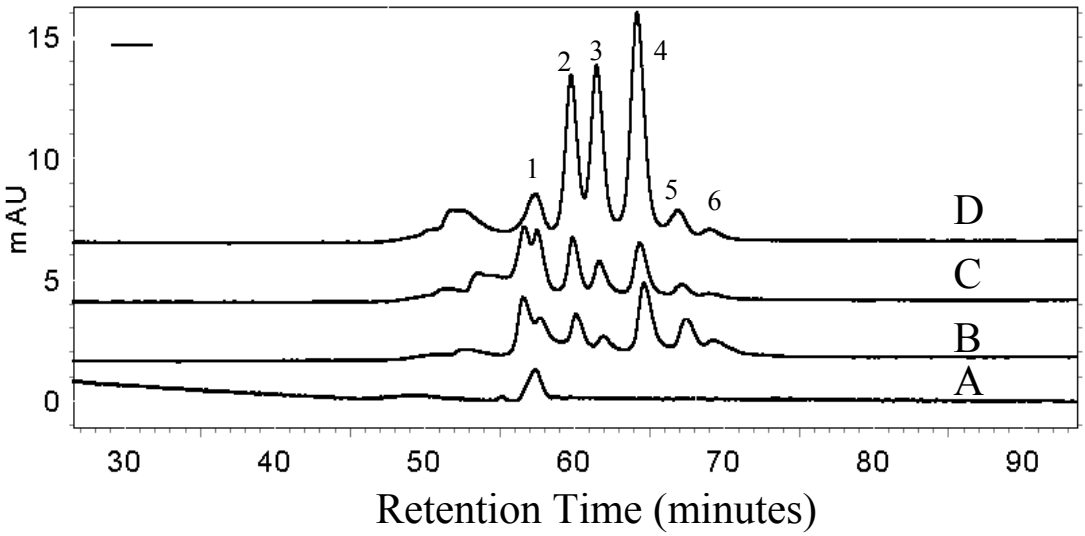
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Figure 1 A:
Folate chromatograms of 5-formyl tetrahydrofolate standards (A) and cell-free extracts of *L. lactis* NZ9000 harboring pNZ8048 (B, control strain with empty vector), or pNZ7010 (C, overexpressing *folKE*), or pNZ7011 (D, overexpressing *folKE* and *folC*), monitored by fluorescent detection. Cells were induced with 2 ng/ml of nisin as described in Material and Methods. Numbers correspond to the polyglutamyl tail length of 5-formyl tetrahydrofolate derivatives. For 5-formyl tetrahydromonoglutamyl folate, both the S and R diastereoisomeres can be distinguished.
Figure 1 B:
Folate chromatograms of 5,10-methenyl tetrahydrofolate standard (A) and cell-free extracts of the same *L. lactis* NZ9000 strains as described in Fig. 1A (B-D), but monitored by UV absorbance at 360 nm.

and loaded on the column that was equilibrated with 5 ml methanol, 5 ml H_2O, and 5 ml 20 mM Na-PO_4 (pH 2.8). After passage of the sample by gravity force, the column was washed with 10 ml 20 mM Na-PO_4 (pH 2.8). Finally, the folates were eluted with 5 ml 10 mM acetic acid - 25% methanol (pH 7.0) and 100 µl of the eluate was analyzed by HPLC as described above.

Folate derivatives used as standards, (6R,S)-5-formyl-5,6,7,8-tetrahydropteroyl mono-, di-, tri-, tetra-, and penta-γ-L-glutamic acid, lithium salt, and (6R,S)-5,10-methylene-5,6,7,8-tetrahydrofolic acid, magnesium salt, were purchased from Schircks (Jona, Switzerland). Small volumes of folate stock solutions were prepared at a concentration of 1 mg/ml and stored at –20°C. Working solutions were prepared by dilution to a concentration within the range 1-100 ng/ml. The tail length of the concentrated 5-formyl polyglutamyl folate samples was analyzed by masspectrometry by using a VG Quattro II mass spectrometer (Micromass UK Ltd., Manchester, U.K.).

Results

The folate distribution and the nature of the polyglutamyl folates produced were studied in the different engineered *L. lactis* strains. Analysis of the intracellular folate pool in late exponential phase cells of *L. lactis* strain NZ9000 harboring pNZ8048 (empty vector) by HPLC showed the presence of 5-formyl tetrahydrofolate with 2, 3, 4, 5, and 6 glutamate residues (Fig. 1A). The total folate production levels were found to be approximately 50 ng/ml/OD_{600}, as was determined by using the folate microbiological assay

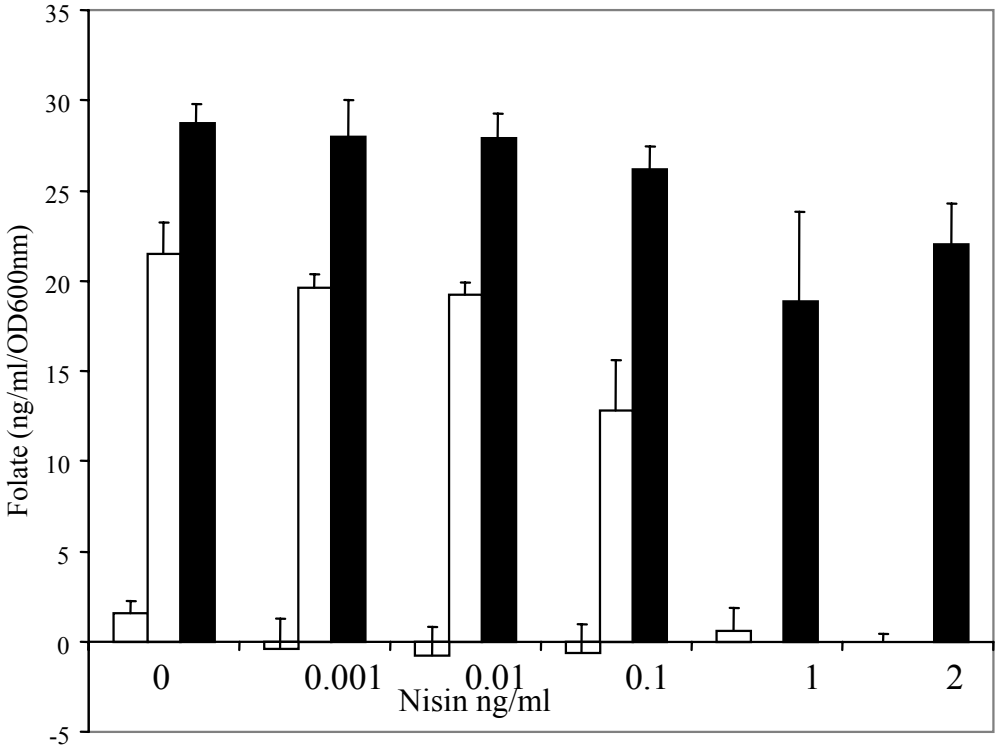

Figure 2:

Folate concentration in different *L. lactis* NZ9000 strains harboring pNZ7016 after step-wise increased overexpression of *folC.* Strains were induced with different concentrations of nisin (0 - 2 ng/ml) at OD_{600} of 0.5. Folate levels were determined at the end of growth, at approximately OD_{600} of 2.5, by using the microbiological assay. (white bars, extracellular folate production; hatched bars, intracellular folate production; black bars, intracellular folate production after deconjugation). The error bars indicate the standard deviation of the microbiological assay (measurement done in duplo).

including deconjugase treatment of long tailed polyglutamyl folates. This assay has a nearly equal response to mono-, di-, and triglutamyl folate, while the response to folates with longer polyglutamyl tails (n-glutamyl more than 3) decreases markedly in proportion to chain length (33). Approximately 90% of the total folate pool was accumulated inside the *L. lactis* cell. The intracellular folate concentration as measured by the microbiological assay, increased after enzymatic deconjugation confirming the presence of polyglutamyl folates. Approximately 10% of the total folate pool was excreted into the environment. Further analysis was done on folate distribution and polyglutamyl tail length in strain NZ9000

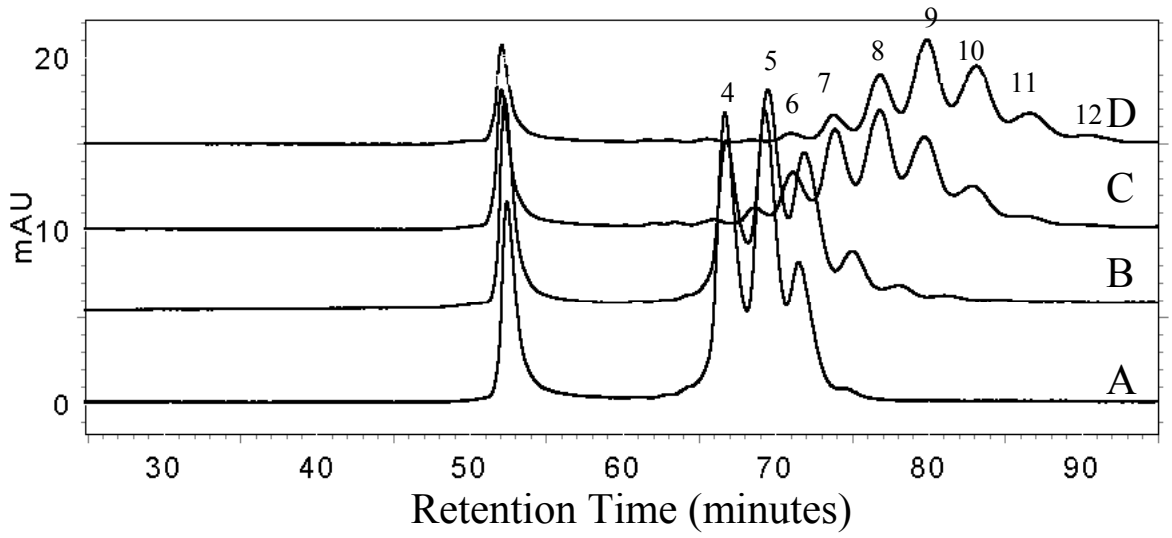

Figure 3:

Folate chromatograms of cell-free extracts of *L. lactis* NZ9000 harboring pNZ7016 with increased overexpression of *folC*, induced with 0 (A), 0.01 (B), 0.1 (C) and 1 (D) ng/ml nisin, monitored at 360 nm. Numbers correspond to the polyglutamyl tail length of 5,10-methenyl tetrahydrofolate derivatives.

harboring pNZ7016, overexpressing *folC*, encoding the bifunctional enzyme folate synthetase/polyglutamyl folate synthetase. The extracellular folate levels in this engineered strain were decreased and the relative accumulation of folate was increased upon induction with nisin resulting in overexpression of FolC (Fig. 2). Growth rate and total folate production decreased maximally 20% upon gradual overexpression of *folC* by using nisin concentrations ranging from 0-2 ng/ml. Moreover, upon high level overexpression of *folC*, intracellular folate could not be measured anymore without enzymatic deconjugation (Fig. 2), suggesting that all folate molecules present have extended polyglutamyl tails. HPLC analysis of the intracellular folate pool of cells overexpressing *folC* confirmed this elongation of the folate polyglutamyl tail. It could be shown that glutamyl tail-length increased step-wise with the increase of the nisin concentration used for *folC* induction. Under maximal induction conditions (2 ng/ml nisin), folates with polyglutamyl tails

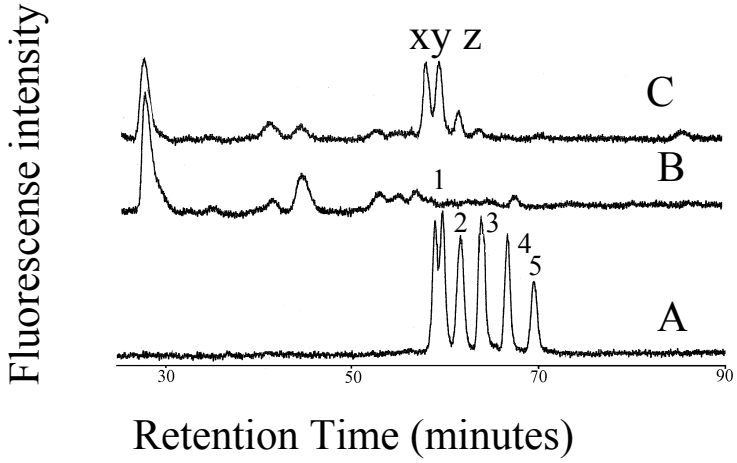

Figure 4 A-C:

Folate chromatograms of 5-formyl tetrahydrofolate standards (A) and concentrated and purified culture supernatant of *L. lactis* NZ9000 harboring pNZ8048 (B, control strain with empty vector) or pNZ7010 (C, overexpressing *folKE*) monitored by fluorescent detection. Legend as in figure 1.

containing up to 12 glutamyl residues could be detected, while in these cells polyglutamyl folates with less than 5 glutamate residues could hardly be visualized (Fig. 3). Based upon the specific UV absorption spectrum of these polyglutamyl folates, characterized by a maximum at 360 nm, we identified these folates as 5,10-methenyl tetrahydrofolate derivatives. Moreover, after enzymatic deconjugation and subsequent chromatographic separation, only 5,10-methenyl tetrahydromonoglutamyl folate could be detected. No extracellular folate could be detected even when the culture supernatant was concentrated (results not shown).

In earlier work we reported about an increase in extracellular- and total folate production upon the overexpression of *folKE*, encoding the biprotein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP-cyclohydrolase I (31). In the present work we show that upon the combined overexpression of *folC* and *folKE* in strain

NZ9000 harboring pNZ7011 the average polyglutamyl tail length decreased. Chromatographic separation of the intracellular folate pool revealed decreased levels of 5-formyl tetrahydrofolate with 5 and 6 glutamate residues and increased levels of 5-formyl tetrahydrofolate with 2, 3, and 4 glutamate residues (Fig. 1A). Moreover, we have previously established that the intracellular folate concentration in cells overexpressing *folKE* and *folC* was more than doubled compared to the control cells, using the microbiological assay (10). In analogy, the chromatograms representing the intracellular accumulated folates in Fig. 1A also show increased levels of the 5-formyl tetrahydrofolate derivatives in the induced cells compared to the uninduced cells. A further reduction of the average folate polyglutamyl tail was accomplished by overexpression of only *folKE* in strain NZ9000 harboring pNZ7010. Chromatographic analysis of the intracellular folate pool showed a further decrease of the levels of 5-formyl tetrahydrofolate with 4, 5 and 6 glutamate residues and an increase of the levels of 5-formyl tetrahydrofolate with 1, 2 and 3 glutamate residues in comparison to a control strain (Fig. 1A). Similar analysis of the purified and concentrated extracellular folate pool produced by cells overexpressing *folKE* showed the presence of 3 components (Fig. 4). Based upon elution time and spectral characteristics compound Y and Z could be identified as 5-formyl monoglutamyl folate and 5-formyl diglutamyl folate respectively. However, compound X, that eluted before 5-formyl monoglutamyl folate, could not be identified. Fraction collection and subsequent analysis of the unknown component by using the microbiological assay showed the absence of biological activity. Quantitative analysis by using the microbiological assay of the fractions containing compound Y and Z showed that they represented more than 90% of the total extracellular folate levels that were injected on the column (results not shown). All obtained folate chromatograms were also analyzed by using UV detection, to evaluate the effect of metabolic engineering on 5,10-methenyl tetrahydrofolate derivatives besides 5-formyl tetrahydrofolate derivatives. These analyses showed that the polyglutamyl tail length of this folate molecule responds similar to the engineering approaches employed. An exemplary set of 5,10-methenyl tetrahydrofolate chromatograms is shown in Fig. 1B for the intracellular folate pools of the control strain and of cells overexpressing *folKE,* and *folKE* and *folC* simultaneously.

Overall, the polyglutamyl tail length as determined by HPLC for all engineered strains appeared to be directly correlated to the intra- and extracellular distribution of folate, as determined by the microbiological assay (Table 2).

Table 2: Average polyglutamyl tail length and ratio between intracellular and extracellular folate concentration in engineered *L. lactis* strains determined at 10 hours after induction with nisin.

strains	Average polyglutamyl tail length[A]	ratio intracelluar folate and extracellular folate[B]
NZ9000-pNZ7010	3.0	0.64
NZ9000-pNZ7011	3.3	1.5
NZ9000-pNZ8048	4.1	9
NZ9000-pNZ7016	>5	>>[C]

A determined by calculating area under curves in chromatogram

B determined by microbiological assay

C depends on detection limit microbiological assay (~1 ng/ml)

Discussion

All living cells contain folate, mostly in the polyglutamyl form. Many folate dependent enzymes have a higher affinity for polyglutamyl folates than the corresponding monoglutamyl folates (2). The enzyme responsible for polyglutamyl folate synthesis and corresponding chain length elongation is polyglutamyl folate synthetase (EC 6.3.2.17) encoded by *folC*. So far, all organisms of which the entire genome sequence has been determined, appear to have a homologue of *folC.* Nevertheless, different species are known to contain a large variety of actual folate polyglutamyl chain-length ranging from 1 to 10 glutamate residues (29). It is generally assumed that polyglutamyl folates determine the retention of folate molecules inside the cells. However, in bacteria this assumption is based on observations that the polyglutamyl tail length of folates externally found in the growth medium is shorter than the intracellularly found tail length (3). Here we provide experimental evidence for the correlation between polyglutamyl tail length and intracellular accumulation or extracellular release of folate in bacterial cells by engineering this property in a directed manner. The overexpression of *folKE* resulted in conversion of intracellular polyglutamyl folates with predominantly 4, 5, and 6 glutamate residues in the wild type, to folates with predominantly 1, 2, and 3 glutamyl folates. Concomitantly, increased levels of 5-formyl monoglutamyl folate could be measured in the culture supernatant of these *folKE* overexpressing strains. Simultaneous overexpression of *folC* and *folKE* can partially counteract the *folKE* overexpression effect, generating a partial recovery of the

polyglutamyl tail length and spatial folate distribution towards the wild-type situation. These results are in agreement with the observation that overexpression of *folC* alone increases the polyglutamylation of folate enormously and polyglutamyl folates with up to 12 glutamyl residues could be detected. As a consequence, the retention of folate in the cells is increased and release of folate to the environment is below detection limits. We conclude that the capacity of folate synthetase and polyglutamyl folate synthetase to add glutamate residues to the folate precursor dihydropteroate and subsequent mono- and polyglutamyl folates is limited when the flux through the folate biosynthesis pathway is increased, for instance by overproduction of the first enzyme of the pathway, GTP cyclohydrolase I. As a consequence, production of short tailed polyglutamyl folates is favored over the synthesis of long tailed polyglutamyl folates. The elongation of the intracellular polyglutamyl tail affected growth rate and total folate production slightly, which could be caused by a reduced affinity of the folate-dependent enzymes for folates with longer polyglutamyl tails. A comparable strategy was used to show the relation between expression of *folC* and retention of folate in human T-lymphoblastic leukemia cells (19). The authors show that decreased activity levels of the human FolC, which was achieved by anti-sense RNA expression, resulted in decreased levels of intracellular folate. However, in this study, the actual length of the glutamyl tails of these folate molecules was only estimated densitometrically from immunoblots of electrophoretically separated ternary complexes.

The chromatographic separation of *L. lactis* cell-free extracts and subsequent analysis of folate species by UV absorption or fluorescence, enables the detection and quantification of several forms of folate with different polyglutamyl tail lengths. Although the maximum polyglutamyl tail length of the used 5-formyl tetrahydrofolate standards does not exceed five glutamyl residues, the appearance of 5-formyl monoglutamyl folate after enzymatic deconjugation of the polyglutamyl folates, justifies the conclusion that the produced polyglutamyl folates are also derivatives of 5-formyl tetrahydrofolate. A similar conclusion can be made for the detection of 5,10-methenyl tetrahydrofolate derivatives with their unique and characteristic absorption maximum at 360 nm (21). Other folate derivatives that might be accumulated by the cells, like tetrahydrofolate- and dihydrofolate derivatives, could not unambiguously be distinguished by using the chromatographic conditions described. However, their presence is assumed to be required for active one-carbon metabolism. Therefore, reliable quantification of the total folate pool can only be achieved by application of a microbiological assay. An alternative explanation for not detecting other folate derivatives might be degradation during sampling due to instability of certain folates that are known to be extremely labile (21), although the folate standards

5-formyl tetrahydrofolate and 5,10-methenyl tetrahydrofolate remained stable during analysis.

Controlled intracellular accumulation or extracellular release of folate by lactic acid bacteria could provide an added value to certain food products. Cells that accumulate intracellular folate, but that are removed from the fermented end product, or that do not lyse during passage through the gastro-intestinal tract, may reduce the bioavailability of the produced folates. Production of folates with short polyglutamyl tail length and subsequent release to the environment could improve actual folate bioavailability for the consumer. By contrast, bacterial strains used as probiotics that are generally consumed in large amounts in relatively small absolute volumes, may deliver higher folate concentrations when the produced polyglutamyl folates are accumulated intracellularly during growth. However, a prerequisite of efficient folate delivery by using such probiotic strains is a limited survival of the cells during intestinal passage. Human trials have indicated that the majority of *L. lactis* cells actually lyse during passage through the gastro-intestinal tract (15).

In the human diet, folate is consumed in the polyglutamyl form. However, folate is absorbed in the small intestine in the monoglutamyl form. Polyglutamyl folates are available for absorption and metabolic utilization only after enzymatic deconjugation in the small intestine by a mammalian deconjugase enzyme (9,26). In animal and human trials (6,24), it has been reported that the bioavailability of monoglutamyl folate is higher than that of polyglutamyl folate. Moreover, the activity of the mammalian deconjugase enzymes to produce monoglutamyl folates is susceptible to inhibition by alcohol or by various constituents found in some foods (1,25,28,36). Folate absorption can be further inhibited by a defective glutamate carboxypeptidase II, resulting from a genetic polymorphism characterized by a H475Y transition (7). Therefore, diets containing elevated levels of monoglutamyl folates could facilitate folate absorption when the intestinal deconjugase activity is hampered.

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Chapter 6

Expression of a Heterologous γ-Glutamyl Hydrolase Gene in *Lactococcus lactis* for Increased Production of Bioavailable Folate

Wilbert Sybesma, Erwin van den Born, Marjo Starrenburg, Igor Mierau, Michiel Kleerebezem, Willem M. de Vos and Jeroen Hugenholtz

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

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Abstract

The functional expression of the gene encoding human or rat γ-glutamyl hydrolase in the dairy starter bacterium *Lactococcus lactis* results in deconjugation of intracellular polyglutamyl folates and subsequent release of bioavailable monoglutamyl folate. The use of these engineered *L. lactis* strains may also include application as delivery vectors for glutamate hydrolase activity *in situ*.

Introduction

Folate deficiency, that occurs throughout the world including the western hemisphere (16,22) is correlated to numerous physiological disorders, like neural tube defects (20) and early spontaneous abortion (9). Moreover, low folate homeostasis is associated with higher risks of cardiovascular diseases (3,15), several types of cancer (5,14,18), and mental disorders such as psychiatric syndromes among elderly and decreased cognitive performance (4,12).

In most biological material folate in the cells is present in a conjugated form, containing a polyglutamyl tail. Folate absorption only occurs in the monoglutamyl form. Folate auxotrophic organisms such as humans, contain a polyglutamyl folate hydrolase activity to remove the glutamyl tail and make the folate available for uptake by the tissue cells. This conversion is catalyzed by intestinal γ-glutamyl hydrolases (11,23). The effective intake level of dietary folate is therefore strongly influenced by the degree of bioavailability of folate, characterized by the level of polyglutamylation and the level of glutamyl hydrolase enzyme activity. Several authors have suggested that the bioavailability of monoglutamyl folate is higher than that of polyglutamyl folate (6,10,21).

We reported previously on changes in the folate polyglutamyl tail length as a result of metabolic engineering on the level of the folate gene cluster in the dairy starter bacterium *Lactococcus lactis*. The results demonstrated a clear relationship between polyglutamyl tail length and the degree of intracellular folate retention (27). Here, we describe a different strategy to convert polyglutamyl folate into monoglutamyl folate and to improve excretion of bioavailable monoglutamyl folate into the fermentation broth, by controlled expression of mammalian γ-glutamyl hydrolase genes in *L. lactis*.

Materials and methods, results and discussion

A PCR product of 885 basepairs encoding the mature human γ-glutamyl hydrolase (HGH) was generated from the corresponding full length cDNA (32) cloned in vector pCR3 kindly provided by The Laboratory of Molecular Diagnostics, Wadsworth Center, Albany, New York, by using sense primer HGH-f (5'-CATGCCATGGGACCCCACGGCGACACCGCCAAG-3') and antisense primer HGH-r (5'-GCTCTAGATCAATCAAATATGTAACATTGCTG-3'). The PCR product was digested with *Nco*I and *Xba*I (sites are underlined in the PCR primers) and cloned in similarily digested

pNZ8048 (17). The resulting plasmid was designated pNZ7001 and contains the human glutamyl hydrolase gene translationally fused to the *nisA* gene and under *nisA* promoter control, allowing nisin controlled HGH expression in an appropriate lactococcal NICE-expression host like NZ9000 (7,17). The introduction of a *Nco*I site resulted in the insertion of an additional methionine at postion 1 and a substitution of arginine by glycine at position 2 of the encoding HGH protein, resulting in the synthesis of MG-HGH. *L. lactis* strain NZ9000 harboring pNZ7001 was grown in SA medium (13).

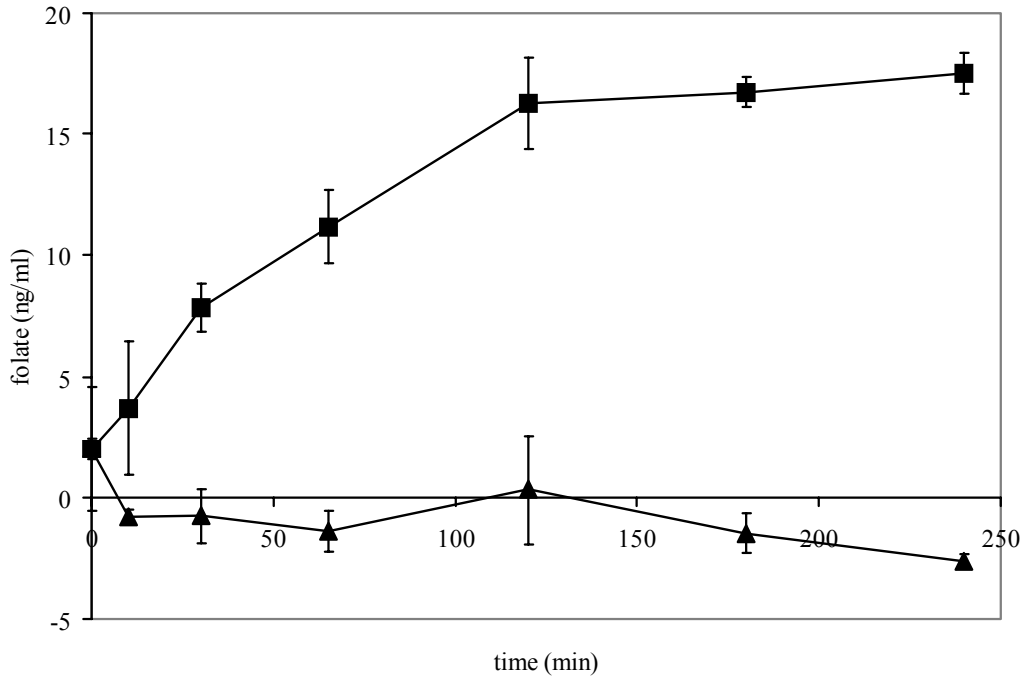


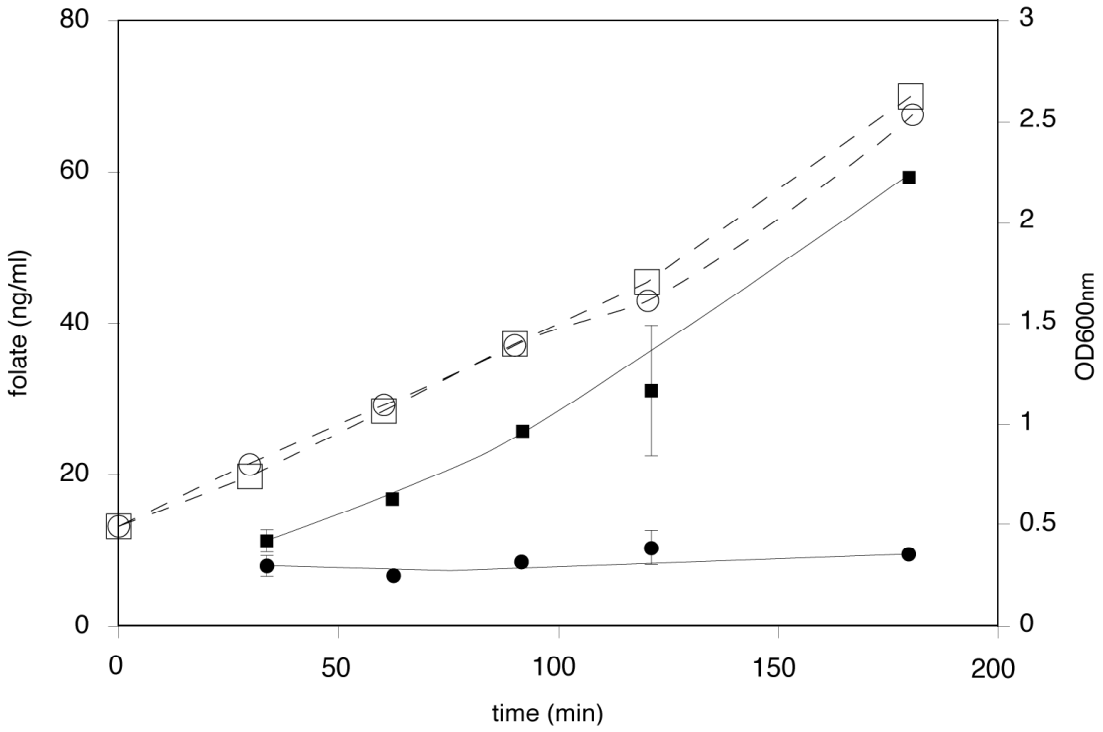
Fig. 1:
Functional expression of the gene coding for human γ-glutamyl hydrolase (MG-HGH) in a cell-extract of *L. lactis* pNZ9000 harboring pNZ7001 (■) or pNZ8048 (negative control) (▲) monitored in vitro using yeast extract as a source of polyglutamyl folate at pH 7.0. Folate concentrations are measured with a microbiological assay and corrected for the production of folate by the *L. lactis* strain. The error bars indicate the standard deviation of the microbiological assay (measurement done in duplo).

Upon induction with nisin the production of the MG-HGH in growing *L. lactis* NZ9000 harboring pNZ7001, could be visualized on a western blot using polyclonal anti-γ-glutamyl hydrolase antibody (25). The detected protein had an estimated molecular mass of 33 kDa consistent with the predicted MW of γ-glutamyl hydrolase (results not shown). Yeast extract, containing mostly heptaglutamyl folate (1), which can only be detected in folate microbiological assay after deconjugation (29), was used to test the MG-HGH production and activity in *L. lactis*. Progressive deconjugation of polyglutamyl folate in yeast extract was observed following incubation with a cell-free extract of *L. lactis* NZ9000 harboring pNZ7001 induced with 1 ng/ml of nisin (Fig. 1). The optimal enzyme activity was found at pH 7.0. In the cell-free extract of control strain NZ9000 harboring pNZ8048 no deconjugase activity was found after addition of nisin.

The strains producing γ-glutamyl hydrolase were further analyzed for their capacity to deconjugate polyglutamyl folates from different sources with different polyglutamyl tail length in *in vitro* experiments: A cell-free extract of cells producing MG-HGH was mixed with a cell-free extract of *L. lactis* strain NZ9000 harboring pNZ7016 (27) that produces polyglutamyl folates. After incubation at 37°C for 3 hours, chromatographic analysis of the mixed cell extracts showed the conversion of polyglutamyl folate into monoglutamyl folate (results not shown). These results indicate that the engineered *L. lactis* strains may have a potential application as delivery vectors for glutamate hydrolase activity *in situ*.

Both intra- and extracellular folate levels produced by the wild type and engineered strain were measured using a microbiological assay including enzymatic deconjugation (28) (Fig. 2). In the MG-HGH producing cells the extracellular folate levels increased from approximately 0 to 60 ng/ml, while intracellular folate levels remained constant at approximately 10 ng/ml. In the control strain NZ9000 harboring pNZ8048, folate was accumulated within the cell and the production increased during growth from approximately 10 to 80 ng/ml. The extracellular folate production remained at a constant level of approximately 10 ng/ml in this strain. Detailed chromatographic analysis of 5-formyl polyglutamyl folate standards (Fig. 3A) and of the fermentation broth produced by cells producing MG-HGH showed an increase of 5-formyl monoglutamyl folate compared to cells not producing MG-HGH (Fig. 3B and C). The intracellular folate pool in cells producing MG-HGH changed from polyglutamyl folates with 4,5, and 6 glutamyl residues to monoglutamyl folate that was partially excreted by the cells (Fig. 3E and D). It can be concluded that by the expression of the gene encoding MG-HGH in *L. lactis*, polyglutamyl folate is deconjugated, retention of folate is decreased, and the monoglutamyl folates formed are excreted into the environment. The efflux of folate does not lead to altered

A



growth rate or to increased folate production, so the remaining intracellular folate levels are sufficient for normal growth of the bacteria. The expression of the gene coding for rat γ-glutamyl hydrolase (31) in *L. lactis* (NZ9000 harboring pNZ7002) gave similar results as described for the MG-HGH, except for the increased rate of deconjugation (data not shown).

The production of γ-glutamyl hydrolase in food grade micro-organisms and the increased production of monoglutamyl folate may have a positive influence on the bioavailability of folate for three reasons: 1. The total monoglutamyl folate levels will increase and consequently, the need for intestinal hydrolase activity to deconjugate polyglutamyl folate is relieved and may be advantageous under the conditions that hydrolase activity is reduced by certain food components present in the diet (2,24,26,30) or by genetic polymorphism (8); 2. The intracellular deconjugation of endogenously produced folate to monoglutamyl folate will result in increased excretion of folate to the environment, resulting in improved bioaccessibility. Especially when micro-organisms tend to survive the passage through the gastro-intestinal tract, release of folate by these cells increases the effective folate consumption and 3, conversion of polyglutamyl to

B

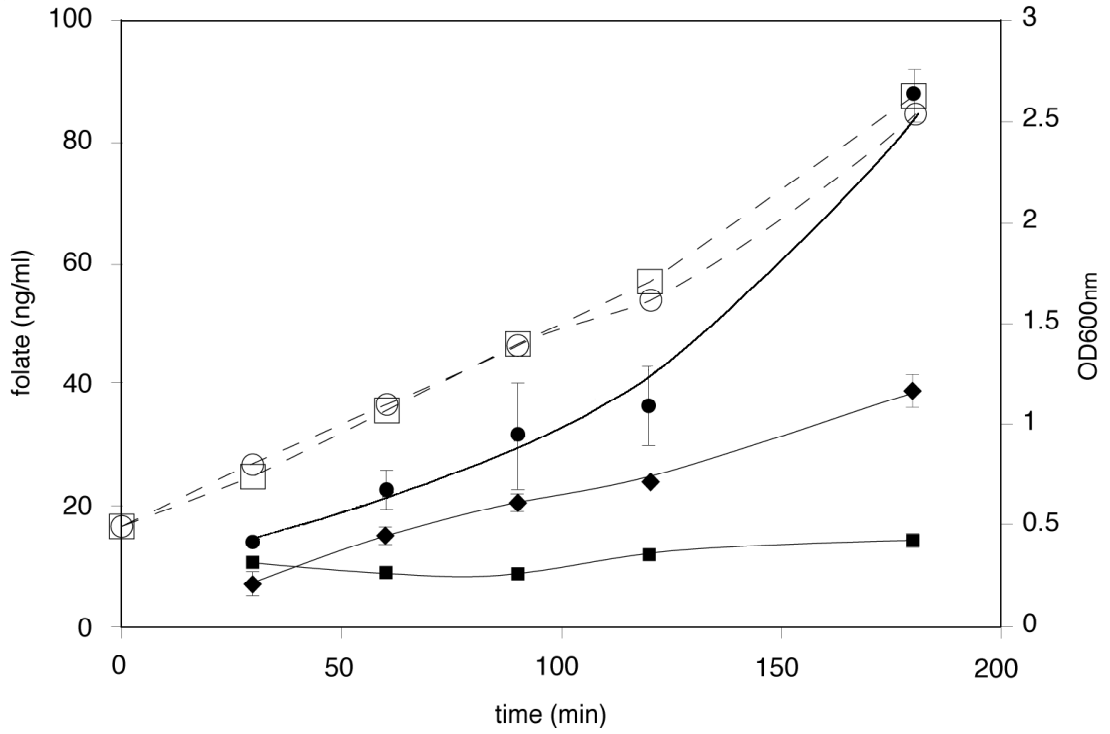


Fig. 2:

A. Growth curves (dashed lines) and functional expression of human γ-glutamyl hydrolase (MG-HGH) gene monitoring the extracellular concentration of folate (solid lines) in growing cells of *L. lactis* NZ9000 harboring pNZ7001 (■) or pNZ8048 (negative control) (●).

B. Growth curves (dashed lines) and functional expression of human γ-glutamyl hydrolase gene by monitoring intracellular concentration of folate (solid lines) in growing cells of *L. lactis* NZ9000 harboring pNZ7001 (■) or pNZ8048 (♦). Higher intracellular folate levels were detected in the control strain after deconjugation of polyglutamyl folate (●). Cells were induced with nisin at $OD600_{nm}$ of 0.5. Folate concentrations were measured with a microbiological assay. The error bars indicate the standard deviation of the microbiological assay (measurement done in duplo).

monoglutamyl folates will be enhanced within the gastro intestinal tract by delivery of extra active γ-glutamyl hydrolase.

The industrial application of food grade micro-organisms expressing a gene encoding γ-glutamyl hydrolase from mammalian origin may have a long term perspective. However, the use of γ-glutamyl hydrolases from (food grade) bacterial origin may

accelerate the market introduction of fermented foods naturally enriched with bioavailable monoglutamyl folate. In many eubacteria, including lactic acid bacteria, enzymes capable of hydrolyzing γ-glutamyl peptide bonds are necessary for cell wall turn over. In *Bacillus* spp. carboxypeptidases have been described that may have the γ-glutamyl hydrolase activity (19). However, further research is required to determine whether these or other bacterial carboxypeptidases can hydrolyze the γ-glutamyl tail of folate.

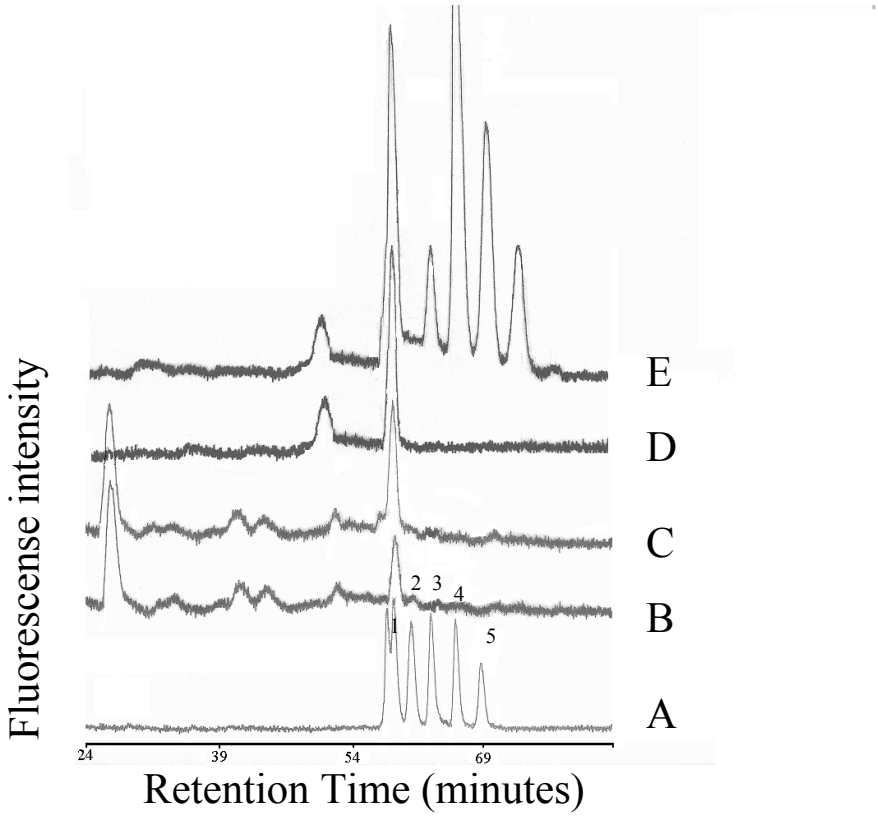


Fig. 3:

Chromatography of 5-formyl tetrahydrofolate standards (A), folates extracted at the end of exponential phase from fermentation broth of *L. lactis* harboring pNZ7001 induced with nisin (C) or not induced with nisin (B), and intracellular folates present in cell extracts of *L. lactis* harboring pNZ7001 induced with nisin (D) or not induced with nisin (E). Fluorescence detection: λ_{ex} 310 nm, λ_{em} 352 nm. Numbers correspond to the polyglutamyl tail length of 5-formyl tetrahydrofolate derivatives. From 5-formyl tetrahydromonoglutamyl folate the S and R diastereoisomeres can be distinguished.

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Chapter 7

Multivitamin Production in *Lactococcus lactis* Using Metabolic Engineering

Wilbert Sybesma[1]*, Catherine Burgess[2]*, Marjo Starrenburg[1], Douwe van Sinderen[2], and Jeroen Hugenholtz[1]

1. Wageningen Centre for Food Sciences, Wageningen, The Netherlands
2. Department of Microbiology. National Food Biotechnology Centre. Department of Food Science, Food Technology and Nutrition, National University of Ireland, Cork, Ireland.
* Equal contribution of both authors

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Abstract

The dairy starter bacterium *Lactococcus lactis* has the potential to synthesize both folate (vitamin B11) and riboflavin (vitamin B2). In the biosynthetic pathways of these two vitamins GTP acts as a precursor molecule. A spontaneous mutant of *L. lactis* strain NZ9000 isolated following exposure to the riboflavin analogue roseoflavin, was changed from riboflavin consumer into riboflavin producer. This mutant contained a single base change in the regulatory region upstream of the riboflavin biosynthetic genes. The constitutive overproduction of GTP cyclohydrolase I in this strain, deregulated in riboflavin biosynthesis, was shown to result in increased production of folate as well. Novel foods, enriched through fermentation using these multivitamin-producing starters, could compensate the B-vitamin-deficiencies that are common even among population groups in highly-developed countries.

Introduction

Folate (vitamin B11) and riboflavin (vitamin B2) are essential nutrients in the human diet. Folate is a general term for a large number of folic acid derivatives that differ by their state of oxidation, one-carbon substitution of the pteridine ring, and by the length of the polyglutamate tail. These differences are associated with different physicochemical properties, which may influence folate bioavailability through variable absorption abilities in the gastro-intestinal tract. Folate serves as an enzymatic co-factor in a variety of one-carbon transfer reactions involved in the *de novo* biosynthesis of nucleotides and in remethylation of homocysteine to methionine. Folate deficiency is correlated with numerous physiological disorders, such as neural tube defects (Lucock, 2000) and early spontaneous abortion (George *et al*., 2002). Moreover, low folate homeostasis is associated with a higher risk of cardiovascular diseases (Boushey *et al*., 1995, Klerk *et al*.,2002), several types of cancer (La Vechia *et al*., 2002, Kim, 1999, Choi and Mason, 2002), and mental disorders, such as psychiatric syndromes among elderly and decreased cognitive performance (Calvares and Bryan, 2001, Hultberg *et al*., 2001). The daily recommended intake of dietary folate for an adult is 400 µg, while for pregnant women this is 600 µg.

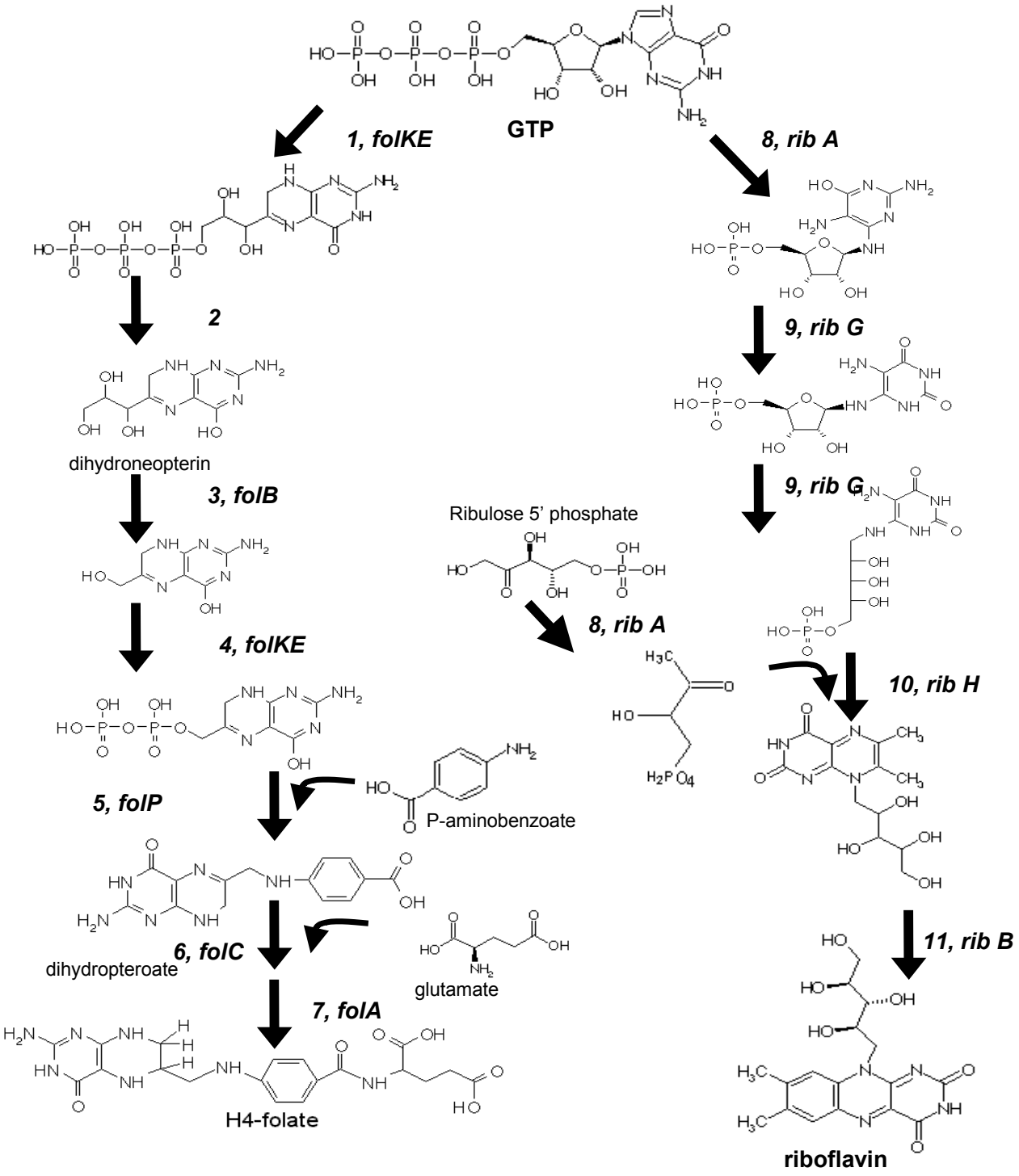
Riboflavin is a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), that are required in reactions such as the enzymatic oxidation of carbohydrates. Riboflavin deficiency in humans is correlated with loss of hair, inflammation of the skin, vision deterioration, and growth failure. This vitamin has also been found to be successful in the treatment of migraine (Krymchantowski *et al*., 2002) and malaria (Akompong *et al*., 2000). The daily recommended intake of dietary riboflavin for an adult is 1.6 mg. Riboflavin was traditionally manufactured using chemical processes, but in recent years biotechnological processes have become more popular using organisms such as *Bacillus subtilis, Ashbya gossypii* and *Candida famata* (Stahmann *et al*., 2000). Folate and riboflavin are commonly obtained in the diet from meat (liver), vegetables, milk and fermented (dairy) products, eggs, and fortified foods such as bread and cereal products. Annually, thousands of tonnes of both folate and riboflavin are produced commercially for fortification of both food and feed.

In previous work we reported on increased folate production by metabolic engineering of the lactic acid bacterium *Lactococcus lactis* (Sybesma *et al*., 2003). In the present work we combine the metabolic engineering approach in the dairy starter bacterium *L. lactis* with a mutagenesis approach to develop a bacterium with increased

synthesis of both folate and riboflavin. The enzymes and corresponding genes for folate and riboflavin biosynthesis in *L. lactis* are known (Bolotin *et al.*, 2001, Sybesma *et al.*, 2003). Both vitamins are synthesized from the precursor GTP in the first step of the folate and riboflavin biosynthesis pathway by GTP cyclohydrolase I and GTP cyclohydrolase II, respectively (Fig. 1). *L. lactis* is by far the most extensively studied lactic acid bacterium and an ideal model organism for metabolic engineering strategies that aim at inactivation of undesired genes and/or (controlled) overexpression of existing or novel ones (Hols, *et al.*, 1999, Hugenholtz *et al.*, 2000, Boels *et al.*, 2003). The presented results are an important step in the development of functional foods with increased vitamin levels that can contribute to more individualized recommendations on the basis of genetic variation. Moreover, recent reports from studies done in The Netherlands and Ireland have indicated that folate and riboflavin deficiency (Konings *et al.*, 2001, O'Brien *et al.*, 2001) is common among various population groups including women of childbearing age even in such developed countries.

Figure 1:

Riboflavin and folate biosynthesis pathway. Indicated are the genes and enzymatic reaction steps: 1. GTP cyclohydrolase I (EC 3.5.4.16), 2. phosphatase reaction, 3. dihydroneopterin aldolase (EC 4.1.2.25), 4. 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.5.1.15), 5. dihydropteroate synthase (EC 2.7.6.3), 6. folate synthetase (EC 6.3.2.12), 7. dihydrofolate reductase (folA, EC 1.5.1.3), 8. GTP cyclohydrolase II / 3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 3.5.4.25), 9. diaminohydroxyphosphoribosylaminopyrimidine deaminase / 5-amino-6-(5-phosphoribosylamino) uracil reductase (EC 3.5.4.26), 10. riboflavin synthase beta chain (EC 2.5.1.9), 11. riboflavin synthase alpha chain (EC 2.5.1.9).


GTP
1, folKE
8, rib A
2
dihydroneopterin
9, rib G
3, folB
9, rib G
Ribulose 5' phosphate
8, rib A
4, folKE
10, rib H
5, folP
P-aminobenzoate
dihydropteroate
6, folC
glutamate
11, rib B
7, folA
H4-folate
riboflavin

Materials and methods

Bacterial strains, media, and culture conditions.

All *L. lactis* MG1363 NZ9000 derivatives (Kuipers *et al.*, 1998) were grown at 30°C in chemically defined medium (CDM) as described previously (Otto *et al.*, 1983, Poolman and Konings, 1988) supplemented with 19 g/L of β-glycerophosphate and 0.5% glucose and depleted of folic acid, riboflavin and nucleotides. When appropriate the media contained chloramphenicol (10 μg/ml). Plasmids were generated and transformed to *L. lactis* as described below.

Isolation of roseoflavin resistant mutants.

Spontaneous roseoflavin resistant *L. lactis* mutants were isolated by plating an undiluted *L. lactis* NZ9000 culture on CDM containing 100 mg/L roseoflavin (Toronto Research Chemicals, Toronto, Canada). Potential roseoflavin resistant mutants were streaked on CDM containing the same analogue to confirm the stability of the resistant phenotype and a number of these were chosen for further characterization. One of the mutant strains, *L. lactis* CB010, was chosen for further use in this study.

Sequence analysis of roseoflavin resistant mutants

Primers were designed to amplify *ribC,* a regulator of the riboflavin biosynthesis operon, and the deduced transcriptional control region immediately upstream of the structural genes for riboflavin biosynthesis. The PCR products obtained were purified using GIBCO PCR purification kit (Invitrogen, Groningen, The Netherlands) and were subjected to sequence analysis (MWG Biotech AG Ebersberg, Germany).

DNA manipulations, construction of plasmids , and transformations.

Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Isolation of plasmid DNA from *L. lactis* and introduction of plasmid DNA into *L. lactis* was performed as previously described (De Vos *et al.*, 1989). Restriction enzymes and T4 DNA ligase were purchased at Life Technologies BV, Breda, The Netherlands.

The *folKE* gene, encoding amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I, was amplified from *L. lactis* chromosomal DNA by PCR using 25 ng of template DNA in a final volume of 50 μl containing deoxyribonucleoside triphosphates (0.25 mM – 0.5 mM each), oligo nucleotides (50 pM)

(FolKE2-F, 5'- ATACATGCATGCAAACAACTTATTTAAGCATGGG-3'; FolKE2-R, 5'-ATACATGCATGCGATTCTTGATTAAGTTCTAAG-3') and 1 U of *Pfx* polymerase (Invitrogen, Paisley, Great Britain). Amplification was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following regime: 30 cycles denaturation at 95°C for 30 seconds (3 minutes in first cycle), annealing at 50°C for 30 seconds and elongation at 68°C for 1 minute. The *folKE* gene was cloned in pNZ8161 under the control of the constitutive promoter of *pepN* as described previously (Sybesma *et al*., 2003) to generate the plasmid pNZ7017. *L. lactis* CB010 was used as a host for pNZ7017.

Analysis of intra- and extracellular vitamin concentration.

Folate was quantified using a *Lactobacillus casei* microbiological assay (Horne and Patterson, 1988) including post sampling enzymatic deconjugation as described previously (Sybesma *et al.*, 2003). For analysis of folate and riboflavin concentrations by HPLC cells were separated from the fermentation broth as follows: *L. lactis* CB010 cells harboring pNZ7017 were grown in 50 ml CDM. Cells were harvested at early stationary phase (OD_{600nm} is 2.5) by centrifugation (12,000 g, 10 min, 4°C) and washed with 20 ml of 50 mM $NaPO_4$ buffer, 1% ascorbic acid, pH 2.3. The cell suspension was resuspended in 1 ml of the same buffer. A cell extract was obtained by adding 1 g of silica beads to the cell suspension followed by disruption of the cells in an FP120 Fastprep™ cell disrupter (Savant Instruments inc., Holbrook, NY, USA). Release of folate from folate binding proteins and precipitation of proteins was achieved by heating the cells at 100°C for 3 min. After centrifugation (two times at 12,000 g, 3 min, 4°C) 100 µl of cell-free extract was injected onto the column as soon as possible after extraction. For analysis of extracellular folate and riboflavin concentrations by HPLC, cells were grown and harvested as described above and 100 µl of undiluted culture supernatant was injected onto the column immediately after extraction.

Folate derivatives were purchased from Schircks (Jona, Switzerland). Riboflavin, was purchased from Sigma (Zwijndrecht, The Netherlands). Small volumes of folate stock solutions were prepared at a concentration of 1 mg/ml and frozen. Working solutions were prepared by thawing microliter volumes and diluting to a concentration within the range 10-1000 ng/ml according to need. The concentrated polyglutamyl folate samples were analyzed by masspectrometry using a VG Quattro II mass spectrometer (Micromass UK Ltd., Manchester, U.K.). The high performance liquid chromatograph consisted of a Waters 600E pump (Waters assoc., Watford, UK), Waters 767 plus autosampler injector,

and on line a Waters 470 fluorescence detector and a SpectroPhysics FL2000 fluorescence detector (Spectro Physics, San Jose, CA). Different mono- and polyglutamyl folates and riboflavin derivatives were discriminated with the aid of a betasil phenyl column (250mm x 3mm ID, 3μm) (Keystone Scientific inc. Bellefonte, PA) protected with a betasil phenyl guard column. Freshly prepared mobile phase consisting of 9% methanol and 1.5% formic acid, pH 3.0, was filtered through a 0.45 μm millipore filter (type durapore) and degassed. Chromatography was performed at 50°C using a flow rate of 0.5 ml/min which produced a back pressure of 1200 psi. Detection was performed by fluorescence with an excitation wavelength of 310 nm and emission setting of 352 nm for detection of folate derivatives and 440 nm and 520 nm for detection of riboflavin derivatives. The optimal signal to noise ratio for sensitive detection varied between 4 and 256 and was dependent on measurement of intra- or extracellular vitamins. The gain value was 100 with a filter value of 4 s.

Results

It has been shown previously in *Bacillus subtilis* that resistance to the riboflavin analogue, roseoflavin, results in riboflavin overproduction (Pero *et al.,* 1991). With the aim to isolate riboflavin overproducing *L. lactis* mutants, the NZ9000 strain was exposed to roseoflavin and spontaneous roseoflavin resistant variants were analyzed for riboflavin production (data not shown). *L. lactis* CB010 was chosen for use in this study and this strain was transformed with pNZ7017 overexpressing the gene *folKE* that codes for the bifunctional protein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophospho-kinase and GTP cyclohydrolase I (Sybesma *et al*., 2003).

Folate and riboflavin were analyzed in a growing culture of *L. lactis* CB010 harboring pNZ7017. The extracellular riboflavin levels were greater than 1200 μg/L as determined by using HPLC. This is in contrast to the wild type strain NZ9000 in which extracellular riboflavin production was below detection limits (Fig. 2). Moreover, further analysis of riboflavin concentrations in cultures growing in medium containing riboflavin showed that NZ9000 acts as a riboflavin consumer while CB010 acts as a riboflavin producer (Fig. 2). The inset of Figure 2 shows the chromatographic separation of riboflavin of the culture supernatant of strain CB010 harboring pNZ7017 (C) or of the control strain (B). For *B. subtilis* roseoflavin resistant and riboflavin overproducing mutants it has been demonstrated that such mutants carry mutations in either the RFN element, that represents a conserved RNA secondary structure with a base stem and four hairpins

involved in the regulation of bacterial riboflavin synthesis genes (Gelfand *et al*., 1999, Vitreschak *et al.,*2002), or *ribC*, a supposed regulator for the riboflavin biosynthesis operon (Mack *et al.,* 1998, Kil *et al.,* 1992, Coquard *et al.,* 1997). In order to see if the same is true for *L. lactis* these regions were sequenced in CB010. No mutations were found in *ribC,* but in the RFN element a guanine to cytosine substitution was detected (Fig. 3). This position is part of the first stem in the predicted RNA stem loop regulatory element and is denoted a conserved base between various species (Vitreschak *et al*., 2002).

Analysis of extracellular folate levels in *L. lactis* CB010 harboring pNZ7017 during growth using the microbiological assay showed a strong increase from approximately 10 ng/ml to 100 ng/ml, while the extracellular folate levels in a control strain, CB010 harboring pNZ8048, remained at a constant value of approximately 10 ng/ml (Fig. 4). The intracellular folate levels in both *L. lactis* strains showed a similar pattern. During growth folate was accumulated in the cells reaching a level of 80 ng/ml (results not shown). Overproduction of FolKE in CB010 resulted in more than two-fold increase in total folate levels (Fig. 4). Analysis of the intracellular folate pool using HPLC confirmed a decrease in the average polyglutamyl tail length in strain CB010 harboring pNZ7017 compared to the control strain. The levels of 5 formyl tetrahydrofolate with 4, 5, and 6 glutamate residues shifted towards the synthesis of 5-formyl tetrahydrofolate with 1, 2, and 3 glutamate residues, in analogy to the overexpression of *folKE* in *L. lactis* strain NZ9000 as was described previously (Sybesma *et al.,* 2003). Like wise, the chromatographic separation of the same culture supernatant as shown in Fig. 3 of the strain CB010 overexpressing *folKE* showed an increase of levels of 5-formyl tetrahydrofolate with 1 glutamate (Sybesma *et al.,* 2003). The overproduction of riboflavin and folate did not change the growth characteristics of the engineered *L. lactis* strains. Moreover, increased folate production did not affect riboflavin production and increased riboflavin production did not affect the folate production, although both vitamins use GTP as a substrate (results not shown).

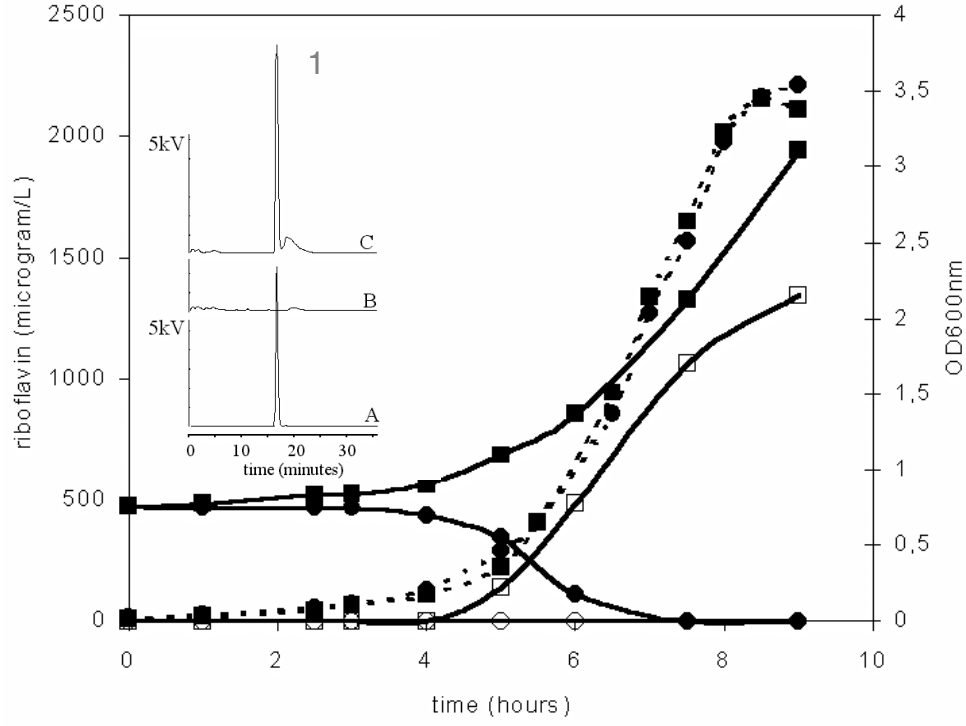


Figure 2:

Growth and extracellular riboflavin levels detected in growing strains of *L. lactis* NZ9000 harboring pNZ8048 (negative control), or strain CB010 harboring pNZ7017 (deregulated in riboflavin biosynthesis and overexpressing *folKE*).

Legend: The spheres indicate strain NZ9000 and the squares indicate strain CB0110. The dashed lines follow growth of the strains. The solid lines follow riboflavin levels in the medium (as measured by HPLC). The open symbols indicate riboflavin production in CDM and the solid symbols indicate riboflavin production in CDM containing 5 μM riboflavin.

(inset)

Chromatograms of riboflavin standard (A) and extracellular riboflavin levels by *L. lactis* NZ9000 harboring pNZ8048 (B, negative control), or strain CB010 harboring pNZ7017 (C, deregulated in riboflavin biosynthesis and overexpressing *folKE*).

Legend: 1, riboflavin peak at 17.5 minutes

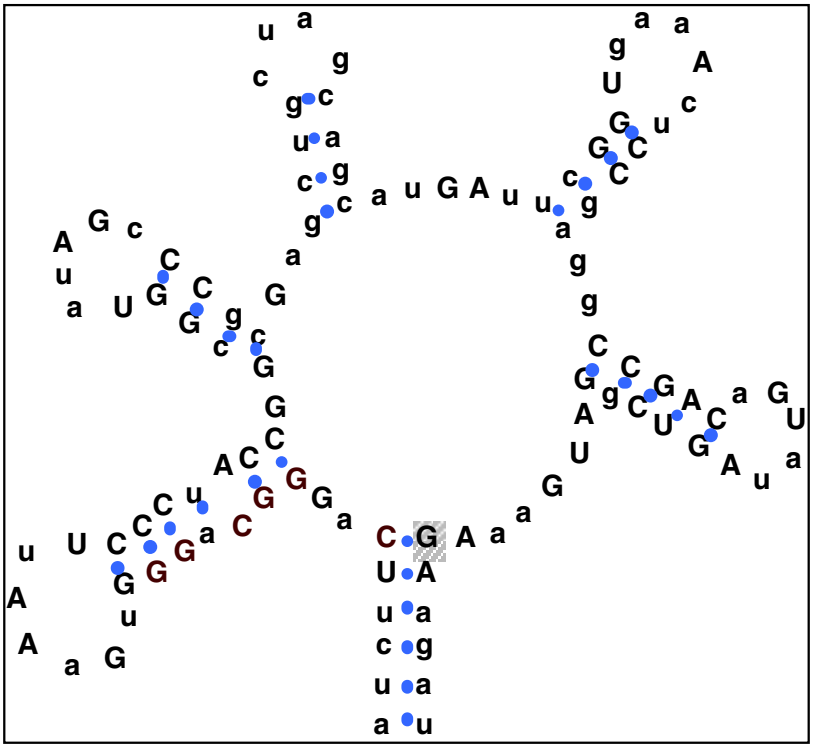


Figure 3:

L. Lactis NZ9000 RFN element. The capital letters indicate conserved bases between species and the dots illustrate base pairing. The grey box indicates where a guanine to cytosine substitution occurs in the roseoflavin resistant L. lactis CB010. The RNA secondary structure was predicted using Zuker's algorithm of free energy minimization (Lyngso *et al.,* 1999) which is utilised in the Mfold program (http://bioinfo.math.rpi.edu/~mfold/rna).

Discussion

In this work we describe a successful combination of directed mutagenesis and metabolic engineering of the complex biosynthetic pathways of riboflavin and folate. The *L. lactis* MG1363 derivative NZ9000 was exposed to the riboflavin analogue roseoflavin. The resistant strain, CB010, that was isolated had a deregulated riboflavin biosynthesis resulting in production of riboflavin instead of consumption, as was seen in the original *L. lactis* MG1363 derivative strain NZ9000. Molecular analysis of the riboflavin biosynthesis genes of the roseoflavin resistant strain revealed a DNA polymorphism in the RFN element, a putative regulatory region upstream of the riboflavin biosynthesis operon.

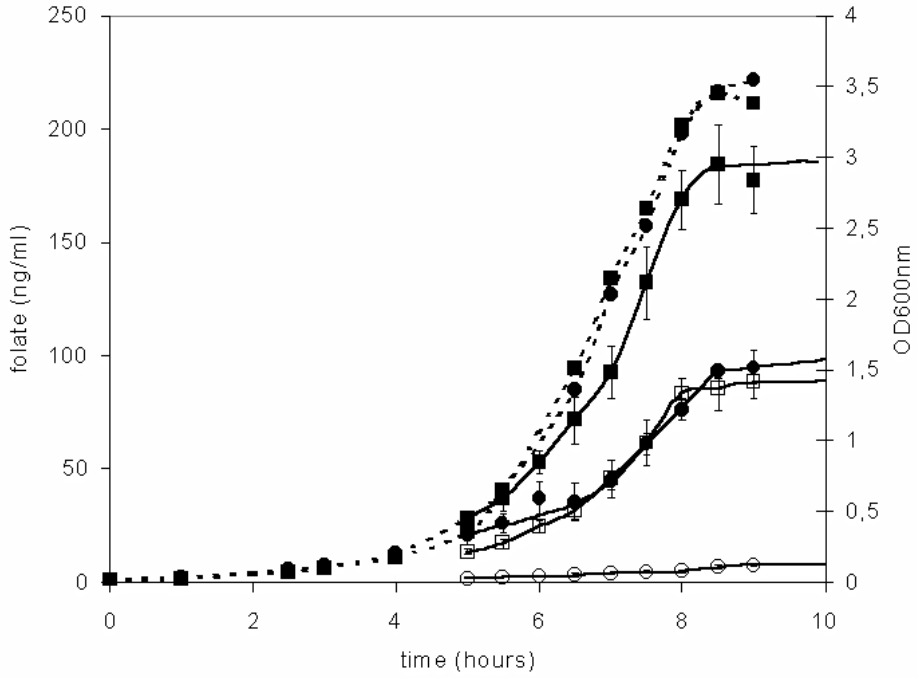


Figure 4:

Growth, intra-, and extracellular folate levels detected in growing strains of *L. lactis* NZ9000 harboring pNZ8048 (negative control), or strain CB010 harboring pNZ7017 (deregulated in riboflavin biosynthesis and overexpressing *folKE*).

Legend: The spheres indicate *L. lactis* strain NZ9000 and the squares indicate *L. lactis* strain CB0110. The dashed lines follow growth of the strains. The solid lines with the solid symbols follow total folate levels and the solid lines with open symbols follow extracellular folate levels. Measurement was done by using a microbiological assay. Error bars indicate standard deviations.

The mutation found is present in a highly conserved position of the regulatory element that is part of the first of the five stems of the stem-loop structure (Vitreschak *et al*., 2002). This may be a contributing or main factor in the altered riboflavin biosynthesis phenotype in CB010, because the binding of flavine mononucleotide to the native RFN element terminates transcription and consequently controls riboflavin biosynthesis (Mack *et al*., 1998, Winkler, *et al*., 2002). In similar work using *B. subtilis* strains with resistance to roseoflavin and increased production levels of riboflavin, mutations were found at different positions on the RFN (Kil *et al.,* 1992, Coquard, *et al*., 1997).

The constitutive overexpression of *folKE* encoding the biprotein amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I in the strain deregulated in riboflavin biosynthesis resulted in more than ten-fold increase of extracellular folate levels and more than two-fold increase in total folate levels. The HPLC data show that the overexpression of *folKE* leads to a reduction of the polyglutamyl tail length, as a consequence the retention of the intracellular folate is decreased. We assume that the capacity of *folC*, encoding the biprotein folate synthetase and polyglutamyl folate synthetase, to elongate the polyglutamyl tail of the extra produced folate generated by overexpression of *folKE* is limited. Besides the increase in folate production, the formation of folate with decreased polyglutamyl tail length could improve the folate bioavailability of folate, because in humans only monoglutamyl folate derivatives can be directly absorbed in the human gut. Polyglutamyl folates are available for absorption and metabolic utilization only after enzymatic deconjugation in the small intestine by a mammalian deconjugase enzyme. In animal and human trials (Clifford *et al*., 1991, Melse-Boonstra *et al*., 2003), it has been reported that the bioavailability of monoglutamyl folate is higher than that of polyglutamyl folate.

During the biosynthesis of both folate and riboflavin, GTP is used as an initial precursor by GTP cyclohydrolase I and II, respectively. The increased production levels of either riboflavin or folate did not effect growth. Moreover, despite the increased demand for GTP, the overproduction of one of the two vitamins did not influence the induced production of the other vitamin. Therefore, we conclude that the synthesis of the pterins, riboflavin and folate, is not limited in strain CB010 by the GTP-supply.

It was already known that folate and riboflavin contribute both to the prevention of diseases like megaloblastic anaemia (Fishman *et al*., 2000). Recent studies established a further important link between folate and riboflavin in individuals with a mutation in methylenetetrahydrofolate reductase (MTHFR) (McNulty *et al*., 2002, Jacques *et al*., 2002, Hustad *et al*., 2000). Between 10-15% of the Caucasian race are homozygous for the C6777T transition in the MTHFR gene and may suffer from high plasma homocysteine levels. Hyperhomocysteinemia is considered a potential risk factor for cardiovascular disease. It has also been associated with birth effects, pregnancy complications and Alzheimer disease. Both folate and FAD appear to protect the thermolabile MTHFR from destabilization (Yamada *et al*., 2001, Guenther *et al*., 1999). The importance of higher vitamin requirement due to genetic polymorphisms was already reported before. Persons with vitamin-D resistant rickets (Abrams, 2002) or with homocystinuria due to cystathionine

β-synthase (Walter *et al.*, 1998, Van Guldener and Stehouwer, 2001) deficiency need supplementation with vitamin D or vitamin B-6, respectively.

This study has demonstrated that metabolic engineering and directed mutagenesis followed by selection can be used for controlling secondary metabolism, such as the more complex folate and riboflavin biosynthesis pathways. The industrial application of the described multivitamin producing strain CB010 harboring pNZ7017 during the production of fermented foods could significantly contribute to acquiring the daily recommended intake of 1.6 mg and 400 μg for riboflavin and folate, respectively. Moreover, the results described here, may be beneficial for health of the general population especially when vitamin deficiency is common among a large part of the population (Konings *et al.*, 2001, O'Brien *et al.*, 2001) or for sub-populations diagnosed with altered genotypes. As an alternative for development of fermented foods with increased levels of naturally produced vitamins, foods could also be fortified with vitamins. However, legislation or limited industrial development may limit the use of fortified foods. Moreover, in all foods that are enriched with vitamins, the consequences of potential overexposure should be acknowledged and further studied. Stover and Garza (2002) summarize some (controversial) reports that suggest that high levels of maternal folate during gestation may have adverse unintended consequences.

Acknowledgements

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Chapter 8

Transformation of the folate-consuming *Lactobacillus gasseri* into a folate-producer

Arno Wegkamp, Marjo Starrenburg,Willem M. de Vos, Jeroen Hugenholtz, and Wilbert Sybesma

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

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Abstract

Five genes essential for folate biosynthesis in *Lactococcus lactis* were cloned on a broad host-range lactococcal vector and transferred to the folate auxotroph *Lactobacillus gasseri*. As a result *L. gasseri* changed from a folate-consumer into folate-producer.

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 (8). Food products like green vegetables, meat and fermented dairy products contain significant folate levels. Still folate-deficiency occurs throughout the whole world, including several well-developed countries. Recently, it has been shown that metabolic engineering can be used to increase folate levels in fermented foods (10).

Lactic acid bacteria such as *Lactococcus lactis* and *Lactobacillus plantarum* have the ability to synthesize folate that is a biological cofactor involved in their amino acid and nucleotide metabolism (6,9). The genes for folate biosynthesis have been identified (5,10). The biosynthetic pathway includes seven consecutive steps, in which the precursor guanosine triphospate (GTP) is converted into tetrahydrofolate (12). However, some lactic acid bacteria, such as *Lactobacillus gasseri* strain ATCC 33323, can not 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 (3).

The folate biosynthesis genes of *Lactococcus lactis* MG1363 are organized in a folate gene cluster, consisting of six genes (*folA*, *folB*, *folKE*, *folP*, *ylgG* and *folC*) (Fig. 1) (10). 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 *Lactococcus lactis*.

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

The plasmid pNZ7017 (10) was used as template for PCR amplification using high fidelity *Pwo* polymerase (Invitrogen, Paisley, Great Britain) of a 5-kb fragment consisting of the constitutive *pepN* promoter (11), multiple cloning site, chloroamphenicol resistance marker, and replication genes that originate from pNZ12 (1). The forward primer pEB8Xba-F (5'-TACTGCAGGCGGTACCACTAGTTCTAGAGAGCTCAAGC-3') was extended at the 5'-end creating a *Xba*I restriction site. The reverse primer used was pEB8-R (5'-GCCTTCTCCTAAATATTCAGTATTAATATAATTATATC-3') (Fig. 1). Similarly, the folate gene cluster (*folB*, *folKE*, *folP*, *ylgG*, and *folC*) of *Lactococcus lactis* was amplified by PCR using the forward primer folBATG-F

(5'-ATGTACAAAATAAAACTTAATAATATGAAATTTAGAGC-'3), which started at the start-codon of the *folB* gene, and the reverse primer folCXba-R (5'-TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG-'3), which was extended at the 5'-end creating a *Xba*I restriction site and overlapped the stopcodon of *folC* (Fig. 1). Both amplified PCR fragments (Fig. 1) were restricted with *Xba*I (Invitrogen). Subsequently, the non-digested ends were phosphorylated by T4 DNA kinase (Gibco BRL, Life Technologies, Grand Island, N.Y) and the two fragments were ligated by using T4 ligase (Invitrogen), generating a translational fusion between the constitutive promoter of the *pepN* gene (11) and the folate gene cluster (Fig. 1). The resulting plasmid was designated pNZ7019. After transformation to *Lactococcus lactis* MG1363 and subsequent cultivation of the strain, the plasmid was harvested as described previously (2).

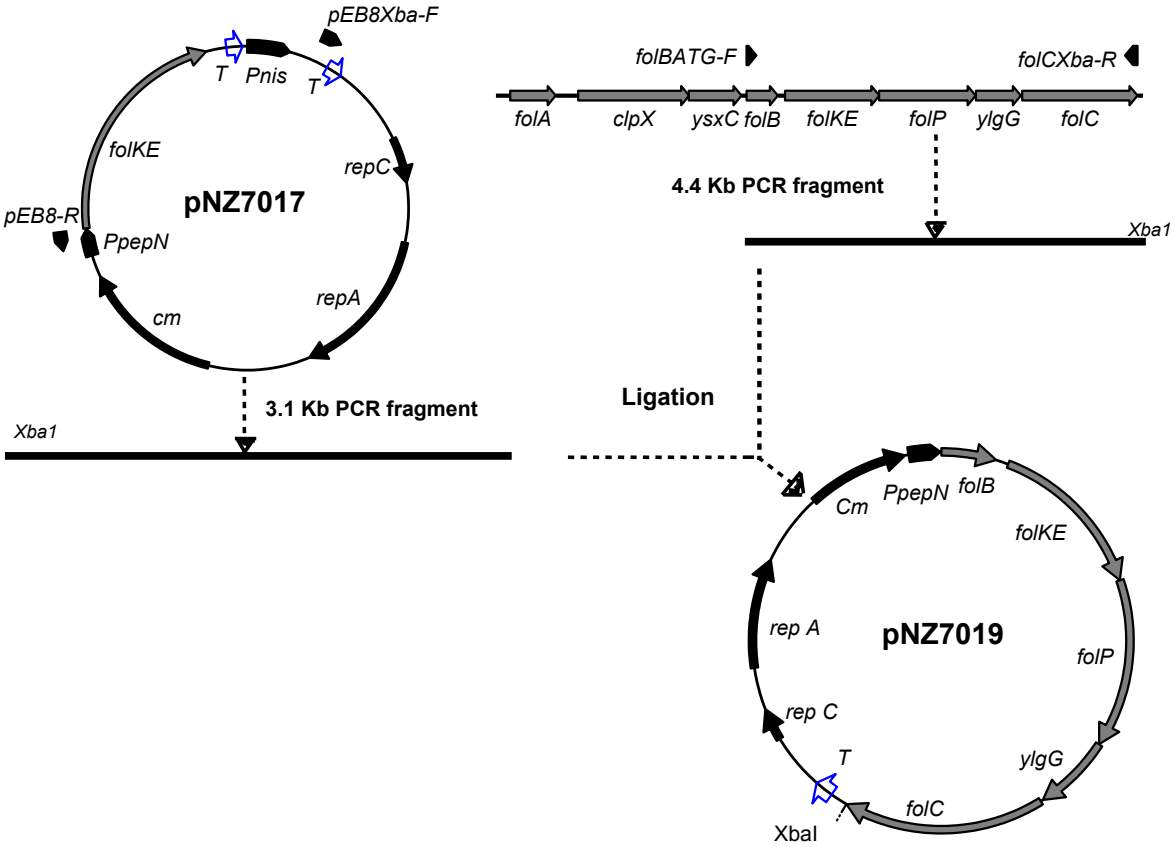

Figure 1:

Construction of pNZ7019 by amplification of linear vector DNA of pNZ7017 and the folate gene cluster from chromosomal DNA of *Lactococcus lactis* MG1363.

L. gasseri (ATCC 33323) was transformed with purified pNZ7019 using an established procedure (7) and plated on MRS containing 10 µg/ml chloramphenicol. After incubation for 40 h at 37ºC, chloramphenicol resistant colonies were examined for the presence of pNZ7019 using restriction analyses. A *L. gasseri* colony harboring pNZ7019 was used for renewed cultivation using the same growth conditions as previously described. RAPD-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 FACM medium (Difco, Becton Dickinson Microbiology Systems, Sparks, MD, USA) was developed for growth and subsequent folate analysis of the *L. gasseri* wild-type strain, and the *L. gasseri* strain harboring pNZ7019. The FACM medium was enriched with 1 mg/l vitamin B12 (Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany) and 1 ml/l Tween 80 (Merck, Darmstadt, Germany). The wild-type strain could not grow at 37 ºC unless folate was added (1.0 mg/l), 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 (3,9,10). 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 harboring pNZ7019 was cultivated for approximately 30 generations on MRS medium supplemented with 10 µg/ml chloramphenicol at 37 ºC. Next, the culture was plated on MRS agar plates supplemented with 10 µg/ml chloramphenicol. Subsequently, hundred colonies were transferred to folate-free FACM plates. Since all colonies grew on these plates, it appears that plasmid pNZ7019 is stably maintained and hence, folate is produced.

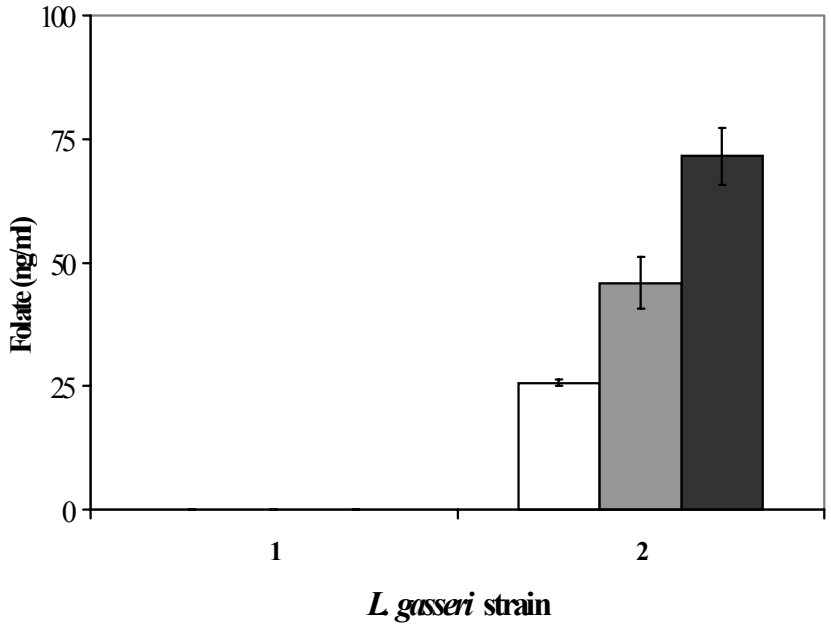

Figure 2:
Folate production of the *L. gasseri* wild-type strain (1) and the *L. gasseri* strain harboring pNZ7019 (2), grown on modified FACM. Legend: extracellular folate levels; (white bar), intracellular folate levels; (grey bar), total folate levels; (black bar). The *L. gasseri* wild-type strain 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, *folB*, *folKE*, *folP*, *ylgG* and *folC,* directing the folate biosynthesis in *Lactococcus lactis* were transferred into *Lactobacillus 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.

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Chapter 9

Hyper Folate Production in *Lactoccocus lactis* by Metabolic Engineering and its Control by *p*-Aminobenzoic Acid

Wilbert Sybesma[1], Arno Wegkamp[1], Marjo Starrenburg[1], Anne de Jong[2], Jan Kok[2], Oscar Kuipers[2], Willem de Vos[1], and Jeroen Hugenholtz[1]

1. Wageningen Centre for Food Sciences, Wageningen, The Netherlands

2. Department of Molecular Genetics, University of Groningen, Haren, The Netherlands

Abstract

More than 50 times increased folate production levels are obtained in the lactic acid bacterium *Lactococcus lactis* by the overproduction of the complete folate biosynthesis pathway consisting of FolB, the biprotein FolKE, FolP, FolQ, and the biprotein FolC. Folate production is controlled by the biosynthesis of the folate precursor p-aminobenzoic acid (*p*ABA) via the shikimate pathway and additional presence of *p*ABA in the medium. The global effects of the applied metabolic engineering approaches are studied by using microarrays. The generation of a hyper folate-producing dairy starter bacterium may contribute to the production of fermented foods that reduce the occurrence of natural folate deficiency within the population.

Introduction

The daily recommended intake of folate for humans varies between 200 and 400 μg per day. Folate is present in various foods that are part of the normal human diet, like orange juice, dark green leafy vegetables, asparagus, strawberries, legumes, meat (liver) and fermented (dairy) products. Still, many people experience folate deficiency (Konings *et al.*, 2001, O'Brien *et al.*, 2000) that may lead to increased risks for the occurrence of neural tube defects in newly borns, cardiovascular diseases, cognitive diseases, and some forms of cancer (Lucock, 2000). It has been established that fermented dairy products, like yogurt, cheese, and others, belong to the top 15 of food products that contribute most to acquiring the daily intake for folate (Konings *et al.*, 2001).

In previous work we have shown that folate can be synthesized by the dairy starter bacterium *Lactococcus lactis* and that folate production levels can be increased several fold by overexpressing genes of the folate biosynthesis pathway (Sybesma *et al.*, 2003a). However, it was also shown that the folate biosynthesis is controlled by the availability of p-aminobenzoic acid (pABA). In a medium lacking pABA the folate production levels of *L. lactis* are two-fold decreased compared to a medium with excess of pABA (Sybesma *et al.*, 2003b).

In many bacteria, including *L. lactis*, pABA is derived from the same pathway as the aromatic amino acids tyrosine, phenylalanine, and tryptophane. Phosphoenol-pyruvate, synthesized in the glycolysis, and erythrose-4-phosphate, synthesized in the pentose phosphate pathway, are the main substrates for synthesis of pABA and these aromatic amino acids (Fig. 1). It has also been shown that the folate production by *L. lactis* is reduced upon the addition of tyrosine, probably because of the feed back inhibition of tyrosine on phospho-2-dehydro-3-deoxyheptonate aldolase, as occurs in *Escherichia coli* (Jossek *et al.*, 2001). Addition of pABA could increase folate production levels and compensate the negative effect of tyrosine. Hence, the contribution of the shikimate pathway to the biosynthesis of pABA could be studied by genetic or physiological approaches.

In the present work we extend the metabolic engineering strategy for increasing the flux through the linear part of the folate biosynthesis pathway, starting with GTP and finishing with tetrahydrofolate, and study the potential metabolic routes for biosynthesis of the folate precursor pABA. In addition, we analyze the global effects of gene expression of these metabolic engineering approaches.

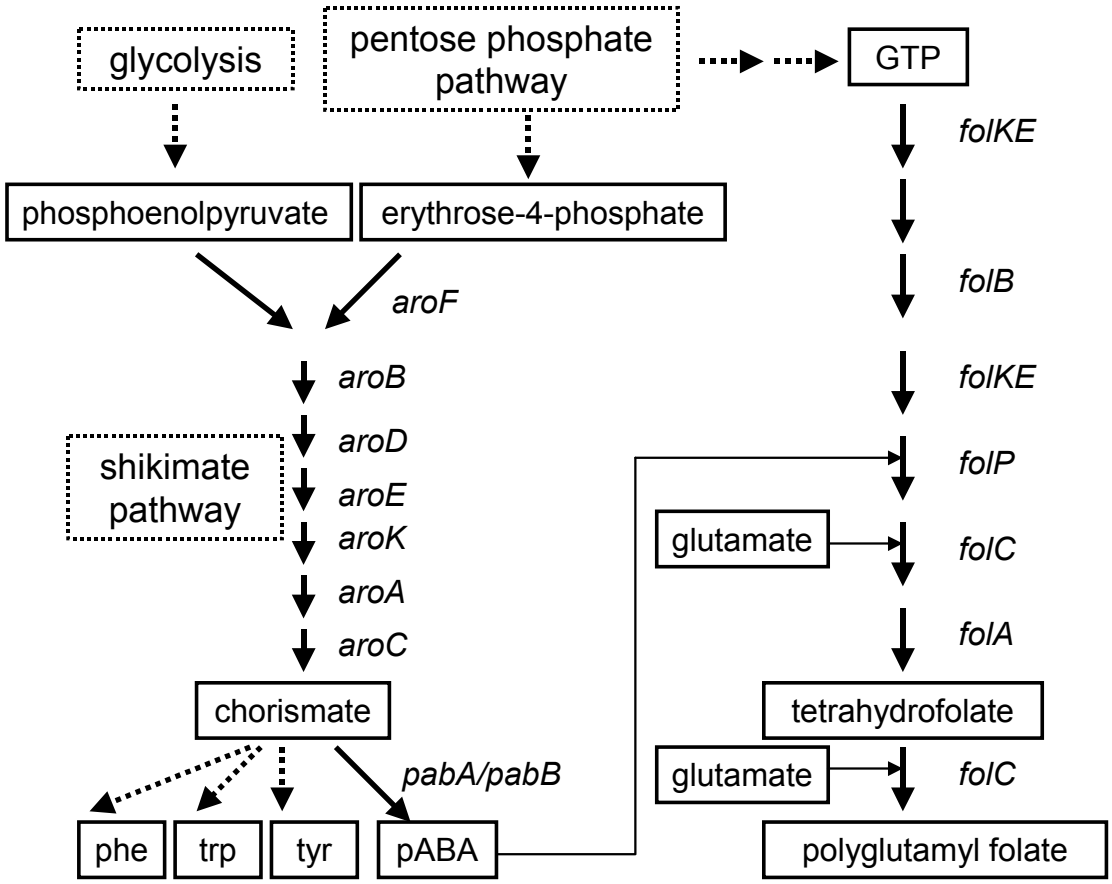


Figure 1: p-aminobenzoic acid and folate biosynthesis pathways.
In a condensation reaction, catalyzed by phospho-2-dehydro-3-deoxyheptonate aldolase (AroF, EC:2.5.1.54), erythrose-4-phosphate and phosphoenol-pyruvate, are converted to orthophosphate and phospho-2-dehydro-3-deoxyheptonate. Next, 3-dehydroquinate and orthophosphate are formed by the action of 3-dehydroquinate synthase. (Aro*B*, EC:4.2.3.4). Subsequent hydrolysis by 3-dehydroquinate dehydratase (AroD, EC:4.2.1.10) generates 3-dehydroshikimate that is reduced by shikimate 5-dehydrogenase (AroE, EC:1.1.1.25). Shikimate-3-phosphate is subsequently formed by the action of 3-phosphoshikimate kinase (AroK, EC:2.5.1.19). The next intermediate is 5-O-(1-carboxyvinyl)-3-phosphoshikimate that is synthesized by the activity of 3-phosphoshikimate 1-carboxyvinyltransferase. (AroA, EC:2.5.1.19). Chorismate is formed by the activity of chorismate synthase (AroC, EC:4.2.3.5) that requires FMN as a cofactor. Chorismate is the branch point for further biosynthesis of pABA and the aromatic amino acids. Finally, pABA is synthesized by the activity of para-aminobenzoate synthetase (PabA / PabB, EC:4.1.3.-) that consists of component I and II. (Genes coding for the enzymes involved are indicated). The folate biosynthesis pathway is described in detail by Sybesma *et al.*, 2003a.

Materials and methods

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *E. coli* was grown in TY medium (Rottlander and Trautner, 1970), and *L. lactis* was grown in M17 medium (Terzaghi and Sandine, 1975) or in chemically defined medium (Poolman, *et al.;* 1988, Otto *et al.,* 1983) containing 0.5% or 1% glucose respectively. *E. coli* cells were transformed by heat shock (Sambrook *et al.,* 1989) and *L. lactis* cells were transformed by electroporation as described before (de Vos *et al.,* 1989). Chromosomal DNA and plasmid DNA were isolated by using established procedures (Sambrook *et al. 1989*). *L. lactis* was plated onto GM17 agar after electrotransformation. Kanamycin and erythromycin were used at final concentrations of 50 and 200 μg/ml, respectively for *E. coli*. Erythromycin and chloroamphenicol were used at a final concentration of 2 μg/ml and 10 μg/L, respectively for *L. lactis*. 5-bromo-4-chloro-3-indoyl-galactopyrano-side (X-gal) was used at a final concentration of 80 μg/ml. The added concentrations of *p*-aminobenzoic acid ranged between 0 and 70 mM.

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
L. lactis strains		
NZ9000	*L. lactis* MG1363 *pepN:nisRK*	Kuipers *et al.*, 1998
E. coli strains		
EC1000	Cloning host: RepA$^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	Leenhouts *et al.,* 1996
Plasmids		
pORI280	EM^r, LacZ$^+$, ori$^+$ of pWV01, replicates only in strains providing *repA* in trans	Leenhouts *et al.,* 1996
pORI280-tyrA-aroA-aroK-tyr	pORI280 with *aroA* and *aroK* and flanking regions of *tyrA* and *pheA* inserted in multiple cloning site	This study
pORI280-tyrA-aroK-pheA	PORI280-tyrA-aroA-aroK-pheA containing a 1174 basepairs in frame deletion of *aroA*	This study
pORI280-tyrA-pheA	PORI280-tyrA-aroA-aroK-pheA containing a 1225 basepairs in frame deletion of *aroA* and a 310 basepairs in frame deletion of *aroK*	This study
PNZ7019	Cm^r, pNZ8161 derivative containing functional lactococcal *folB, folKE, folP, folQ, and folC* genes behind the constitutive *pepN* promoter	Wegkamp *et al.*, 2003
pNZ8048	Cm^r, Inducible expression vector carrying the *nisA* promoter	Kuipers *et al.*, 1998

Table 2: Oligonucleotides used for DNA amplification by PCR. Nucleotides represented in non-capitals represent modifications with regard to the mature gene.

Primer	Sequence 5' - 3'
PORIcntr-F	CGACCCGTGCTATAATTATACTAA
TyrA-F1	TGCTGGaTCcACCAAGAGTGAGATTGTCGAGC
PheA-R1	GCAGGTGGAAATTACTGAGTCgAaTTCGGC
AroA-F1	GCAATTGCTGCAgTTTTGGTTGAAAATGGTG
AroA-R1	GGGCGTTTAAAGGTTCCtGcAGATAAATCAATTTCA
TyrA-F2	GCAACGTTTGATTTAAAAGATGG
PheA-R2	GCAGCGGTGGCAAATTCTTTTGC
AroK-R1	TTTGACTGCaGATTTTGATACTTTATGGAAGCG

The plasmids used in this study are listed in Table 1. The pre-integration vector for inactivation of *aroK,* encoding 3-phosphoshikimate 1-carboxyvinyltransferase, was constructed by using pORI280 (Leenhouts *et al.,* 1996) carrying an amplified linear DNA fragment (3.1 kb) consisting of 789 basepairs of the 3'-end of *tyrA*, the complete *aroA* and *aroK* genes*,* and 385 basepairs of the 5'-end of *pheA.* This fragment was obtained by using primers tyrA-F1 and pheA-R1 (Table 2) that contained at the 5'-end a *Bam*HI site and an *Eco*RI site, respectively. After restriction of the resulting pORI280 derivative by using *Bam*HI and *Eco*RI, the two fragments were ligated by using ligase (Invitrogen, Paisley, UK). The new generated plasmid, pORI280-tyrA-aroA-aroK-pheA, was transformed to *E. coli* strain EC1000 and used to construct the final integration vector by PCR using pORI280-tyrA-aroA-aroK-pheA as a template and primers aroA-F1 and aroA-R1 (Table 2). Both primers were extended at the 5'-end with a *Pst*I site. The amplified DNA was digested with *Pst*I and ligated by using ligase, generating plasmid pORI280-tyrA-aroK-pheA that carries an in frame deletion of almost the entire *aroA* gene. The plasmid was transformed to *E. coli* and isolated for subsequent transformation to *L. lactis*. Transformants of *L. lactis* in which plasmids had integrated via single crossing-over event were grown overnight in GM17 with erythromycin and plated on GM17 agar containing erythromycin and X-gal. Integration of the plasmid was confirmed by the presence of blue colonies and by PCR performed directly on blue colony cell material. Next, positive blue colonies were grown in medium without antibiotics and diluted to a density of 1-10 cells/ml of the same medium, followed by growth for approximately 30-40 generations. Dilutions of the culture were spread onto agar plates containing X-gal. After 48 h of incubation at 30°C, white colonies were selected for further analysis. The presence or absence of *aroA* from the selected colonies was determined by using PCR directly on their cell material by using primers tyrA-F2 and pheA-R2 that are located on *tyrA* and *pheA,* respectively, but not

present on the integration vector (Table 2). A strain with the expected configuration, designated NZ9000ΔaroA, was stored for further experimentation.

The inactivation of both *aroA* and *aroK,* encoding 3-phosphoshikimate kinase, was achieved by PCR using pORI280-tyrA-aroA-aroK-pheA as a template and primers aroK-F1 and aroA-R1 (Table 2). Both primers were extended at the 5'-end with a *Pst*I site. The amplified DNA was digested with *Pst*I and ligated by using ligase, generating plasmid pORI280-tyrA-pheA, that had an in frame deletion of almost the entire *aroA* and *aroK* genes. Plasmid transformation and subsequent selection for *L. lactis* colonies lacking *aroA* and *aroK* on the genome were done as described above using the same primers tyrA-F2 and pheA-R2 for confirmation of the desired genotype of the mutant strain NZ9000ΔaroA-aroK. All PCR reactions were performed by using 1.0 U *Pfx* polymerase (Invitrogen) according to the manufacturers protocol.

Plasmid pNZ7019, containing the folate genes *folB*, *folKE*, *folP*, *folQ* and *folC*, under the control of the constitutive *pepN* promoter, was described previously (Wegkamp *et al.*, 2003).

Folate analysis. Intracellular folate levels were determined, including enzymatic deconjugation, by using a microbiological assay and by HPLC as described previously (Sybesma *et al.,* 2003bc). Extracelllar folate levels were determined as follows. From a fully grown culture, with an optical density at 600nm of approximately 2.5, cells were removed by centrifugation (10 min., 5000g), and the supernatant was diluted 1:1 with 0.1 M NaAc buffer, 1% ascorbic, pH 4.8. Hundred micro liter of this mixture was directly injected on the HPLC column. 5.10-methenyl tetrahydrofolate and 5-formyl polyglutamyl folate standards were purchased from Schircks (Jona, Switzerland) and prepared as described before (Sybesma *et al.,* 2003b).

Transcriptome analysis. The isolation of total RNA of *L. lactis* MG1363, subsequent DNase treatment of RNA samples and cDNA synthesis, followed by labeling of cDNA, hybridization to the DNA array, and data analysis, were done as described previously for *L. lactis* IL1403 (Kuipers *et al.*, 2002).

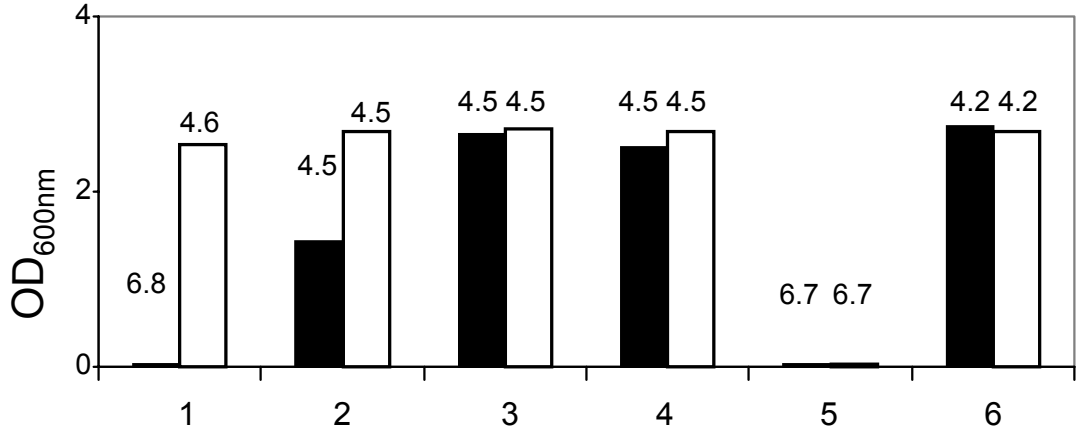


Figure 2:
Effect of simultaneous deletions of *aroA* and *aroK* on growth and pH in *L. lactis*. Optical density measured at 600 nm at the end of growth of *L. lactis* NZ9000ΔaroA-aroK (black bars) and NZ9000 (white bars) grown under different medium conditions. pH, measured at the end of growth, is indicated on top of bars. (The growth characteristics strain of NZ9000ΔaroA were identical as NZ9000). Legend: 1. CDM (without phe, trp, tyr, pABA, FA); 2. CDM with phe, tyr, trp; 3. CDM with phe, tyr, trp, pABA; 4. CDM with phe, tyr, trp, FA; 5. CDM with chorismate; 6. GM17.

Results

Inactivation of shikimate pathway. By using the replacement strategy described previously (Leenhouts *et al.*, 1996), two isogenic mutants were obtained from *L. lactis* NZ9000 that contained a defined deletion of the complete *aroA* or *aroA-aroK* genes. The growth and folate production of these strains were compared with the parental strain NZ9000 (Fig. 2). The growth characteristics and folate production in chemically defined medium of strain NZ9000•aroA were similar as in the *L. lactis* wild-type strain NZ9000. However, growth of the strain with the double deletion of two genes of the shikimate pathway was dependent on the presence of tyrosine, tryptophane, and phenylanaline. The addition of these aromatic amino acids to the medium enabled growth of NZ9000•aroA-aroK till a final optical density that was approximately half of that obtained by the wild-type strain NZ9000. Further addition of pABA or folic acid restored growth completely (Fig. 2). The presence of only one of the aromatic amino acids in the medium

did not enable growth. Addition of the pABA precursor chorismic acid to the chemically defined medium was growth inhibitory to mutant and wild-type strains (Fig. 2). Besides the optical density, also the acidification of the medium of the full grown strains was determined. Similar to the growth characteristics, it appeared that only in the presence of all three aromatic amino acids (with or without pABA, or folic acid) the pH was decreased to 4.5 (Fig. 2). The growth characteristics of the double mutant strain in the rich M17 medium were similar to those of the wild-type and NZ9000ΔaroA strain. Growth rate of all strains in chemically defined medium containing the aromatic amino acids and pABA was reduced approximately 50% compared to growth rate in M17. Without the presence of pABA the growth rate was even further reduced (Table 3).

The folate production in strain NZ9000ΔaroA was not influenced by inactivation of 3-phosphoshikimate 1-carboxyvinyltransferase. However, folate production in the double mutant strain NZ9000ΔaroA-aroK was dependent on the presence of all of the aromatic amino acids, and on the presence of pABA. The addition of excess of pABA, 1 μg/l, to the chemically defined medium, led to a similar folate production as observed in the wild-type strain. Without pABA, folate production remained below detection levels (< 1ng/ml) (Fig. 3).

Table 3: Growth rate of NZ9000ΔaroA-aroK and NZ9000 in different media. (The growth characteristics of strain NZ9000ΔaroA were similar as strain NZ9000). Concentrations are as described for CDM by Poolman, *et al.,* 1988, and Otto *et al.,* 1983.

Growth rate (h^{-1}) NZ9000	Growth rate (h^{-1}) NZ9000ΔaroA-aroK	Medium
0.27	0	CDM, without pABA, folic acid, phe, tyr, trp
0.27	0.04	CDM with phe, tyr, trp
0.29	0.22	CDM with phe, tyr, trp, and pABA
0.27	0.11	CDM with phe, tyr, trp, and folic acid
0	0	CDM with chorismic acid
0.45	0.45	M17

Folate overproduction. To study the effect of overproducing all enzymes involved in the linear part of the folate biosynthesis pathway, starting with GTP and finishing with tetrahydrofolate (Fig. 1), plasmid pNZ7019 was constructed carrying *folB*, *folKE*, *folP*, *folQ* and *folC* under the control of the constitutive *pepN* promoter. The functionality of this plasmid was proven by the introduction of pNZ7019 into the folate auxothrophic *Lactobacillus gasseri*, which transformed this folate-consuming bacterium into a folate-

producing one (Wegkamp *et al.,* 2003). The introduction of pNZ7019 into *L. lactis* NZ9000 grown on M17 resulted in a 3-fold increase of total folate levels compared to a control strain harboring pNZ8148, reaching levels of almost 300 μg/l. Remarkably, growth of this strain in CDM (containing 10 mg/L of pABA) resulted in an enormous increase of folate production capacity, reaching levels of approximately 5,000 μg/l. Growth experiments and analysis of M17 and CDM showed that folate production was critically dependent on pABA addition. The addition of increasing concentrations of pABA to M17 resulted in further increase of folate production levels (Fig. 4). Moreover, folate production levels per cell could be improved, reaching levels of more than 8 mg/L, by increasing the buffer capacity of the medium by the addition of 10 g/l K-PO_4 (Fig. 4). The growth rate of strain NZ9000 harboring pNZ7019 during growth in M17 was 25% lower than the wild-type strain.

HPLC analysis of the different folate derivatives that were produced intracellularly by *L. lactis* harboring pNZ7019 resulted in the identification of 5-formyl tetrahydrofolate and 5.10-methenyl tetrahydrofolate. The polyglutamyl folate tail length varied between 2,3, and 4 glutamyl residues (Fig. 5 left panel), and was clearly smaller than the average polyglutamyl folate tail length of approximately 4 glutamyl residues as reported previously

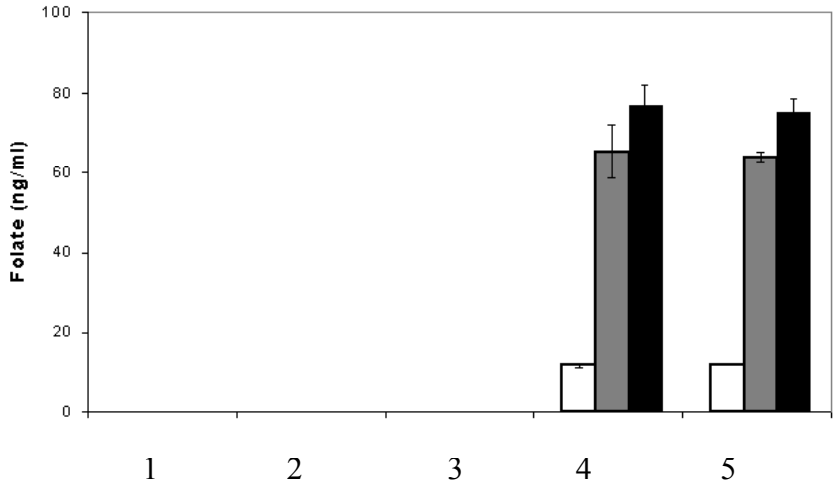
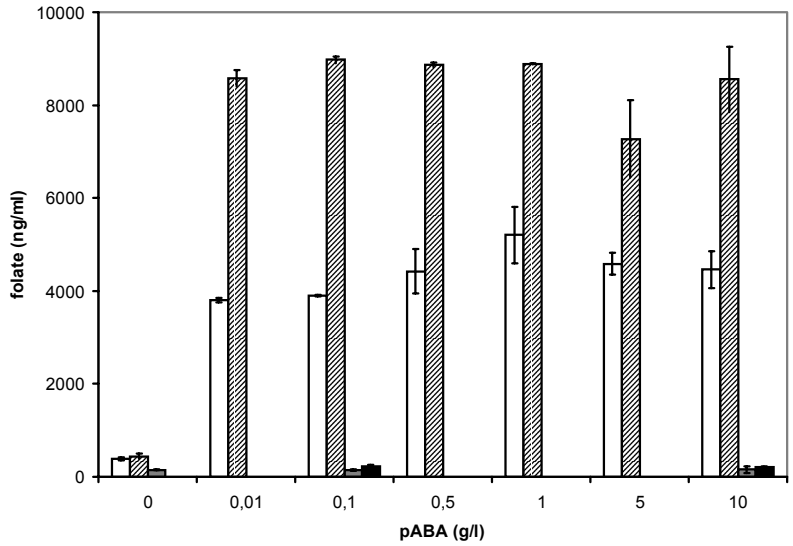


Figure 3:
Effect of simultaneous deletions of *aroA* and *aroK* on folate production levels.
Extracellular folate production levels (white bars), intracellular folate production levels (gray bars), and total folate production levels (black bars) in *L. lactis*ΔaroA-aroK strain grown in chemically defined medium, with phenylalanine (17 mM), tryptophane (2.4 mM) and tyrosine (1.3 mM) (1), with 5x phe, 5x trp, and tyr (2), 10x phe, 10x trp, and tyr (3), with phe, trp, tyr and pABA (7 mM) (4), idem with 10x pABA (5). Concentrations are as described for CDM by Poolman, *et al.,* 1988, and Otto *et al.,* 1983. Folate is determined by using a microbiological assay.

Figure 4:
Folate production in *L. lactis* harboring pNZ7019 or pNZ8048
Total folate production levels in strain NZ9000 harboring pNZ7019 (white bars) and NZ9000 harboring pNZ8148 (gray bars) in the presence of increasing concentrations of p-aminobenzoic acid and in the absence or presence of 10 g/l K-PO4 (pNZ7019, hatched bars; pNZ8148 black bars).



for *L. lactis* (Sybesma *et al.*, 2003c). The extracellular folate levels showed the presence of 5-formyl tetrahydrofolate and 5.10-methenyl tetrahydrofolate with predominantly a single glutamyl residue in the folate-overproducing strain and could not be detected in the wild-type strain (Fig. 5 right panel). The folate distribution over the inside and outside of the cell membrane was also changed by increasing the folate production capacity. In the folate-overproducing strain NZ9000 harboring pNZ7019 approximately half of the total folate produced was excreted from the cell, while in the wild-type strain approximately 5% of the produced folate was found to be present as extracellular polyglutamyl folate (Sybesma *et al.*, 2003a). During the chromatographic separation of intracellular folate, fractions of 10 ml were collected and analyzed for folate concentrations. In Fig. 6 it is shown that only in the fractions collected after approximately 60 – 80 minutes folate could be detected. Compared to the total amount of folate present in the injected sample, more than 80% of the folate could be recovered from these collected fractions in the form of 5-formyl tetrahydro(polyglutamyl)folate and 5.10-methenyl(polyglutamyl)tetrahydrofolate.

These results indicate that most of the folates produced by the *L. lactis* strain could be separated and detected by this HPLC method.

From previous experiments (Sybesma *et al.*, 2003a) it is known that the overexpression of *folKE* only, *folKE* and *folP* simultaneously, and *folKE* and *folC* simultaneously, in *L. lactis* grown on M17, resulted in approximately 3-fold increased folate production levels. In this work we have further shown that the addition of pABA to the medium does not lead to a further increase of folate production levels in these engineered strains (results not shown).

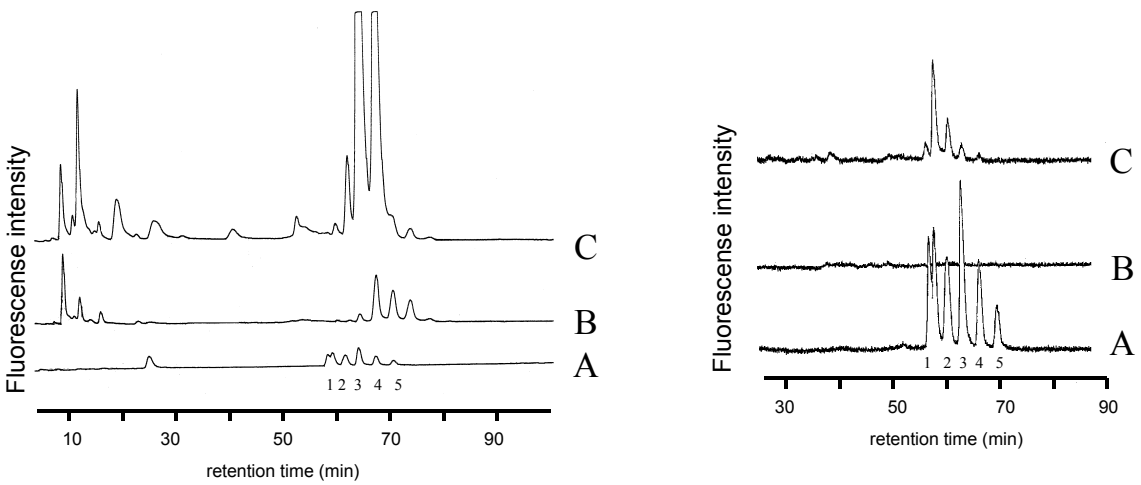


Figure 5:
Folate chromatograms, monitored by fluorescence, of cell-free extracts (left) or extracellular medium (right) of *L. lactis* NZ9000 harboring pNZ7019 (C), control strain with empty vector pNZ8148 (B), and 5-formyl tetrahydrofolate standards (A). Numbers correspond to the polyglutamyl tail length of 5-formyl tetrahydrofolate derivatives. For 5-formyl tetrahydromonoglutamyl folate, both the S and R diastereoisomeres can be distinguished.

Transcriptome analysis. Transcriptome analysis of strain NZ9000 harboring pNZ7019 or pNZ8148 (empty vector) monitored the expression of more than 1800 genes from the genome of *L. lactis* MG1363. Table 4 lists all genes that were more than 2-fold up regulated in strain NZ9000 harboring pNZ7019 compared to the control strain NZ9000 harboring pNZ8048. It is evident that the folate genes are among the genes that have the highest expression levels. Moreover, it can be seen that genes involved in the pentose phosphate pathway and shikimate pathway are upregulated as well, like two genes encoding for transporters. Several other genes that have increased expression are

involved with transcription and translation. A scattered plot of the total expression profile of the folate-overproducing strain relative to the control strain is given in Fig. 7. Remarkably, only a very limited number of genes were slightly down regulated (Table 4).

Table 4: Overview of more than 2-fold increased or decreased expression levels of genes from *L. lactis* NZ9000 harboring pNZ7019 compared to NZ9000 harboring pNZ8048. Experiment was performed in duplo, standard variation of expression levels varied between 1 and 20%

Group	Enzyme	Factor of overexpression of corresponding gene
Transcription/Translation	30S ribosomal protein S15	2.83
	30S ribosomal potein S11	3.01
	arginyl-tRNA synthase	3.23
	methyonyl-tRNA synthetase	2.66
	RNA polymerse B-chain	2.67
	DNA topoisimerase I	5.80
	Exonuclease VII	2.74
	50S ribosomal protein L18	2.68
Transport	Amino acid ABC transporter	2.78
	Multidrug transporter	2.72
	HPr kinase	2.91
Cell wall/polysaccharide	EPS 11 protein	3.01
	Endo-1,4-beta-xylanase D	2.88
Peptidases	Endopeptidase, PepO2	2.65
	Peptide-binding protein	3.82
	Endopeptidase PepO1	5.21
	Dipeptidase, PepV	3.87
Pentose-metabolism	6-P-gluconate dehydrogenase, Gnd	2.88
	P-pentomutase, DeoB	2.70
	3-P-shikimate 1-carboxy-vinyltransferase, AroA	2.99
Regulation	Transcriptional regulator	2.71
	ATP-dependent CLP protease, ClpE	2.65
Miscellaneous	Gamma-glutamyl phosphate reductase	2.65
	Quinone oxidoreductase	2.97
	ORF2 in nrdEF operon	2.70
	Acetyl-CoA carboxylase	2.69
	Alcohol-acetaldehyde dehydrogenase	2.76
	Cysteine synthase, CysK	3.03
Folate gene cluster	Polyglutamylfolate synthase, FolC	3.33
	Dihyroopteroate synthase, FolP	3.82
	Dihydroneopterin aldolase, FolB	4.61
	GTP cyclohydrolase, FolKE	100.43
	Hypothetical dihydroneopterin triphosphatase, FolQ	6.34
Unknown	Unknown (Phage BK5)	2.65
	Conserved Hypothetical Protein	2.73
	Hypothetical protein	2.79
	Hypothetical protein	3.63

Group	Enzyme	Factor of overexpression of corresponding gene
Miscellaneous down regulated genes	Competence protein, ComFC	0.68
	UDP-N-acetylmuramoylalanine-D glutamate ligase	0.68
	Mannitol-specific PTS system IIA component	0.68
	Phosphonate ABC transporter permease protein	0.68
	Cell division protein	0.67
	ORF2 of PepF2	0.67
	Glutamine synthetase	0.71
	Oligopeptide ABC-transporter	0.70
	Hypothetical Protein	0.66

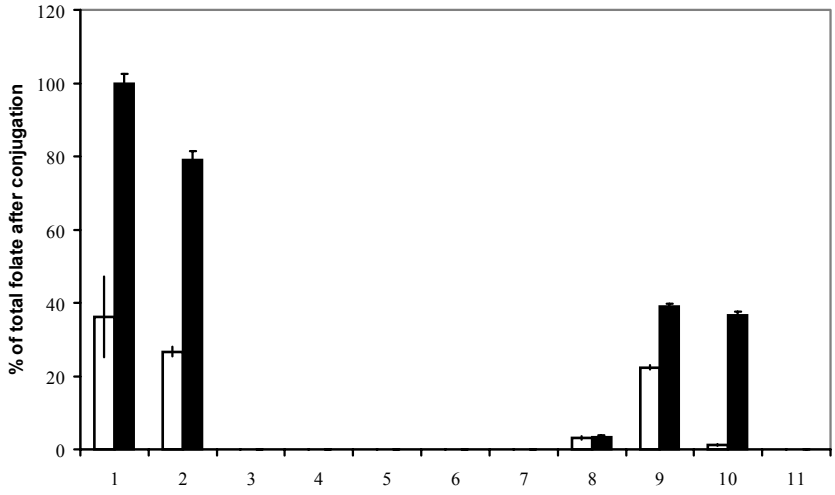


Figure 6:
Separation of folate in *L. lactis* harboring pNZ7019.
Folate analysis of fractions separated by HPLC of the cell-free extracts of *L. lactis* harboring pNZ7019 with (black bars) and without (white bars) enzymatic deconjugation. 1. Total folate in injected volume, 100 µl (100%). 2. Summed total folate determined in all fractions. 3-11. Folate determined in 9 fractions of equal volume, collected between 0 and 90 minutes.

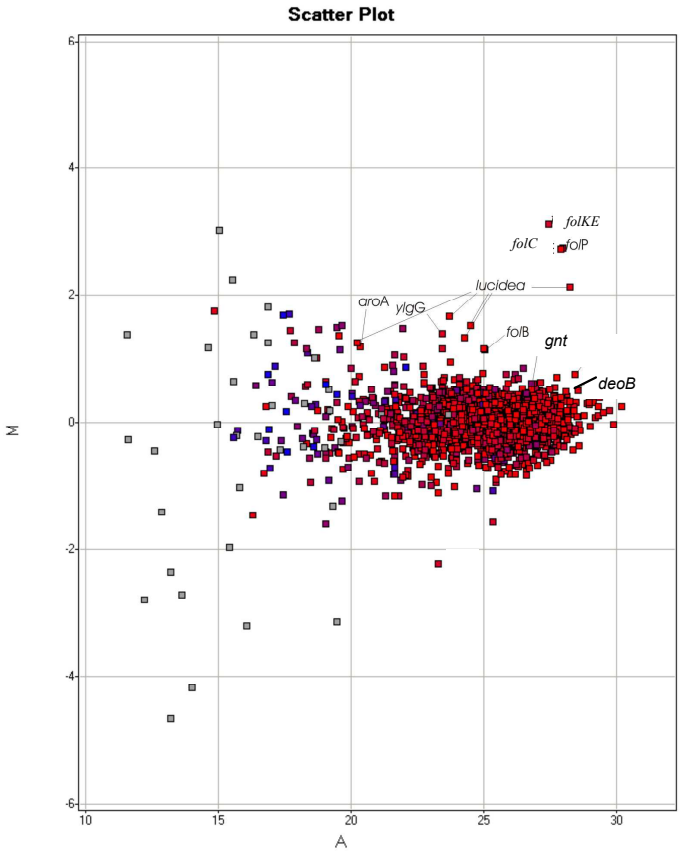


Figure 7:
Global gene expression analysis.
Scattered plot of changes in expression profile of NZ9000 harboring pNZ7019 compared to NZ9000 harboring pNZ8148. Circled dots correspond to the underlined genes/proteins from Table 4. Lucidea DNA and RNA is used as control.

Conclusions

More than 50 times increased folate production levels were obtained in growing *L. lactis* harboring pNZ7019 by the overproduction of the complete folate biosynthesis pathway consisting of FolB, the biprotein FolKE, FolP, FolQ, and the biprotein FolC, in excess of pABA. The medium experiments revealed that folate biosynthesis is controlled by the availability of pABA that is connected to the folate precursor 6-hydroxymethyl dihydropterin-pyrophosphate by the activity of dihydropteroate synthase. pABA can be synthesized in *L. lactis* from glucose via the glycolysis, pentose phosphate pathway and shikimate pathway. It can be concluded that the pABA biosynthesis capacity cannot compete with the increased demand for pABA upon the increased flux through the folate biosynthesis pathway.

It was investigated if pABA could be synthesized via alternative routes. By the inactivation of the shikimate pathway via the combined deletion of *aroA,* encoding 3-phosphoshikimate 1-carboxyvinyltransferase, and *aroK,* encoding 3-phosphoshikimate kinase, the aromatic amino acid biosynthesis route was blocked. As a consequence, cells could only grow in the presence of phenylalanine, tryptophane and tyrosine in the medium. Moreover, all these three amino acids were required to restore partial growth indicating that no sufficient conversion of one of the aromatic amino acids to the other was possible. The addition of the folate precursor pABA or folic acid itself was necessary to restore growth completely. Detection of folate production was only possible in the presence of the aromatic amino acids and pABA. The observation that growth without pABA was possible, although folate production remained below detection levels, leads to the conclusion that a minimal amount of pABA can be synthesized via conversion of one of the aromatic amino acids to chorismate and subsequently to pABA. However, the kinetic constants of the aromatic amino acids synthesizing enzymes seem to strongly favor the production of aromatic amino acids from chorismate in *L. lactis*. Therefore, it is unlikely that an alternative biosynthesis route for increased production of pABA and subsequent folate can occur via the inverted biosynthesis pathway of tyrosine, tryptophane, or phenylanaline to chorismate.

The inactivation of only *aroA* in *L. lactis* strain NZ9000ΔaroA does not lead to a different phenotype as the wild-type strain, as can be observed from the growth characteristics and the folate production. We conclude that probably one or more other carboxivinyl transferase enzymes with a low substrate specificity can take over the function of 3-phosphoshikimate 1-carboxyvinyltransferase in *L. lactis*. A search for homologous genes of *aroA* in the genome of *L. lactis* showed that the highest homology

was found with *murA,* encoding UDP-N-acetylglucosamine 1-carboxyvinyltransferase (45% homology on amino acid level). However, enzyme activity and substrate specificity experiments are necessary to confirm the potential of MurA to take over inactivated parts of the shikimate pathway.

The expected outcome of the transcriptome analysis was the visualization of the overexpression of all the genes that were introduced on the multi-copy plasmid pN7019. The overexpression of several genes involved in transcription and translation is probably a result of the constitutive overexpression of five folate synthesizing genes on a multi copy plasmid and the corresponding increased protein synthesis. The up regulation of two genes encoding for proteins involved in transport may be caused by increased uptake of pABA, increased efflux of folate, or both. Apart from this, the overexpression should be noted of the genes encoding 3-phosphoshikimate 1-carboxyvinyltransferase (involved in the shikimate pathway) and phosphopentomutase, and 6-phosphogluconate dehydrogenase (both involved in the pentose phosphate pathway). These results point to a pull strategy, induced by the increased flux through the linear part of the folate biosynthesis pathway, for the pABA biosynthesis, via the shikimate pathway, and for the pABA and GTP biosynthesis, via the pentose phosphate pathway. The transcriptome analysis could lead the way towards a metabolic engineering strategy aimed at increased production of pABA in the cell, facilitating folate overproduction.

High folate production in *L. lactis* depends on the presence of pABA in the medium. However, for application in food fermentations, it would be desirable if the demand for pABA in folate-overproducing *L. lactis* could be generated by the bacterium itself. Therefore, we propose that by metabolic engineering of the glycolysis and pentose phosphate pathway the flux to the shikimate pathway could be increased. Subsequently, the flux through the shikimate pathway could be increased by overexpression of *aroE*, *aroB*, *aroD, aroK,* aroA. Further progress is expected by inactivation of the aromatic amino acid synthesizing enzymes that use chorismate as a substrate. Thus, the inactivation of *trpG*, encoding anthranilate synthase component II (EC:4.1.3.27), and *ywiC*, probably encoding chorismate mutase (EC:5.4.99.5), could lead to accumulation of chorismate. The subsequent overexpression of *pabA* and *pabB* is expected to further increase the flux to pABA. Simultaneously, protein engineering could be applied to further inhibit feed back inhibition of aromatic amino acids on phospho-2-dehydro-3-deoxyheptonate aldolase and the shikimate pathway (Jossek *et al.,* 2001). Knowledge about the kinetic constants of enzymes involved in pABA and folate biosynthesis in combination with metabolic control analysis could be applied to determine precisely which genes should be over expressed or

which genes should be inactivated to increase the intracellular pABA concentrations in *L. lactis*.

The application of the described folate-overproducing strain in a food production process, could lead to the generation of fermented milk products that contain more than 5 mg per liter of folate. Consequently, the daily consumption of 50 ml of this product would already be enough in acquiring the daily recommended intake for folate. The generation of a dairy starter bacterium with more than 50-fold increased folate production has the potential to contribute to reducing the occurrence of natural folate deficiency within the population.

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Chapter 10

Identification of a New Open Reading Frame and Annotation of an Unknown *ylgG* Gene Present in the Folate Gene Cluster of *Lactococcus lactis*

Wilbert Sybesma[1], Arno Wegkamp[1], Marjo Starrenburg[1], Robert Kerkhoven[2], Bas Teusink[1,2], Igor Mierau[1], Willem M. de Vos[1], and Jeroen Hugenholtz[1]

1. Wageningen Centre for Food Sciences, Wageningen, The Netherlands
2. Centre for Molecular and Biomolecular Informatics, University of Nijmegen, The Netherlands

Abstract

The folate biosynthesis pathway in *L. lactis* is encoded by several genes that are all located on a poly-cistronic operon of approximately 8 kb. However, this region is not yet fully characterized. In this paper we will discuss the function of the unknown *ylgG* in folate biosynthesis by combination of advanced bioinformatics, targeted gene deletion, and folate production analysis. Furthermore, we report the presence of a new open reading frame within a 249 bp intergenic region present in the folate gene cluster.

Introduction

Recent publications about folate biosynthesis and increased production of folate by using metabolic engineering of lactic acid bacteria have opened avenues for the development of fermented food products with increased folate levels (Sybesma et al., 2003a,b). *L. lactis* subsp. *cremoris* strain MG1363 contains a folate gene cluster consisting of several genes that are involved in the folate biosynthesis and have the order: *folA, folB, foKE, folP* and *folC* (Sybesma et al., 2003b / GenBank accession number AY156932). Between *folP* and *folC* exists the *ylgG* gene, encoding a hypothetical protein, that is transcribed coordinately with *folB*, *folKE, folP*, and *folC* (Sybesma et al., 2001).

Within the folate gene cluster at least two transcriptional units have been identified (Sybesma *et al*., 2001, Leszczynska *et al.,* 1995). The first gene, *folA* is part of a bigger transcriptional unit that is assumed to contains also *clpX* and *ysxc*, which are not involved in folate biosynthesis, but probably involved in temperature stress responses (Skinner *et al.,* 2001). Between *folA* and *clpX,* there exists an unidentified intergenic region consisting of 249 basepairs. Although the region compassing *folA* and *clpX* has been sequenced by several groups (accession number X60681, AF236863, AE006347, AY156932), this intergenic region has not yet been annotated and its potential involvement in folate biosynthesis is presently unknown.

The longest transcriptional; unit is formed by *folB-folKE-folP-ylgG-folC*. While all these *fol* genes are involved in folate biosynthesis, as is concluded from homology studies with known enzymes and genetic experiments performed in *L. lactis*, the function of *ylgG* is unknown and its relation within the biosynthesis of folate remains to be established.

In this paper we will discuss the function of the unknown *ylgG* in folate biosynthesis by combination of advanced bioinformatics, targeted gene deletion, and folate production analysis. Furthermore, we report the presence of a new open reading frame within the intergenic region between *folA* and *clpX* and we describe the results of an *in silico* analysis.

Materials and methods

Bacterial Strains. The bacterial strains used in this study are listed in Table 1. *E. coli* was grown in TY medium (Rottlander and Trautner, 1970), and *L. lactis* was grown in M17 medium (Terzaghi and Sandine, 1975) containing 0.5% glucose. *E. coli* cells were transformed by heat shock (Sambrook *et al.,* 1989) and *L. lactis* cells were transformed by electroporation as described before (De Vos *et al.,* 1989). Chromosomal DNA and plasmid DNA were isolated by using established procedures (Sambrook *et al.,* 1989). *L. lactis* was plated on GM17 agar after electrotransformation. Kanamycin and erythromycin were used at final concentrations of 50 and 200 μg/ml respectively for *E. coli*. Erythromycin was used at a final concentration of 2 μg/ml for *L. lactis*, 5-bromo-4-chloro-3-indoyl-galactopyranoside (X-gal) was used at a final concentration of 80 ug/ml.

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
L. lactis strains		
NZ9000	*L. lactis* MG1363 *pepN:nisRK*	Kuipers *et al.,* 1997
E. coli strains		
EC1000	Cloning host: RepA$^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	Leenhouts *et al.,* 1996
Plasmids		
pORI280	EM^r, LacZ$^+$, ori$^+$ of pWV01, replicates only in strains providing *repA* in trans	Leenhouts *et al.,* 1996
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 basepairs in frame deletion of *ylgG*	This study

Double cross-over recombination. The plasmids used in this study are listed in Table 1. The pre-integration vector for inactivation of *ylgG* was constructed by using pORI280 (Leenhouts, *et al.,* 1996) and amplified linear DNA fragment (2287 nucleotides) consisting of 450 basepairs of the 3'-end of *folP*, the entire *ylgG* gene, and the entire *folC* gene, by using primers folP*Nco*I-F and folCXbaI-R as listed in Table 2. The forward primer was extended at the 5'-end with an *Nco*I site and the reverse primer was extended at the 5'-end with a *Xba*I site. After restriction of pORI280 and amplified DNA with *Xba*I and *Nco*I,

the two fragments were ligated by using ligase (Invitrogen, Paisley, UK). The new generated plasmid was pORI280-folP-ylgG-folC. The plasmid was transformed to *E. coli* and harvested for further processing. 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 ylgG*Pst*I-F and ylgG*Pst*I-R (Table 2). Both primers were extended at the 5'-end with a *Pst*I site. The amplified DNA was digested with *Pst*I and ligated by using ligase, generating plasmid pORI280-folP-folC. The plasmid was transformed to *E. coli* and isolated for subsequent transformation into *L. lactis*.

Table 2: Oligonucleotides used for DNA amplification by PCR. Nucleotides represented in non-capitals represent modifications with regard to the mature gene.

Primer	Sequence
FolP*Nco*l-F	GATATTATTAATGATATccATGGTTTTGACACAGCG
FolC*Xba*-R	TCTCTAGACTACTTTTCTTTTTTCAAAAATTCACG
YlgG*Pst*I-F	GAAGAAGctgCAGAAGAGTTTAAAAAGTATTATCG
YlgG*Pst*I-R	CCACTGCAGTTATTTGAGAAATCAAATCC
FolP-F	ATGAAAATCTTAGAACTTAATC
PORIcntr-F	CGACCCGTGCTATAATTATACTAA

Transformants of *L. lactis* in which plasmids had integrated via single cross-over were grown overnight in GM17 with erythromycin and plated on GM17 agar containing erythromycin and X-gal. The orientation of the single cross-over event was tested by performing PCR amplifications directly on cell material from a blue colony by using pORIcntr-F (sequence present downstream of multiple cloning site of pORI280) and folP-F (sequence present on *folP*, but not present on the integration vector) (Table 2). Subsequently, colonies 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 approximately 1-10 cells/ml. After growth for approximately 30-40 generations, dilutions of the culture were spread onto agar plates containing X-gal. After 48 h white colonies were selected for further analysis. The presence or absence of *ylgG* from the selected colonies was determined by using the PCR amplification directly on cell material from a white colony by using primers folP-F and folCXbaI-R (Table 2). All PCR reactions were performed by using 1.0 U *Pfx* polymerase (Invitrogen) according to the manufacturers protocol.

Folate analysis. Folate analysis was performed by using a microbiological assay, including enzymatic deconjugation, as described previously (Sybesma *et al.,* 2003b).

***In silico* analysis**. Homology searches with the amino acid sequences and nucleotide sequences of *ylgG* and *orfX* were done by using advanced bioinformatics tools, including BLASTX, PSI BLAST, ALIGN, STRING, COGNITOR and SOSUI, in combination with published (NCBI) and unpublished (ERGO) databases.
(See also http://www.ncbi.nlm.nih.gov/COG/xognitor.html, http://www.bork.embl-heidelberg.de/STRING/, http://www.integratedgenomics.com/genomic.html, http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html, http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Inactivation of *ylgG.* The gene *ylgG,* encoding a hypothetical protein, present in the folate gene cluster between *folP* and *folC*, was inactivated in strain NZ9000ΔylgG by double cross-over recombination. The mutant was created by modifications of a previous described method (Leenhouts *et al.,* 1996). One modification is the design of the integration vector pORI280-folP-folC via partial amplification of a pre-integration vector pORI280-folP-ylgG-folC. This procedure saves one restriction and ligation step compared to established procedures that involve two independent ligation and cloning steps of the up-stream region and down-stream region flanking the gene of interest. The other modification concerns a deliberate asymmetry in the inactivated gene. The flanking DNA regions around the inactivated *ylgG*, which were introduced in the integration vector, pORI280-folP-folC, were of different length. The plasmid contained 450 bp of *folP* up-stream of the *ylgG* gene and 1400 bp of *folC* down-stream of the *ylgG* gene. As a consequence, the single cross-over event is not expected to occur randomly anymore at either flanking region, but is favored via the long *folC*-flanking region. This is reflected in the outcome of the analysis of 20 blue colonies which revealed that 17 colonies were integrated over the long *folC* flank and only 3 colonies over the short *folP* flank. By subsequent cultivation of the blue colonies integrated over the *folP*-flanking region, the reversion to a wild-type strain genotype could only occur by double cross-over via the same *folP*-flanking region. However, the desired inactivation of the gene of interest could occur by cross-over via the long *folC* flank, which has a higher probability to occur. Subsequent analysis of 20 white colonies revealed that more than 12 colonies had the expected mutant genotype.

Growth of the newly generated *L. lactis* mutant strain NZ9000ΔylgG lacking 95% of the *ylgG* gene was identical to the wild-type strain. However, the folate analysis revealed that no extracellular folate was produced by the mutant strain and that intracellular folate levels were 3-4 times lower compared to the wild-type strain, that produced approximately

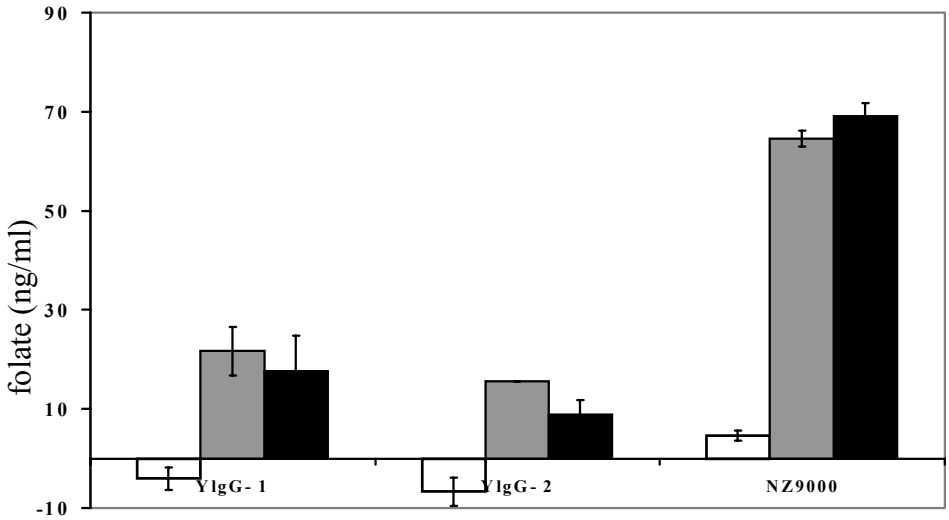

Figure 1:
Folate production levels in *L. lactis* strains.
Extracellular folate production levels (white bars), intracellular folate production levels (gray bars), and total folate production levels (black bars) in *L. lactis* NZ9000ΔylgG (duplo experiment, ylgG-1 and ylgG-2) and the control strain *L. lactis* NZ9000 grown in GM17. Folate is determined by using a microbiological assay.

70 ng/ml of folate (Fig. 1). The total folate levels in the wild-type strain were also 3-4 times higher than in the mutant strain, indicating that folate production capacity of strains with inactivated *ylgG* was strongly reduced.

YlgG has a molecular weight of 19.156 Da and an isoelectric point of 5.97. A direct homology search of the YlgG present in *L. lactis* MG1363 with other organisms only identifies the same protein in the related *L. lactis* IL1403. *In silico* comparison of the amino acid sequence and predicted structure of YlgG shows homology with nucleotide pyrophosphatases. Analysis with COGnitor reveals homology with COG0494, a group of NTP pyrophosphohydrolases, that also includes oxidative damage repair enzymes. Analysis with ERGO indicates homology with COG0127, a xanthosine triphosphatase. Further analysis reveals the presence of a nudix domain that can also be found in the *E. coli* protein MutT, a nucleoside-tripohosphate phosphatase belonging to protein group PF0023 (Bergman *et al.,* 1996).

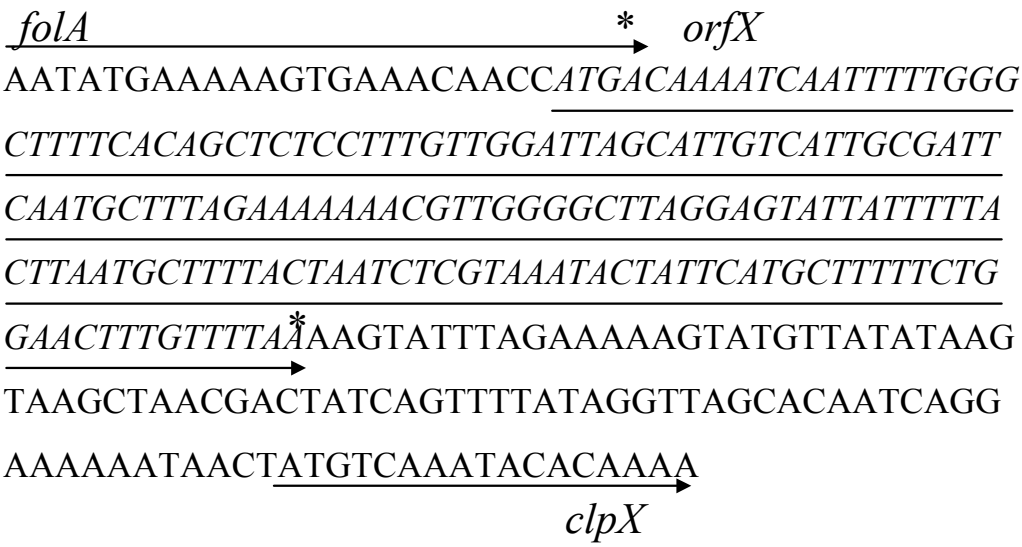

Figure 2:
Partial visualization of the sequenced folate gene cluster (GenBank accession number AY156932) with new identified open reading frame, *orfX* (nucleotides represented in italics). Asterisks indicate stop codons of *folA* and *orfX*. Arrows indicate the 3'-end of *folA*, *orfX*, and 5'-end of *clpX*.

Identification of *orfX*. Careful analysis of the intergenic region of 249 bp between *folA* and *clpX* revealed the presence of an open reading frame, designated *orfX*. The newly identified open reading frame starts with an ATG codon, overlapping the stop codon of *folA*, and encodes a protein consisting of 56 amino acid residues (Fig. 2). The alignment of *orfX* with the genome of other sequenced bacteria reveals strong identity with unknown proteins in *L. lactis* IL1403 (100%), *Streptococcus pyogenes* (82%), *Streptococcus agalactiae* (80%), *Streptococcus mutants* (78%), *Streptococcus pneumoniae* (76%) (Fig. 3). The homology search of the protein sequence versus translated nucleotide sequences did not match with a genome sequence of any other sequenced organism, besides the streptococcal genomes. Just as in the genome of *L. lactis*, the homologous genes in the different streptococcal strains are located between *folA* and *clpX*, *ysxc*. Classification of the gene and secondary structure prediction using SOSUI suggests that *orfX* encodes a membrane protein that has two trans-membrane helices. A similar outcome was obtained by analyzing *orfX* from the streptococcal strains.

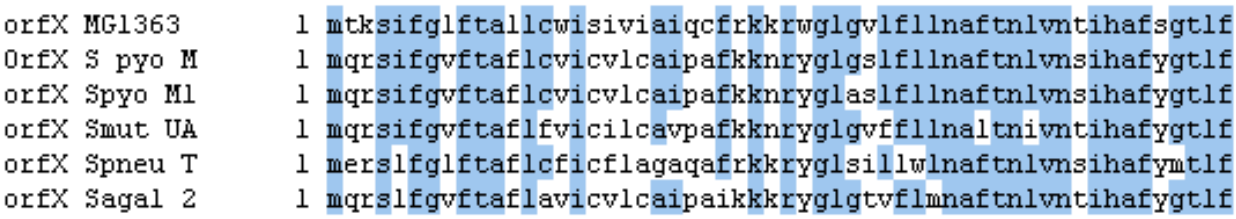

Figure 3:
Alignment of amino acids of the protein encoded by *orfX* of *L. lactis* MG1363 or IL1403 with the protein encoded by *orfX* identified in 5 streptococcal strains. Conserved amino acid residues are highlighted.

Conclusions

The folate biosynthesis pathway in *L. lactis* consists of 7 consecutive steps starting with the conversion of GTP into dihydroneopterin triphosphate and finishing with the synthesis of tetrahydrofolate (Sybesma *et al.* 2003b). All, but one of the enzymatic reactions have been annotated on the genome of *L. lactis*. The conversion of dihydroneopterin triphosphate into dihydroneopterin is supposed to be catalyzed by a yet undefined triphosphate phosphatase. The *in silico* analysis and homology studies suggest that YlgG may have a nucleoside triphosphatase activity. However, no information about substrate specificity can be derived from *in silico* data. The results in this work show that disruption of *ylgG* from the genome of *L. lactis* results in decreased folate production in the organism with almost a factor four. The design of the in frame deletion of *ylgG* renders polar effects of down-stream located genes that could influence the folate production levels highly unlikely. Moreover, the structure of dihidroneopterin triphosphate, the substrate of the not yet defined enzymatic step in the folate biosynthesis pathway, resembles the structure of nucleosides triphosphates. Hence, we propose to annotate the YlgG protein as a dihydroneopterin triphosphate phosphatase, encoded by *folQ*. The observation that some folate is still produced by the mutant strain suggests that the activity of FolQ can be partly taken over by other non-specific nucleotide triphosphatase or non specific pyrophospatases and phospho monoesterases. Enzymatic activity assays with purified

FolQ could confirm its function and provide further information about the precise nature and substrate specificity of this enzyme.

The newly discovered *orfX* that is part of the folate gene cluster of *L. lactis* and is predicted to encode a hypothetical membrane protein of 56 amino acid residues and two supposed trans-membrane helices, shows strong sequence and predicted structure homology, respectively, with several unknown genes and encoding proteins in streptococcal strains. OrfX seems to be a unique protein in *L. lactis* and related streptococcal strains. Moreover, the genetic organization of the flanking genes of *orfX*, *folA*, *clpX* and *ysxc*, was identical in all the identified strains. At the moment we do not have sufficient data to predict a physiological function for the encoding protein of *orfX*. It was shown by northern blot analysis of the up-stream located *folA* of *L. lactis* that *folA* was part of a bigger transcriptional unit (Leszczynska *et al.,* 1995). Therefore, it is assumed that *orfX* is transcribed together with *folA*, but data about the occurrence of translation are still missing. Currently we are setting up experiments involving the overexpression and inactivation of *orfX* in order to study the functionality of this unknown gene in the folate gene cluster of *L. lactis*.

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Chapter 11

Bioavailability of Folates from Engineered *Lactococcus lactis* Incorporated into Amino Acid Based Diets Determined by a Rat Bioassay

Wilbert Sybesma[1]*, Jean Guy LeBlanc[2]*, Marjo Starrenburg[1], Fernando Sesma[2], Graciela Savoy[2,3], Willem M. de Vos[1], and Jeroen Hugenholtz[1]

1. Wageningen Centre for Food Sciences, Wageningen, The Netherlands
2. Centro de Referencia para Lactobacillos, Tucumán, Argentina
3. Universidad Nacional de Tucumán, Argentina

*Contributed equally to the research

Abstract

In the present study the bioavailability of folate generated by genetically modified lactic acid bacteria producing folates with a different polyglutamyl tail length, is evaluated in a rat bioassay. After completion of an amino acid based folate depletion – repletion diet, folate concentrations are determined in various organs and blood components. The results indicate that, while there is no significant difference in delivering folate with 3 or 8 glutamyl residues, the folate-overproducing *L. lactis* strains can compensate very well for the folate depletion.

Introduction

Folate has an important function in human C1-metabolism. While folate is produced by different plants and by some micro-organisms, vegetables and dairy products are the main source of folate for humans. Although folate is omni-present in a normal human diet, folate deficiency still may occur (Konings *et al.,* 2001; O'Brien *et al.,* 2001). Folate shortage may increase the risks of developing diseases like neural tube defects, cardiovascular disease, anemia, and some forms of cancer (Herbert, 1967; Hines *et al.,* 1968; MRC Vitamin Study Research Group, 1991; Ma *et al.,* 1997; Czeizel *et al.,* 1992; Danesh *et al.,* 1998; Boushey *et al.,* 1995). Lactic acid bacteria, such as the industrial starter bacteria *Lactococcus lactis* and *Streptococcus thermophilus* have the ability to synthesize folate (Lin *et al.,* 2000; Sybesma *et al.,* 2003a). For this reason, some fermented dairy products, including yoghurt, are reported to contain even higher amounts of folate than unfermented milk. Recently, it has been shown that metabolic engineering can be used to increase folate levels in *L. lactis* (Sybesma *et al.,* 2003b).

Apart from the total amount of folate that is consumed, the form of folate also determines the effective uptake. Many food products are currently enriched with folic acid (pteroyl glutamic acid) that does not exist in nature. Too high intake of folic acid may mask the diagnosis of a vitamin B12 deficiency, but this is not expected to occur with food folates (Scott, 1999). However, it has been reported that the relative bioavailability of dietary folate is estimated to be only 50% compared with synthetic folic acid (Sauberlich *et al.,* 1987). Bioavailability is defined as the proportion of a nutrient ingested that becomes available to the body for metabolic processes or storage. The bioavailability of dietary folate may be hampered by the polyglutamate chain to which most of the natural folate is attached. This polyglutamate chain must be removed in mammals, except for the proximal glutamate moiety, by glutamate carboxypeptidase II that is present in the brush border of the small intestine. Subsequently, folate can be absorbed and transported as a monoglutamyl folate into the portal vein. In the past decades, several attempts have been made to assess the bioavailability of monoglutamyl folate compared to polyglutamyl folate. The available data suggest that the polyglutamate form is 60–80% bioavailable compared with the monoglutamate form (reviewed by Gregory, 1995).

Recently we have generated *L. lactis* strains that produce intracellularly folates with a short glutamyl tail length (average polyglutamyl tail length of 3) or with a long polyglutamyl tail length (average polyglutamyl tail length of 8), which generates an increased retention of folate in the cells (Sybesma *et al*., 2003c). In the present work we

describe a feeding trial with folate-depleted rats that have been fed these genetically modified bacteria with the aim to determine (i) whether folate-overproducing bacteria can be used as alternative sources of folate, and (ii) whether there is an effect of the folate glutamyl tail length on the bioavailability. The results indicate that while there is no significant difference in delivering folate with 3 or 8 glutamyl residues, the folate-overproducing *L. lactis* strains can compensate very well for the folate depletion. These results open the way for using these folate-overproducing bacteria in human trials.

Materials and methods

Bacterial strains, media and culture conditions. *L. lactis* strains NZ9000 harboring pNZ7010, pNZ7011, or pNZ7016 were used (Sybesma *et al.,* 2003c). Plasmid pNZ7016 contains the gene *folC*, encoding the bifunctional protein folate synthetase / polyglutamyl folate synthetase, under the control of the *nisA* promoter. The intracellular folates produced have an average polyglutamyl tail length of 8, as was reported previously (Sybesma *et al.,* 2003c). pNZ7011 contains the genes *folC* and *folKE*, encoding the bifunctional protein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophospho-kinase and GTP-cyclohydrolase I, under the control of the *nisA* promoter. The intracellular folate levels are higher than in the wild type strains and have an average polyglutamyl tail length of 3.3, as was reported previously (Sybesma *et al.,* 2003c). pNZ7010 contains *folKE* under the control of the *nisA* promoter and the intracellular folates have an average polyglutamyl tail length of 3 (Sybesma *et al.,* 2003c).

L. lactis strains were grown at 30°C in M17 medium (Merck, Darmstadt, Germany) (Terzaghi *et al.,* 1975) supplemented with 0.5% (w/v) glucose (GM17) and 10 μg/ml chloramphenicol. An overnight culture was diluted 1:100 in 5 l of the medium. Nisin induction was performed as described previously (Sybesma *et al.,* 2003b). Several of such batch fermentations were performed in order to obtain the desired amount of cells with folate intracellularly accumulated. Cells were harvested by centrifugation (30 min., 6000*g*) and washed with 0.1 M NaAc, 1% ascorbic acid, pH (4.75). Subsequently, cells were freeze dried in darkness during 48 h.

Folate analysis. Intracellular folate levels in a sample of the lyophilized cells were quantified by using a microbiological assay (Horne *et al.,* 1988, Sybesma 2003a) Folates were identified and the average polyglutamyl tail length was determined by HPLC as

described previously (Sybesma *et al.,* 2003a). Finally, cells were mixed with an amino acid-based diet to a final concentration of 250 µg per kg diet. Several samples were analyzed in duplo in two different laboratories (Table 3).

Table 1: FOLIC ACID DEFICIENT DIET COMPOSITION

FOLIC ACID DEFICIENT DIET			
Component	per	10.0	kg
GELATIN		800.0	g
DEXTROSE		4550.0	g
CORN OIL		200.0	g
HYDROGENATED VEGETABLE OIL		1850.0	g
SALT MIX BRIGGS		600.0	g
VITAMIN FREE CASEIN		2000.0	g
VDFM (OMIT FOLIC ACID)			
VITAMIN DIET FORTIFICATION MIXTURE	per	10.0	kg
DRY VITAMIN A ACETATE (500.000 U/GM)		396.0	mg
CALCIFEROL (GRAN.) 850.000 U/g		26.0	mg
ALPHA TOCOPHEROL POWDER 250 I.U./g		4.840	g
INOSITOL		1.10	g
CHOLINE CHLORIDE		16.72	g
MENADIONE		0.493	g
BIOTIN		5.0	mg
P AMINO BENZOIC ACID		1.1	g
ASCORBIC ACID		9.9	g
NIACIN		0.981	g
RIBOFLAVIN		220.0	mg
PYRIDOXINE HCL		220.0	mg
THIAMINE HCL		220.0	mg
CALCIUM PANTOTHENATE		0.660	g
FOLIC ACID		***	
B-12 TRITURATION (0.1%)		299.0	mg

Experimental design. The overall experimental protocol is summarized in Figure 1. One-hundred weanling Wistar rats (60 +/- 3g) were obtained from the closed-colony of Universidad Nacional de Tucumán, Argentina. Sixty-four rats were randomly selected for the study. Rats were individually housed in a room with a 12 h light cycle at 22ºC +/-2 and were allowed free access to food and water throughout the study. Normal Balanced Rodent Food (balanced/autoclaved Rodent Diet (Batistela, Buenos Aires, Argentina) and a Folic Acid Deficient Diet (ICN Biomedicals Inc. Irvine, CA, USA) containing 1% Succinylsulfathiazole (ICN) were used in this study. The composition of the Folic Acid Deficient Diet is detailed in Table 1.

The 64 rats were divided into three groups of 4, 8 and 56 rats of equal mean weights. The smallest group was fed Folic Acid Deficient Diet with 2 mg folic acid/kg diet and the second group was fed Normal Balanced Rodent Food throughout the study (control groups). The 56 remaining rats were fed Folic Acid Deficient Diet for 30 days (depletion period) followed by a 28 day repletion period where animals were fed Folic Acid Deficient Diet supplemented with different levels of folic acid (pteroyl glutamic acid, Sigma, Buenos Aires, Argentina, 125, 250 or 500 µg/kg diet) or with engineered lactic acid bacteria containing folates (250 µg folates/kg diet from *L. lactis* NZ9000 harboring pNZ7016, pNZ7011, or pNZ7010). After the depletion period, 4 rats were randomly selected and sacrificed. The 48 remaining rats were divided into six groups of 8 rats consisting of the experimental groups.

Blood and organ samples collection. At day 30 of the start of the trial, 2 rats from each of the 8 groups (2 control groups and 6 experimental groups) were randomly selected, placed into a homemade sampling chamber, and whole blood was collected from the tail and transferred into a tube without anticoagulant or used directly for hematological evaluation. Afterwards, animals were returned to their respective groups for the duration of the study. At the end of the trial, animals were anesthetized with an i.p. injection of 3.0 ml/kg animal weight ketamin (10%) – xylacin (2%) (40:60 vol/vol, Alfasan, Woerden, The Netherlands) and bled by cardiac puncture. Blood was transferred into tubes with anticoagulant (Heparin, Rivero, Buenos Aires, Argentina) and without anticoagulant.

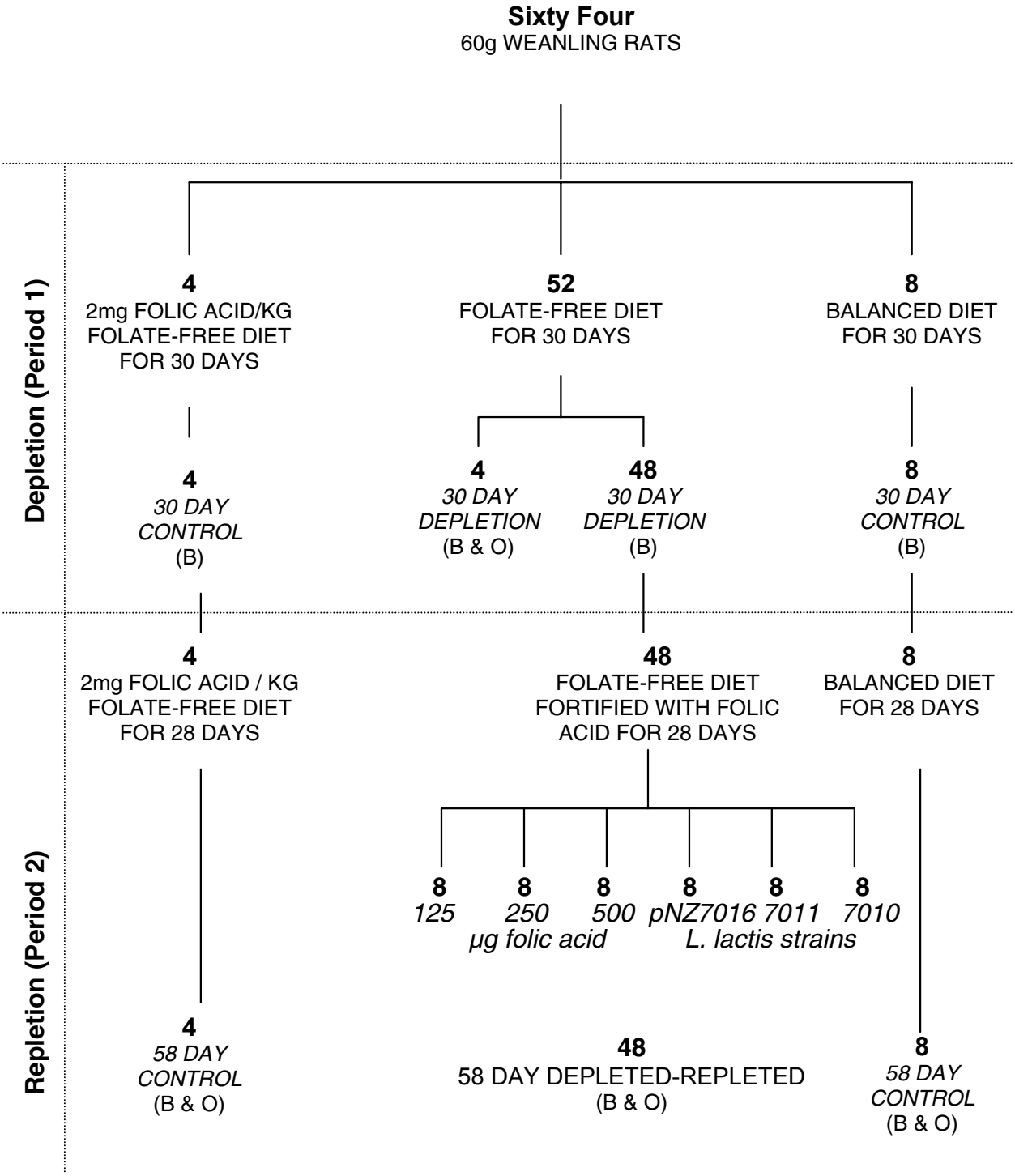

Figure 1. Folate depletion-repletion experimental protocol. Italicized text indicates the time since the trial began and folate depletion-repletion status of the various groups. The parenthesis indicate when samples of blood (B) or organs (O) were taken. Italicized numbers represent µg folic acid per kg of Folic Acid Deficient Diet or the different lactococcal strains (*folC, folKE* and *folC, folKE*) that were added to the same diet (250 µg/kg diet).

Table 3: Overview of folate concentrations determined in several tissues and blood collected from rats that were fed a depletion – repletion diet with well defined folic acid concentrations. (FA is folic acid, SD is standard deviation).

Reference	This study, WCFS		This study, CERELA		O'Leary *et al.*, 2002		O'Leary *et al.*, 2001		Walzem *et al.*, 1988		Walzem *et al.*, 1983		Clifford *et al.*, 1990a		Semchuk *et al.*, 1994		Abad *et al.*, 1987		Clifford *et al.*, 1990b		Clifford *et al.*, 1989	
	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD	FA	SD
Serum (ng/ml)																						
0	15.0	3 .0	13.6	3.6					20.0	1.0	15.0	5.0	0.9	0.2								
125µg	26.0	1.5	22.2	2.5									2.6	0.4					1.1	0.1	4.0	1.0
250µg	21.0	2.7	15.3	3.0															2.5	0.2	6.0	1.0
500µg	19.0	3.1	19.2	3.6									12.5	0.9					4.2	0.2	7.0	1.0
		13.							150													
>2mg	39.0	0	45.0	12.1					0	5.0	96.0	2.0	61.4	4.0							8.0	1.0
																					40.0	1.0
Plasma (ng/ml)															14.6	6.0	13.2	2.0				
0			12.2	5.1	4.7	0.1																
125µg	12.1	6.0	13.4	5.1																		
250µg	12.5	4.6	14.5	2.9			11.4	1.5									33	7.0				
500µg	14.1	3.3	12.8	3.1																		
>2mg	32.9	8.7	41.2	7.9	8.0	0.3									81	4.0	72	8.0				
RBC (ng/ml)																						
0	151	25	133	21					700	200	377	47	300	10	569	37						
125µg	154	31	169	28									300	20					1060	60		

250µg	166	28	220	54															1600	140		
500µg	327	168	350	54									420	30					2590	100		
>2mg	245	36	141	53					900	200	695	146	770	60	1190	91						
Liver (µg/g)																						
0	6.6	1.7	4.8	1.1	2.6	0.3			2.0	1.0	1.3	0.2	0.7	0.1	4.2	0.5	8.8	1.5			1.0	0.5
125µg	10.4	1.8	9.5	1.8									1.0	0.1					4.7	1.0	1.0	0.5
250µg	12.3	2.3	13.4	2.9			0.8	0.1											10.7	0.4	2.0	0.5
500µg	15.9	2.6	16.1	3.1									2.6	0.2			11.4	3.0	16.4	0.9	3.0	0.5
>2mg	21.9	6.5	19.8	8.1	4.3	0.3			12.0	4.0	25.3	2.0	8.2	0.4	8.3	1.0	14.1	0.5			6.0	0.5
Spleen (µg/g)																						
0	4.1	0.4	4.1	1.1					3.0	1.0			0.1	0.0								
125µg	5.1	0.5	4.9	2.1									0.1	0.0								
250µg	4.3	0.4	5.0	1.6																		
500µg	4.9	0.3	4.8	0.9									0.5	0.0								
>2mg	5.5	0.3	4.9	1.5					5.0	1.0			0.9	0.1								
Kidney (µg/g)																						
0	4.7	0.7	4.4	1.1	4.1	0.2							0.4	0.0								
125µg	4.8	1.9	5.2	1.8									0.5	0.0								
250µg	7.6	1.9	7.5	2.1			0.5	0.1														
500µg	10.1	2.5	11.6	1.7									1.3	0.1								
>2mg	8.5	2.6	8.1	2.8	6.5	0.3							3.2	0.1								

For serum samples, blood without anticoagulant was allowed to clot, serum was separated by centrifugation (1500*g,* 10 minutes) and diluted 1/10 with Assay Buffer consisting of 0.1 M K_2HP0_4 /KH_2PO_4 buffer containing 0.3% (w/v) ascorbic acid.

For preparation of whole-blood sample for erythrocytes folate analysis, an aliquot of blood containing anticoagulant (100 µl) was diluted in 9 volumes distilled water (900 µl) and incubated for 30 minutes at 37ºC to allow serum conjugase to convert folate polyglutamates released from the lysed erythrocytes to the assayable monoglutamate forms. These lysates were then further diluted (500 µl in 4.5 ml Assay Buffer).

For plasma samples, remaining blood containing anticoagulant was separated by centrifugation (1500*g* / 10 minutes) and diluted 1/10 with Assay Buffer. The diluted blood samples were further processed as described previously (Clifford *et al.,* 1989).

For organ samples, aliquots (0.5 g) of freshly excised organs (liver, spleen and kidneys) were added to 9 volumes (wt/v) of Assay Buffer, homogenized for 1 min and incubated at 37ºC for 24 h to allow serum conjugase to convert folate polyglutamates released from the lysed erythrocytes to the assayable monoglutamate forms. Samples were further processed as described previously (Clifford *et al.,* 1989). All samples were analyzed for folate content by using a microbiological assay as described above.

Results

In order to prepare for an alternative folate source we grew *L. lactis* harboring pNZ7010, pNZ7011, or pNZ7016 in large amounts. After harvesting and washing, approximately 15 g of cell pellets were lyophilized. The concentration of folate in lyophilized cells of the three different *L. lactis* strains was determined and cells were homogenously mixed with the amino acid based diet (250 µg lactococcal folate / kg diet). After a 30 days folate depletion phase the different composed diets were fed to three different groups of 8 rats as illustrated in Fig. 1. Folate analysis at the end of the repletion phase showed that the folate concentrations in the lyophilized lactococcal cells were not deteriorated (results not shown). HPLC analysis confirmed the expected polyglutamyl tail length and identified 5-formyltetrahydrofolate and 5.10-methenyltetrahydrofolate as the predominant folate derivatives produced by the lactococcal strains (Sybesma *et al.,* 2003c).

Fig. 2 shows the mean folate concentration in liver, kidney, and spleen, determined in rats that were fed a repletion diet during 28 days containing 125, 250, and 500 µg folic acid / kg diet (standards), or lyophilized *L. lactis* cells with a concentration of 250 µg

intracellular folate / kg diet. Fig 2. also shows the mean folate concentration in the mentioned organs after 30 days of a folate depletion phase, and of two control groups, that did not pass a depletion phase, but were fed the amino acid based diet supplemented with 2 mg folic acid / kg diet, or a Normal Balanced Rodent Food diet. Mean liver and kidney folate concentrations of rats fed repletion diets all showed an increase in tissue folate concentrations, compared to measurement right after the depletion phase. The mean liver and kidney folate concentrations of rats fed the Normal Balanced Rodent Food diet were highest, nearly 30 µg / g liver and 15 µg / g kidney, respectively, followed by the rats fed with 2 mg folic acid / kg diet. The rats fed with 250 µg folate / kg diet derived from the three different *L. lactis* cells, each producing folate with a different polyglutamyl tail length, did not show a significant difference in folate concentrations in the specific organs. However, the absolute values corresponded to the upper values of the standards groups fed with 125, 250, or 500 µg folic acid / kg diet.

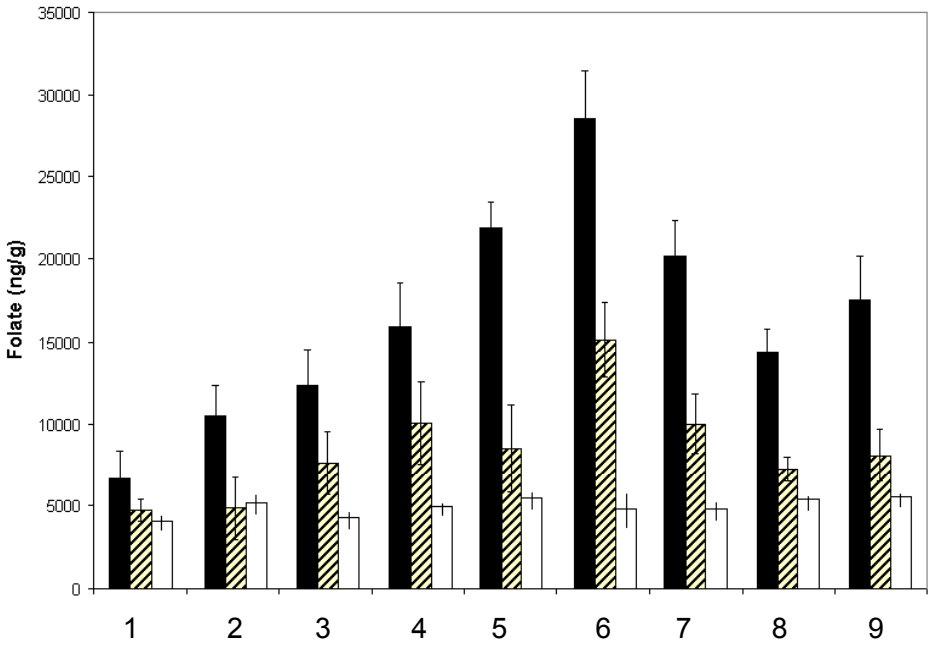

Figure 2: Folate concentrations determined in rat organs after folate depletion-repletion diet in a rat bioassay. Folate concentrations in liver (black bars), kidney (hatched bars), and spleen (white bars). Legend: 1. 30 days depletion; 2. 28 days repletion 125 µg FA / kg diet; 3. 28 days repletion 250 µg FA; 4. 28 days days repletion 500 µg FA; 5. no depletion, 58 days repletion 2 mg FA; 6. no depletion, Normal Balanced Rodent Food; 7. 28 days repletion, 250 µg lactococcal folate (*folC* overexpression); 8. 28 days repletion, 250 µg lactococcal folate (*folKE* overexpression); 9. 28 days repletion, 250 µg lactococcal folate (*folC* and *folKE* overexpression).

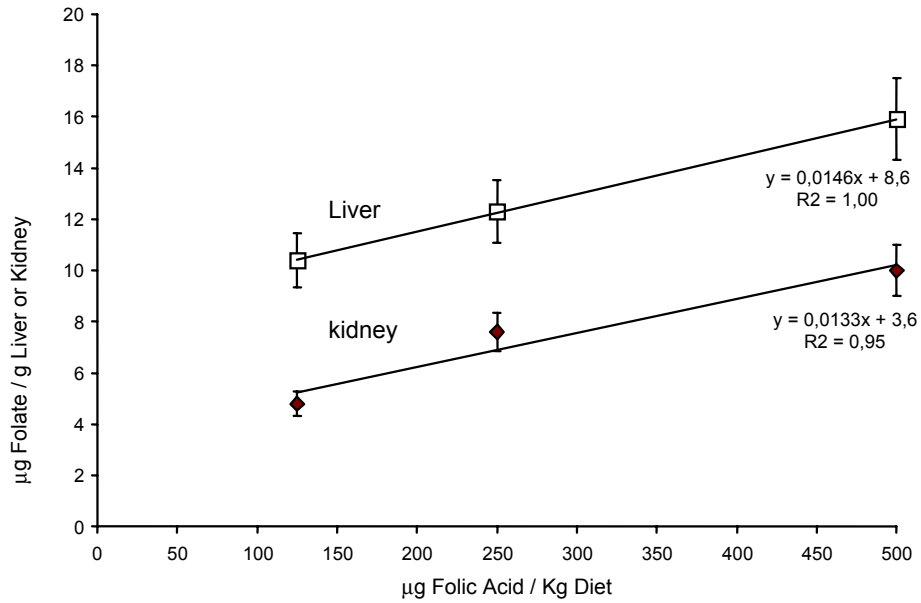

Figure 3: Folate concentrations in liver and kidney in folate depleted rats that were repleted with an amino acid based diet supplemented different concentrations of folic acid.

Fig. 3 shows the relation between the amount of folate that was found in the liver and kidney, versus the amount of folic acid that was added to the three standard diets. The linear relationship between the level of folic acid in the standard diets and the mean liver and kidney folate concentration can be described by the equations $y = 0.0146x + 8.62$ ($R^2 = 1$) and $y = 0.0132x + 3.64$ ($R^2 = 0.95$), respectively, where y is the expected concentration of micrograms of folate per g organ, and x is the concentration of micrograms of folic acid per kg diet. Based on this relation, the folate concentration in the diets with the lactococcal cells can be estimated (Table 2). On average, the response by liver was 1.6 times as high as the response by kidney. Mean folate concentrations in spleen, serum, plasma, and erythrocytes, of rats fed 125, 250, or 500 µg folic acid / kg diet were not different within the same sample group (Table 3). Therefore, corresponding regression equations were not calculated and no further interpretations could be made. Nevertheless, besides liver and kidney, also plasma and serum showed an increase of folate levels after the completion of the repletion phase. Table 3 also summarizes the folate concentrations determined in various tissues or blood by different groups after feeding rats with various concentrations of folic acid per kg diet. Folate analysis including

Table 2: Folate levels added to folate free amino acid based diet calculated by the microbiological assay or by liver folate or kidney folate of rats repleted with amino acid based diet supplemented with *L. lactis* strains and standardized with folic acid, which was similarly incorporated into the same diet. (Standard deviation is represented in percentages).

Diet supplementation	Added amount of folic acid / folate in µg based on microbiological assay	Added amount of folic acid / folate in µg based on rat assay (liver)	Added amount of folic acid / folate in µg based on rat assay (kidney)
folic acid	125	125 (17%)	125 (40%)
folic acid	250	250 (18%)	250 (25%)
folic acid	500	550 (16%)	500 (25%)
L. lactis- pNZ7016	250	793 (11%)	480 (18%)
L. lactis-pNZ7010	250	394 (10%)	269 (10%)
L. lactis-pNZ7011	250	610 (15%)	337 (20%)

post enzymatic deconjugation was repeated on 20 samples. The results confirmed that no polyglutamyl folates were present anymore in the sampled tissues and different blood components (data not shown).

The growth responses of all rats were determined for a period of 58 days (Fig.4). The results do not show a correlation between growth rates or final growth weight of the rats fed 125, 250, or 500 µg folic acid / kg diet. Growth rates were similar and final growth weight varied between 250 and 300 g. Moreover, the growth characteristics of the other groups, including the rats that were fed the Normal Balanced Rodent Food, were not different from the standard groups. The average amount of diet mixed with folic acid or with lactococcal cells that was consumed during the repletion phase was identical for all the rats in the different groups.

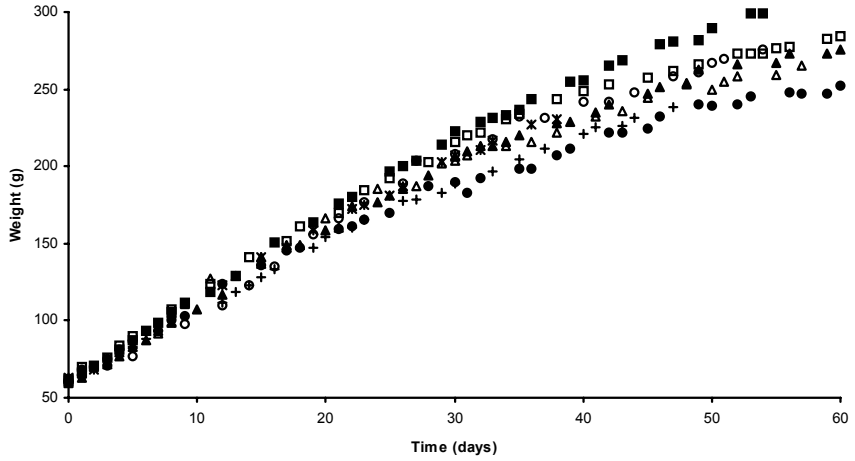

Figure 4: Growth response curves of different groups of rats during a folate depletion-repletion diet.
Legend: ■ 28 days repletion 125 µg FA / kg diet; ▲ 28 days repletion 250 µg FA; ● 28 days repletion 500 µg FA; □ 28 days repletion, 250 µg lactococcal folate (*folC* overexpression); ∆ 28 days repletion, 250 µg lactococcal folate (*folC* and *folKE* overexpression); ○ 28 days repletion, 250 µg lactococcal folate (*folKE* overexpression);
+ no depletion, Normal Balanced Rodent Food; * no depletion, 58 days repletion 2 mg FA.

Conclusions

The present study was conducted to assess in a rat bioassay the response of folate produced by genetically modified lactic acid bacteria producing folates with a different polyglutamyl tail length. The set up of the rat bioassay was described previously (Clifford *et al.,* 1989) and was based upon a folate depletion – repletion diet including three standard groups fed with 125, 250 and 500 µg folic acid / per kg diet during the repletion

phase. Although clear effects of the repletion diets were found in all samples, linear responses were found in kidney and liver. The analysis of the groups that were fed the different lactococcal cells also gave a clear response in all the samples tested, however, the values can be only assessed well in liver and kidney. It can be concluded that no significant difference is found between the cells containing polyglutamyl folate with an average tail length of 8, 3, or 3.3, even though the absolute values suggest a slight increase in the accumulation of folates in the organs of the rats fed with the long

polyglutamyl folates. In general it may be concluded that folate produced by engineered lactic acid bacteria gives a clear response in the folate concentrations in various organs and blood of rats after a depletion- repletion diet. The rat carboxypeptidase II enzyme that is required for transforming polyglutamyl folate into monoglutamyl folate, which can subsequently be adsorbed by the blood, obviously does not limit the folate absorption of polyglutamyl folates with different glutamyl tail length. Alternatively, the affinity of this enzyme for long polyglutamyl folates might even be higher than for short polyglutamyl folates. It is remarkable that the absolute folate levels determined in the organs seem to indicate that more than earlier determined 250 µg folate from lyophilized cells was added to each kg diet (Table 2). An explanation may be that the microbiological assay that was used to determine the folate levels in the different bacterial cells, underestimates the natural folates compared to folic acid (pteroyl glutamic acid) that is traditionally used as a standard in this assay. Indeed we have recently found a 46% decreased response for 5-formyltetrahydrofolate using folic acid as a standard in the microbiological (Starrenburg, unpublished results). Alternatively, the bioavailability of the natural folates is higher than that of folic acid, but this is not supported by previous studies (Sauberlich *et al.,* 1987, Cuskelly *et al.,* 1996, Brouwer *et al.,* 1999).

The folate concentrations determined in various tissues or blood of the rats that were fed with various concentrations of folic acid per kg diet, were analyzed in duplicate by two different laboratories (Table 3). The results are almost identical for all the samples tested. Several studies report a wide range of folate concentrations found in rat tissues after performance of similar rat bio assays (Table 3). Overall, our values are consistent with what has been reported previously (see references in Table 3), except for erythrocytes. The low folate levels found in the erythrocytes may assume an incomplete repletion phase in time and / or in concentrations of folate added to the diet. However, this is not likely because of the absence of such observations in similarly set up folate rat bioassays in the past. Another point of interest is related to the absence in our study of a big response between the serum and plasma values determined in the depleted group and in the control group, fed with 2 mg folic acid / kg diet. In many studies (see references in Table 3) a large difference can be seen before and after repletion. It might be possible that the used amount of 2 mg folic acid / kg diet is not sufficient for a maximum response, because in most diets, the folic acid content is quite higher.

Furthermore, it can be concluded that spleen is not an appropriate organ for detection of intracellularly accumulated folate as a response to the amount of folic acid provided by the diet. The absence of a linear response in plasma, serum, and erythrocytes

may be due to the low concentrations of folate in these samples, compared to folate concentrations in organs, in combination with the sampling method that was used in this study and in earlier work. Recently, we have determined that heating of samples with low folate concentrations partially degrades the folates (Starrenburg, unpublished results). Therefore, for future rat bioassays we recommend to omit extensive sample preparation using autoclavation. Contrarily to earlier work (Clifford *et al.,* 1990b), growth was not a good parameter to assess folate bioavailability.

The outcome of our studies also reveals that *L. lactis* can be used as delivery vehicle for folate. In contrast to monoglutamyl folate, polyglutamyl folates cannot be transported across the bacterial cell membrane. Hence, the release of intracellular polyglutamyl folate depends on the disruption of the cells during passage through the gastro-intestinal tract. The clear responses of lactococcal cells added to a folate free diet on the folate levels in organs and blood indicate that these cells lyse after consumption and that the bacterial folate becomes available for absorption in the gastro-intestinal tract of the rat.

This study has provided the first animal trial with food containing living bacteria that were engineered in order to increase the intracellular accumulation of folate, or to change the average polyglutamyl tail length compared to a wild-type lactococcal strain. These results pave the way for analyzing the effect of these folate-overproducing lactic acid bacteria in human trials.

Acknowledgements

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Chapter 12

Concluding Remarks and Perspectives - Summary

Wilbert Sybesma

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

This thesis describes the metabolic engineering (ME) of folate production in lactic acid bacteria with specific attention for *Lactococcus lactis*. The topic was chosen for three main reasons; 1. to apply the ME strategy to biosynthetic pathways, 2. to analyze the effects of this ME strategy on the producing lactic acid bacteria and 3. to achieve higher levels of relevant B-vitamins in foods through fermentation. All goals were reached successfully and, in addition, valuable knowledge and methodologies were developed in the area of folate detection, folate metabolism and folate bioavailability.

Concluding Remarks

Folate. Folate, vitamin B11, can be synthesized in the dairy starter bacterium *Lactococcus lactis*. Folate has an important function in the physiology of the consumer and is involved in the synthesis of DNA, RNA and several amino acids. Although folate is omni-present in a normal human diet, folate deficiency may still occur. Shortage of folate might increase the risks on diseases like neural tube defects, cardiovascular disease, anemia, and some forms of cancer. An increase in the amount of folate in fermented foods by using folate-overproducing starter bacteria may contribute to increased dietary intake of folate. (Chapter 1).

Higher production by optimal cultivation. Just as *Lactococcus lactis,* more lactic acid bacteria*,* are able to synthesize folate. This potential can be explored to develop starter cultures that have increased folate production capacities. The optimization of the growth conditions of the fermenting bacteria, for instance by performing pH-controlled fermentations in excess of glucose, could lead to increased biomass production and, consequently, increased folate production levels. Moreover, it was reported that the pH optima of the enzymes involved in folate biosynthesis in a large group of different organisms were all above 7.5 (Chapter 3). Therefore, the maintenance of a more alkalic pH during fermentation that may result in an increased intracellular pH, is expected to improve the specific enzymatic activity for folate biosynthesis, leading to increased folate production per cell. Moreover, it has been observed that a decreased growth rate also increased the folate production capacity per cell. This may be explained by the decreased acidification rate of environment that results in a longer exposure of the cells to a more optimal pH for folate biosynthesis. (Chapter 3).

Higher production by altered expression of individual genes. The recent advances in genome sequencing have led to the annotation of the genome of *L. lactis* subsp. *lactis* IL1403 (Bolotin *et al.,* 2001), that is closely related to the genetic and industrial model strain used in this work, *L. lactis* subsp. *cremoris* MG1363. In *L. lactis* IL1403 a folate gene cluster was found containing all the genes required for folate biosynthesis. The high degree of homology between the two strains was used to set up a long range polymerase chain reaction to confirm the presence of a folate gene cluster in *L. lactis* MG1363. The identification of all the genes involved in the biosynthesis of folate formed the basis of the ME approaches described in Chapters 4, 5, 7, 8 and 9, which all follow a strategy to control and increase the folate distribution and production levels in *L. lactis*. The ME experiments were made possible by using previous developed genetic tools and techniques, like transformation of high copy plasmids and controlled gene expression systems (de Ruyter *et al.,* 1996).

The overexpression of *folKE* in *L. lactis*, encoding the bifunctional fusion protein 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I, was found to increase the extracellular folate production almost ten times, while the total folate production increased almost three times. The controlled overexpression of both *folKE* and *folC,* encoding polyglutamyl folate synthetase, increased the retention of more folate in the cell (Sybesma *et al.,* WO02097063). The cloning and overexpression of *folA,* encoding dihydrofolate reductase, decreased the folate production two times, suggesting a feedback inhibition of reduced folates on folate biosynthesis. In accordance to this, the controlled production of antisense RNA of *folA* resulted in decreased activity of the corresponding dihydrofolate reductase, and slightly increased folate production. Recently it was shown that the overexpression in *L. lactis* of a regulating gene in purine metabolism, *purR*, resulted also in a two-fold decrease in folate production levels (Sybesma and Starrenburg, unpublished results). In line with this observation is the outcome of transcriptome analysis of a *purR* mutant *Streptococcus pneumoniae* strain, that showed more than two fold up regulation of genes involved in folate biosynthesis (Ng *et al.,* 2003). (Chapter 4 and 5).

Higher production by overexpressing the gene cluster. Many of ME experiments have been realized in *L. lactis* by using the Nisin Induced and Controlled Expression System (NICE) that allows analyzing the dynamic cellular response in a range of expression conditions (De Ruyter *et al.,* 1996). However, the constitutive expression of genes can be better controlled in industrial settings and hence we studied the effects of

constitutive overexpression of genes involved in folate biosynthesis. The overexpression *in L. lactis* of the complete folate gene cluster, *folB, folKE, folP, folQ*, and *folC* (*folA* was not inserted in this construct) under the control of the constitutive *pepN* promoter grown in the presence of p-aminobenzoic acid (pABA), has resulted in more than 50 times increased folate production levels, reaching levels above 8 mg folate per liter culture medium. In similar experiments, it was shown that the folate production of this strain could be further improved in the presence of excess glutamate, a substrate for folate synthetase as well as polyglutamyl folate synthetase, encoded by *folC*, reaching levels of more than 10 mg folate per liter culture medium (Wegkamp and Starrenburg*,* unpublished results). (Chapter 9).

Crucial role of p-aminobenzoic acid. The folate precursor pABA has shown to be a determining factor in folate production. In the absence of pABA, a two fold decrease in folate production levels was observed. Moreover, the 50-fold increased folate production capacity was only possible in the presence of excess of pABA. In *L. lactis* pABA is synthesized via the same biosynthetic pathway as the aromatic amino acids and involves glycolysis, the pentose phosphate and shikimate pathways. Inactivation of the shikimate pathway resulted in requirement of phenylalanine, tryptophane, and tyrosine. Folate production in this strain remained below detection limits. This indicates, that pABA cannot be synthesized alternatively, via the inverted amino acid biosynthesis route. For application in food industry it would be desirable if pABA in folate-overproducing *L. lactis* could be generated by the bacterium itself. (Chapter 3 and 9).

Functional genomics (global networks). The outcome of transcriptome analysis may enhance successful ME approaches aimed at the generation of strains with further increased folate or folate precursor production levels. The transcriptome data of a hyper folate producing strain suggest that overexpression of the pentose phosphate pathway and the shikimate pathway may lead to increased pABA production capacity. Moreover, the overexpression of genes encoding transporters in cells with increased pABA uptake and folate efflux, may lead to identification of folate and or pABA transport mechanisms. The potential identification of regulators may enhance further ME strategies to control folate production. (Chapter 9).

Control of polyglutamyl folate tail length. An important task in folate physiology and biosynthesis is fulfilled by the biprotein folate synthetase / polyglutamyl folate synthetase, encoded by *folC*, This enzyme is responsible for the production of

monoglutamyl folate from the precursor dihydropteroate, but also for elongation of the glutamyl tail of folate during the production of polyglutamyl folate. The polyglutamyl folates are the functional cofactor of folate in the cell and guarantee the intracellular retention of folate. The folate derivative produced in *L. lactis* and the determination of the polyglutamyl tail length was possible after the development of a HPLC analysis system. In *L. lactis* the predominantly present polyglutamyl folates have 4,5, and 6 glutamate residues. Moreover, more than 90% of the total folate pool is accumulated in the cell. It was shown that by increasing the flux through the folate biosynthesis pathway by overexpression of *folKE*, the average polyglutamyl tail length was reduced and the efflux of folate into the environment was enhanced. The overexpression of the *folC* resulted in total accumulation of polyglutamyl folate with a polyglutamyl tail length ranging between 5 and 12 glutamate residues. A complete reduction of the polyglutamyl tail of folate and a complete excretion of folate from the cell into the environment was established by the expression of the genes coding for γ-glutamyl hydrolase from rat or human origin in *L. lactis*. The control of the folate polyglutamyl tail length could be applied in food industry. The accumulation of intracellularly stored folate, achieved by the elongation of the polyglutamyl tail, could be used to increase the delivery doses of folate by lactic acid bacteria or probiotics. During the growth of these bacteria, all folate is accumulated in the cell and no folate is lost into the environment. Concentration of the bacteria in the final end product will result in the highest delivery of natural folate. On the other hand, the production of monoglutamyl folate may enhance the folate levels in fermented foods, when the fermenting bacteria are removed from the end product. (Chapters 4 and 5).

Changing bioavailability. Another important feature of monoglutamyl folate is related to folate bioavailability. Polyglutamyl folate is predominantly present in the medium However, folate is absorbed in the human intestine as monoglutamyl folate after the conversion of poly- to monoglutamyl folate by intestinal γ-glutamyl hydrolase. This enzymatic activity may be reduced by some components in the diet, and therefore the bioavailability of monoglutamyl folate may be higher. The use of an engineered *L. lactis* strain producing γ-glutamyl hydrolase, may not only enhance the production of monoglutamyl folate by the bacterium, but it may also include application as delivery vectors for glutamate hydrolase activity in the food matrix or in the GI tract of the consumer. The assessment of the bioavailability of folate with different polyglutamyl tail length produced by engineered *L. lactis* has been analyzed in a folate rat bioassay. Following a depletion diet, without folate, rats were fed for 28 days with a folate free diet

mixed with cells from *L. lactis* producing folate with an average polyglutamyl tail length of 3 and more than 5 glutamate residues. (Chapters 6 and 11).

Transferring folate production to auxotrophic LAB. The transformation of the plasmid containing the folate gene cluster to the folate auxothrophic *Lactobacillus gasseri*, a probioticum, resulted in folate biosynthesis capacity and folate prototrophy. This concept may be applied in food fermentation, for instance during the production of yogurt by the folate producing *Streptococcus thermophilus* and the folate consuming *Lactobacillus bulgaricus*. The introduction of folate biosynthesis capacity into the latter strain avoids folate consumption and results in increased levels of folate in the fermented dairy product. (Chapter 8).

Multivitamin production. *L. lactis* has the ability to synthesize not only folate but also riboflavin. A spontaneous mutant of *L. lactis* strain NZ9000 that was isolated after exposure to the riboflavin analogue roseoflavin, was changed from riboflavin consumer into riboflavin producer (Burgess *et al.,* 2003). Furthermore, the constitutive overproduction of 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase and GTP cyclohydrolase I in this strain was shown to result in increased production of folate as well. The engineered strain may improve the contribution of fermented foods in acquiring the recommended daily intake for B-vitamins. Besides the overall shortage of folate and other vitamins among a big part of the population in both developed and under developed countries, several groups hampered with a genetic polymorphism, may have an increased demand for B-vitamins. Between 10-15% of the Caucasian race are homozygous for the C6777T transition in the MTHFR gene (methylenetetrahydrofolate reductase) and may suffer from high plasma homocysteine levels. To overcome the decreased activity of this enzyme, increased folate levels and riboflavin levels are necessary. The engineering of a dairy starter bacterium with increased production capacity for both folate and riboflavin could contribute in the development of nutrigenomics, facilitating a tailor made diet for individuals that have specific requirements for certain vitamins due to a genetic polymorphism. (Chapter 7).

Extended annotation of the folate gene cluster. Experimental and *in silico* experiments have enabled the annotation of *ylgG*, located on a folate poly cistronic operon in the folate gene cluster (see annex), encoding a hypothetical protein. Based upon the missing step in the folate biosynthesis pathway, a dihydroneopterin triphosphate

phosphatase, the location of *ylgG* in the genome, the *in silico* homology with nucleoside triphosphatases, and the strong decrease in folate production by the inactivation of YlgG, the protein was annotated as dihydroneopterin triphosphate phosphatase, encoded by *folQ* (Chapter 10). Careful analysis of the remainder of the folate gene cluster has resulted in the identification of a previously unknown gene, *orfX* (Chapter 10), encoding a hypothetical protein consisting of 56 amino acids residues. The protein consist of two trans membrane helices and has more than 80% homology with an unknown protein in various streptococci strains. The genetic location of *orfX* is in all case between *folA* and *clpX*. No similar gene was found in the genome of any other organism. (Chapter 10).

The use of genetically engineered lactic acid bacteria. Today the advances described in this thesis to increase the concentration of natural folate in fermented food, thereby contributing in decreasing the number of people that suffer from folate deficiency, are not yet used by the food industry. The reason for this is mainly the negative opinion of a (small) number of people that are opposed to the use of genetically modified organisms in food. However, no clear distinction is made between GMOs derived from, animal, plant, or microbial origin. Moreover, under current legislation, the market introduction of foods with organisms that have been improved via classical breeding or random mutagenesis, without profound safety analysis, is currently acceptable, but the question is whether these foods are more safe than GMO-derived novel foods. A re-evaluation of the nature of genetically modified lactic acid bacteria in relation to the advances that they may bring, and comparison with traditionally used lactic acid bacteria with a long history of safe use in food, may result in improved safety assessment procedures that are proportional to the risks that may arise. This may assure the safe use of food fermented by traditionally or genetically modified lactic acid bacteria and contribute to the acceptance of novel foods by consumers. (Chapter 2).

Perspectives

Increased folate production. Further progress in increasing folate production levels in lactic dairy bacteria is expected by a combination of the successful strategies described so far. The overexpression of the complete folate gene cluster in a strain with a reduced capacity of dihydrofolate reductase might eventually prevent feed back inhibition of reduced folates on the folate biosynthesis pathway. The overproduction of folate in a strain with an inactivated *purR* regulator may also enhance further folate production

capacity. However, it remains to be tested if the enormous increase in the flux through the folate biosynthesis pathway by constitutive overexpression of five genes, is severely limited by the expression of *folA* and *purR*. More effect can be expected from the combination of the folate-overproducing strain with optimal medium and growth conditions. It is expected that during a pH controlled fermentation, the biomass and the folate production per cell, due to maintenance of a more optimal pH for the enzymes involved in folate biosynthesis, will be increased. Evidently, the stimulation of the intracellular production of pABA by using ME will also enhance folate production without the need of external addition of pABA.

Application of engineered strains. The application of the described folate-overproducing strain in food production, could lead to the generation of fermented food products that contain more than 5 mg per liter of folate (WO02097063). Consequently, the daily consumption of 50 ml of this product would already be enough in acquiring the daily recommended intake for folate. The generation of a dairy starter bacterium with more than 50-fold increased folate production capacities may result in products that contribute to reducing the occurrence of natural folate deficiency within the population. Currently Wageningen Centre for Food Sciences is setting up a human trial using a fermented dairy product obtained by fermentation with folate-overproducing bacteria. Such a human test will demonstrate two things. First, the test may demonstrate that food derived from organisms modified by self cloning can be of benefit to consumers, for instance by increasing folate levels and decreasing homocysteine levels in the blood. The test may yield proof-of-concept for a role for genetically changed food bacteria in creating healthy foods rich in folate. Second, the engineered strains overproduce natural folate, which is the folate form found in the human body. This differs from the synthetic folate (folic acid) which is used in food fortification. The advantage of methylated folate over synthetic folate is that the natural folate has less of a tendency to mask vitamin B12 deficiency. Masking of vitamin B12 deficiency has been a major concern in food fortification and it is the reason why e.g. the Dutch government does not allow folate fortification

ME on the level of complicated biosynthetic pathways in lactic acid bacteria. Previous studies have shown that ME can be well applied in rerouting of the lactococcal primary metabolism to end products other than lactic acid. ME of more complicated pathways involved in secondary metabolism has only recently begun by the engineering of exopolysaccharide production in *L. lactis* (Boels, 2003). In this thesis we have shown that

the complicated biosynthesis of folate can be successfully controlled by using metabolic engineering. This biosynthesis includes parts of glycolysis, pentose phosphate pathway and shikimate pathway for the production of the folate building block *p*-aminobenzoate, while the biosynthesis of purines is required for the production of the building block GTP. In addition, a number of specific enzymatic steps are involved in the final assembly of folate and for production of the various folate derivatives. The examples given in this thesis may open a way to introduce or increase the production levels of other vitamins in lactic acid bacteria, like riboflavin (vitamin B2) and cobalamin (vitamin B12) and provide a basis for further development of functional foods with increased nutritional value.

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Annex

Improved efficiency and reliability of RT-PCR using tag-extended RT primers and temperature gradient PCR

Wilbert Sybesma, Jeroen Hugenholtz, Igor Mierau, and Michiel Kleerebezem

Wageningen Centre for Food Sciences, Wageningen, The Netherlands

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In reverse transcription-PCR (RT-PCR) on prokaryotic RNA an often encountered problem is the contamination of RNA with chromosomal DNA which generates false positive reaction products. Over the last decade a number of methods has been developed to improve the reliability of RT-PCR. In some of the methods DNA is degraded by an RNase free DNase. However, extensive DNase treatment at relatively high temperature leads to loss of mRNA and thus a lower specificity and sensitivity of the analysis (2,6). In other methods DNase treatment can be omitted due to the generation of different denaturation temperatures of the dsRNA/DNA hybrid as compared to dsDNA (1) or the development of primers that display differential annealing behavior during RT and the subsequent PCR (4,5,7,10). Shuldiner *et al*. (10) describe the development of primers that generate 5'-tagged cDNA during RT that is subsequently used as a specific template during PCR. Since the sequence of this 5'-tag is not present in chromosomal DNA, no DNA derived product is amplified during PCR. Koo *et al*. (7) describe the development of a primer containing mismatched bases. Consequently, this primer can only anneal at relatively low temperatures, as are applied during RT. Subsequent PCR involves higher annealing temperatures preventing annealing of the primer to the chromosomal DNA due to its mismatched basepairs. Convenient application of both methods is impaired by the development of long primers (47 basepairs) (10) and/or of the laborious optimization of the experimental conditions. Primers often need to be optimized and resynthesized several times and annealing temperatures need to be optimized in many successive PCR runs. Another drawback of these methods is the increased amount of false positive reaction products when increasing concentrations of contaminating chromosomal DNA are present in the RNA samples. Therefore, also the reproducibility of RT-PCR results is limited when different batches of RNA isolates are used. Here we describe the application of temperature gradient PCR to improve the efficiency and reliability of a RT-PCR method using tag-extended primers. The methodology is a valuable extension to the earlier described method. The use of temperature gradient PCR makes it possible to visualize the functionality of the primers in simultaneously performed PCR amplifications with increasing annealing temperatures and thus guarantees that above a critical annealing temperature, RT-PCR products could not have been amplified from contaminating DNA. An additional advantage of the method is that primer length can be reduced far below 47 nucleotides as was described by Shuldiner *et al*. (10). As an example we demonstrate that this technique can be applied for the analysis of a multi-cistronic folate biosynthesis operon of *Lactococcus lactis*. Oligonucleotides were supplied by Amersham Pharmacia Biotech,

Buckinghamshire, England. RNA from *Lactococcus lactis* strain MG1363 was isolated as was previously described (8).

RNA concentration was determined on basis of UV absorbance at 260 nm. Reverse transcriptase reactions were performed using Gibco Superscript™ reverse transcriptase (Life Technologies BV, Breda, The Netherlands) and downstream primer dAB (Table 1) according to the manufacturers protocol. The downstream primer dAB corresponds to the last 21 nucleotides of the *folC* gene of *L. lactis* MG1363, encoding folyl polyglutamate synthase, and is tagged with a 12bp non-specific oligonucleotide (Table 1). For temperature gradient PCR we used a Mastercycler® gradient PCR apparatus (Eppendorf, Hamburg, Germany). 25 cycles of PCR were performed, each cycle consisted of denaturation (94°C, 15 seconds), annealing (temperature gradient between 48 and 68.5°C, 25 seconds) and extension (72°C, 1 minute for primer folc-u, 4 minutes for primer gch-u). In the first cycle the denaturation time was increased to 2 minutes. PCR was carried out with 2µl of RT reaction mixture as template, *Taq* polymerase (Life Technologies), upstream primer (folc-u or gch-u), and downstream primer (dA, or dB) using reaction conditions according to the manufacturers protocol.

Table 1: Primer sequences (5'-3') and theoretically calculated melting temperature (2°C per A/T, 4°C per C/G). (note: The upstream primers are slightly modified or extended at 5' to introduce restriction sites that enhances cloning strategy).

Primer	Sequence	
folC-u	CAAACATGAGTATTGAACAAGCATTAGAATGG	T_M = 84°C (underlined)
gch-u	CATGCCATGGGGCAAACAACTTATTTAAGCATGGG	T_M = 66°C (underlined)
dAB	GGATTTCCCGGGCTACTTTTCTTTTTTCAAAAA	T_M = 50°C (underlined)
dB	GGATTTCCCGGGCTACTTTTC	T_M = 64°C
dA	CTACTTTTCTTTTTTCAAAAA	T_M = 50°C

Upstream primer folc-u is a 32-mer corresponding to the first 32 nucleotides of the coding region of the *folC* gene of *L. lactis* MG1363 (Table 1), encoding folyl polyglutamate synthase. Upstream primer gch-u is a 24-mer corresponding to the first 24 nucleotides of the coding region of the *gch1* gene of *L. lactis* MG1363 (Table 1.), encoding GTP-cyclohydrolase. Downstream primer dA corresponds to the last 21 nucleotides of *folC* of

L. lactis MG1363. Downstream primer dB corresponds to the last 9 nucleotides of *folC* of *L. lactis* MG1363 and is extended with the 12 tag-nucleotides present in primer dAB (Table 1.). PCR products were analyzed using 0.7% agarose gel electrophoresis (9), followed by visualization using ethidium bromide staining and UV transillumination.

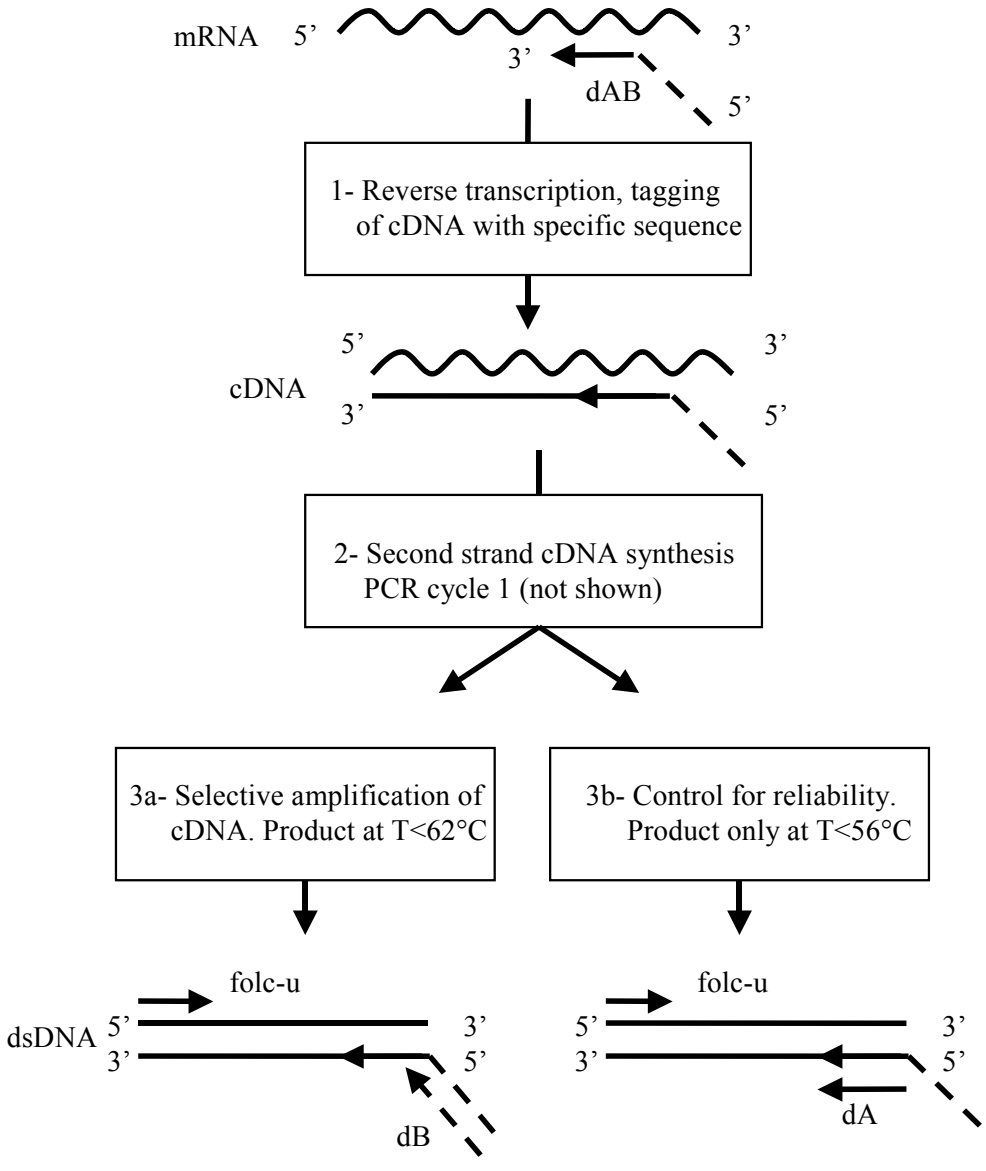

Figure 1: Principle of RT-PCR with tagged primer (dAB), tag specific primer (dB) and control primer (dA). See text for details.

RNA samples prepared for RT-PCR often contain trace amounts of chromosomal DNA which may result in chromosomal DNA derived false positive reaction products. The development of tagged primers displaying differential annealing temperatures during RT and PCR can eliminate these false positive reaction products. To guarantee the reliability of RT-PCR, temperature gradient-PCR was applied to two reaction mixtures (PCR1 and PCR2). During reverse transcription a primer (dAB) was used that is tagged with a specific sequence at its 5' end (Fig. 1). Subsequent PCR reactions (PCR1 and PCR2) were differentiated by the use of a tag-specific primer dB or the non-tag based primer dA. Annealing temperature gradients were set at a range from 48°C to 68.5°C (these temperatures are read outs from the apparatus). The lower temperature allows annealing of both primers, whereas the higher temperature does not allow priming of either primer. In PCR1 we use a temperature gradient to analyze the maximum annealing temperature of the tag specific primer, in this case primer dB. In PCR2 we use a temperature gradient to analyze the maximum annealing temperature of primer dA that contains only the nucleotides in primer dAB which anneal to the original mRNA. PCR2 mimics the annealing of tagged primer dAB that is still present in the RT mixture and that theoretically might be responsible for false positives due to annealing on contaminating DNA. PCR1 generated PCR products at the temperature range of 48-66.1°C, whereas PCR2 only generated PCR products at a shorter temperature range of 48-56.1°C (Fig. 2A). We repeated the experiments with the RT-mixture without reverse transcriptase treatment. PCR1 did not result in any amplification of nucleic acids. PCR2 showed a similar behavior as in the RT treated mixture, however band intensities were reduced (results not shown). From these results it was concluded that direct differentiation is possible between the true RT-PCR product and chromosomal DNA based false positive reaction products when annealing temperatures are raised above a specific temperature. This means that RT-PCR can be carried out in a crude mRNA preparation in the presence of DNA and that therefore steps to remove DNA from the RNA preparation can be omitted. The application of temperature gradient RT-PCR increases efficiency by a direct reliability assessment through simultaneous visualization of RT-PCR products which are amplified with primers exhibiting differential behavior during RT and subsequent PCR reactions. Temperature gradient PCR is a simple and reliable tool to evaluate whether RT-PCR without false positives reaction products can be achieved with the differential primers.

Detection of a multi-cistronic mRNA involved in folate biosynthesis in *Lactococcus lactis*: In *L. lactis* subsp. *lactis* IL1403 the genes involved in folate biosynthesis are clustered in one chromosomal locus (3). In the related *L. lactis* subsp. *cremoris* MG1363

we identified a similar folate biosynthesis locus. The RT-PCR method described was applied to specifically analyze the transcript length derived from this locus. Temperature gradient RT-PCR using upstream primer gch-u and downstream primers dAB during RT, and dB or dA (control reaction) during the subsequent PCR, resulted in amplification of a 3.9 kb RT-PCR product. The application of temperature gradient PCR established that above a temperature of 61.5°C the RT-PCR with tagged primers did not produce a product which could be derived from chromosomal DNA (Fig. 2B). From these results we conclude that in *L. lactis* MG1363 GTP cyclohydrolase and folyl polyglutamate syntehase are present on a multi-cistronic transcript of at least 3.9 kb. Cloning and sequencing of the DNA fragment from *L lactis* MG1363 and comparison with the sequence of *L. lactis* IL1403 identified a folate operon that contains the genes *folKE*, encoding GTP cyclohydrolase I, *folP*, encoding dihydropteroate synthase, *ylgG*, encoding a hypothetical protein and *folC*, encoding folyl polyglutamate synthetase.

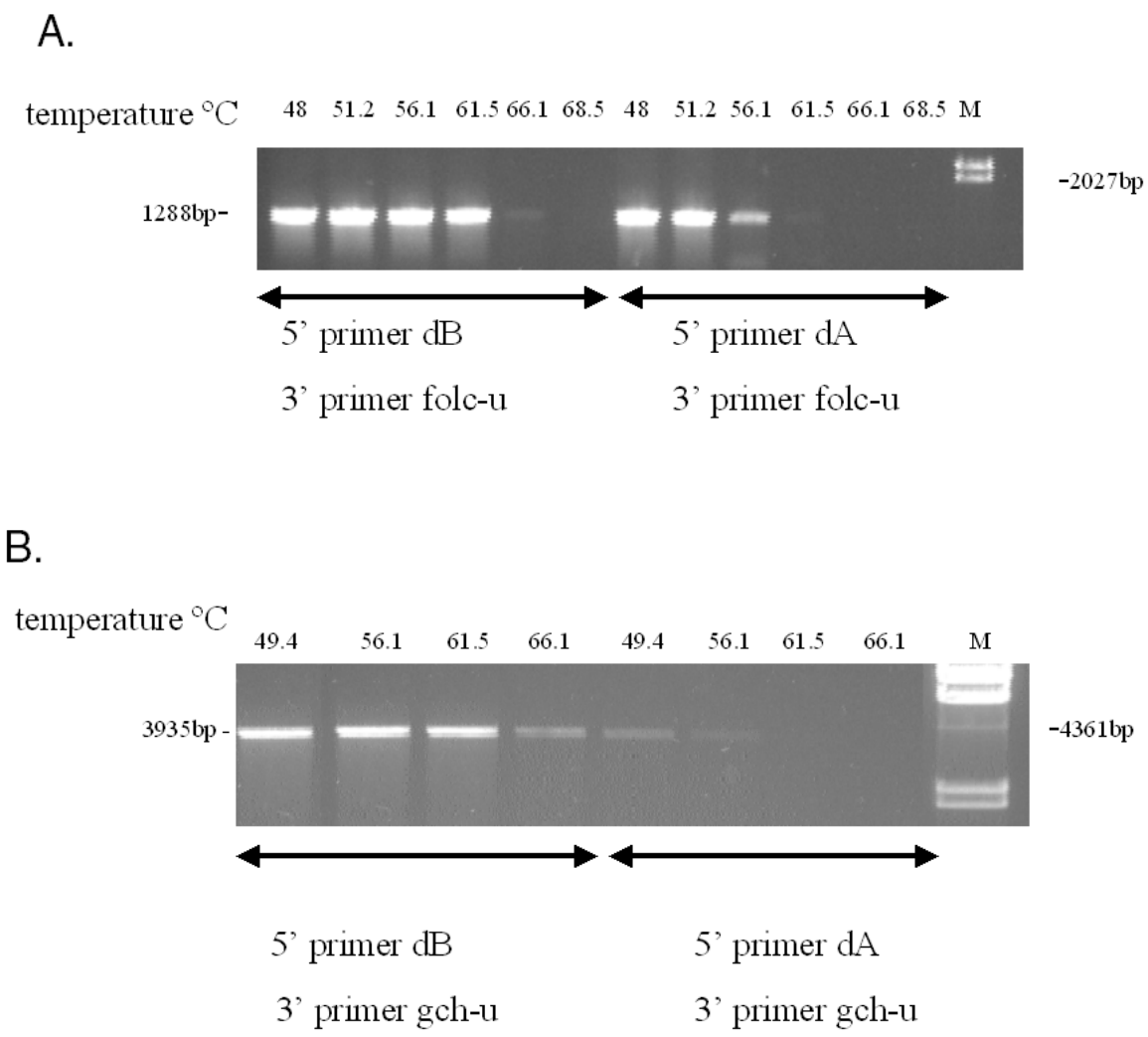

Figure 2:
Temperature gradient RT-PCR with tagged primer in RT step and tag-specific primer or control primer in PCR step. See text for details.
Panel A. Temperature gradient RT-PCR, 48-68.5 °C. In lane 1-6 a combination of upstream primer folc-u and downstream primer dB (tag specific) was used, while in lane 7-12 upstream primer folc-u and downstream primer dA were combined (control experiment to check at what temperature tagged primer used in RT step cannot anneal anymore in PCR step).
Panel B. Temperature gradient RT-PCR, 49.4-66.1 °C. In lane 1-4 upstream primer gch-u and downstream primer dB (tag specific) were used, while in lane 5-8 upstream primer gch-u and downstream primer dA were used (control experiment as described in panel A.).
M represents a lambda-DNA *Hind*III digest DNA marker.

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Samenvatting

Productie van Folaat in Melkzuurbacteriën met behulp van Metabolic Engineering

Dit proefschrift beschrijft de productie van folaat in melkzuurbacteriën met behulp van Metabolic Engineering (ME). De melkzuurbacterie *Lactococcus lactis* is gekozen als modelorganisme. De belangrijkste doelstelling van dit onderzoek is het verhogen van de concentratie folaat in gefermenteerde voedingsproducten. De resultaten van het onderzoek vormen tevens een model voor het verhogen van de productiecapaciteit van andere B-vitamines zoals cobalamine en riboflavine in melkzuurbacteriën.

Folaat

Folaat, vitamine B11, kan worden geproduceerd door de melkzuurbacterie *Lactococcus lactis*, die wordt gebruikt als startercultuur in de zuivelindustrie voor bijvoorbeeld de productie van karnemelk. Folaat heeft een belangrijke functie in de fysiologie van organismen en is betrokken bij de synthese van DNA, RNA en bepaalde aminozuren. Hoewel folaat ruim voldoende aanwezig is in een normaal dieet, zijn er toch bepaalde groepen mensen met een tekort aan folaat. Dit tekort kan voor zwangere vrouwen het risico vergroten op het verkrijgen van kinderen met een open ruggetje. Een te lage folaat status kan ook leiden tot een verhoogde kans op het verkrijgen van hart– en vaatziekten, sommige vormen van kanker en vormen van bloedarmoede. Het verhogen van de folaat productiecapaciteit in bacteriën die gebruikt worden bij de productie van gefermenteerde voedingsmiddelen zou kunnen bijdragen tot het verhogen van de folaatconsumptie onder de bevolking. (Hoofdstuk 1).

Verhoogde folaatproductie door geoptimaliseerde groeicondities

De eigenschap van melkzuurbacteriën zoals lactococcen, streptococcen en sommige lactobacillen, om folaat te kunnen produceren kan gebruikt worden om starter culturen te ontwikkelen met een verhoogde folaatproductiecapaciteit. De optimalisatie van de groeicondities van fermenterende bacteriën, bijvoorbeeld door het controleren van de zuurgraad en het overvloedig aanbieden van substraten, zal leiden tot een verhoging van de biomassa en daardoor tot een verhoging van de absolute folaatproductie. Een bijkomstigheid is dat, zoals in de literatuur staat weergegeven, de enzymen die betrokken zijn bij de productie van folaat in vele organismen een basisch pH optimum hebben.

Dit betekent dat bij het handhaven van een relatief hoge extracellulaire pH, wat leidt tot een hogere intracellulaire pH, de folaat-producerende enzymen efficiënter hun werk kunnen doen met als resultaat een hogere folaatproductie per bacteriële cel. Het vertragen van de groeisnelheid van melkzuurbacteriën is een andere omstandigheid die leidt tot een verhoogde folaatproductiecapaciteit per cel. Dit kan worden verklaard door het feit dat bij een lagere groeisnelheid een langzamere verzuring van het medium optreedt, waardoor de cellen langere tijd zijn blootgesteld aan een meer optimale (hogere) pH. (Hoofdstuk 3).

Verhoogde folaatproductie door een gewijzigde expressie van individuele genen

De opheldering van het genoom van de *Lactococcus lactis* stam IL1403, die nauw verwant is aan de in dit onderzoek gebruikte *Lactococcus lactis* stam MG1363, heeft geleid tot een benoeming van alle genen van dit genoom. In stam IL1403 is een zogenaamd folaatgencluster gevonden dat alle genen bevat die coderen voor de enzymen in de folaatbiosyntheseroute. De grote mate van overeenkomst tussen de beide Lactococcus stammen maakte het mogelijk om met behulp van de polymerase chain reaction (PCR) de aanwezigheid van een soort gelijk gencluster in stam MG1363 aan te tonen. Zo werd de basis gelegd voor het opzetten van een groot aantal ME experimenten in *L. lactis* MG1363 met als doel het verhogen en controleren van de folaatproductie en de folaatdistributie in deze melkzuurbacterie. Hierbij werd gebruik gemaakt van genetische technieken, waarbij nieuwe genetische informatie werd overgebracht, en van gecontroleerde gen-expressie systemen.

De overexpressie van het gen *folKE*, dat codeert voor het bifunctionele enzym uit de folaatbiosyntheseroute met de naam 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrofosfokinase en GTP cyclohydrolase I, leidde tot een tienvoudige verhoging van de extracellulaire folaatproductie en een drievoudige verhoging van de intracellulaire folaatproductie. De gecontroleerde overexpressie van ditzelfde gen en van *folC*, dat codeert voor het enzym dat verantwoordelijk is voor de synthese van polyglutamylfolaten, leidde tot een verhoging van de ophoping van het extra geproduceerde folaat in de cel. De overexpressie van het gen *folA*, dat codeert voor het enzym dihydrofolaatreductase, leidde tot een halvering van de folaatproductie. Deze laatste observatie suggereert dat gereduceerde folaten een negatieve terugkoppeling hebben op de folaatproductie. Dit is in overeenstemming met een ander experiment waarbij de activiteit van het betreffende enzym werd verlaagd waarna een geringe toename van de folaatproductie werd gevonden. (Hoofdstuk 4).

Verhoogde folaatproductie door overexpressie van het hele gencluster

Een aantal van de ME experimenten zijn gedaan met behulp van een nisine gecontroleerd expressie systeem, het zogenaamde NICE systeem, dat de expressie van

bepaalde genen stapsgewijs kan controleren. Voor industriële toepassingen voor verhoogde folaatproductie is het praktischer om gebruik te maken van genen die constitutief tot expressie komen. De constructie en klonering in *L. lactis* van een plasmide met de folaat genen *folB*, *folKE*, *folP*, *folQ* en *folC*, onder de controle van de constitutieve promoter van *pepN* leidde tot een meer dan vijftigvoudige verhoging van de hoeveelheid geproduceerd folaat zolang er maar voldoende van het substraat *para*-aminobenzoaat (pABA) aanwezig was in het medium. Met deze genetisch gemodificeerde melkzuurbacterie werden concentraties van meer dan 8 mg folaat per liter cultuur medium behaald. Extra toevoeging van glutamaat leidde tot nog hogere concentraties folaat (10 mg/L). (Hoofdstuk 9).

De cruciale rol van *p*ABA

In afwezigheid van de folaatprecursor pABA werd de folaatproductie gehalveerd. Bovendien was de vijftigvoudige toename in folaatproductie alleen mogelijk in aanwezigheid van overmaat pABA. In *L. lactis* wordt pABA gesynthetiseerd via dezelfde biosynthese route als die van de en de aromatische aminozuren en hierbij zijn de glycolyse, de pentosefosfaatroute, en de shikimaatroute betrokken. Door het inactiveren van deze laatste route kon de bacterie niet meer groeien zonder de aanwezigheid van de aromatische aminozuren tryptofaan, fenylalanine, en tyrosine. De folaatproductie bleef echter beneden het detectieniveau in de aanwezigheid van deze 3 aminozuren. Dit betekende dat pABA niet kon worden gesynthetiseerd via een alternatieve route vanuit de aromatische aminozuren. Voor de toepassing van folaat-overproducerende bacteriën in de voedingsmiddelenindustrie zou het gewenst zijn als ook voldoende pABA door de bacterie geproduceerd zou kunnen worden. (Hoofdstuk 3 en 9).

Functional genomics

De uitkomst van een transcriptome-analyse van de folaatoverproducerende stam in vergelijking met een wild-type stam kan de opzet van een ME strategie, met het doel om de productie van folaat en folaatprecursors te verhogen, ondersteunen. Het verhoogde expressie niveau van bepaalde genen in de vijftigvoudige folaatoverproducerende bacterie suggereerde dat de overexpressie van genen in de pentosefosfaatroute en de shikimaatroute zou kunnen leiden tot een verhoging de pABA productiecapaciteit. De transcriptoomanalyse toonde ook een verhoogde expressie aan van bepaalde transportmechanismen wat zou kunnen leiden tot identificatie van genen betrokken bij het transport van pABA of folaat in de melkzuurbacterie. (Hoofdstuk 9).

Controle van de polyglutamylstaart-lengte

Een belangrijke taak in de biosynthese en fysiologie van folaat wordt vervuld door het gen *folC* dat codeert voor het eiwit polyglutamylfolaatsynthetase. Dit enzym produceert folaat vanuit de precursor dihydropteroaat, maar het is ook in staat om extra glutamaat residuen te koppelen aan het folaatmolecuul waarna polyglutamylfolaat ontstaat. De polyglutamaat-vorm van folaat is de actieve co-factor en zorgt voor ophoping van het folaat in de cel. Dankzij het ontwikkelen van een HPLC scheidings- en detectiemethode was het mogelijk om verschillende folaten te identificeren en hun polyglutamylstaartlengten in *L. lactis* te bepalen. Meer dan 90% van het geproduceerde folaat in deze bacterie werd intracellulair opgeslagen en bestond voornamelijk uit 5 formyl-polyglutamylfolaten en 5.10-methenyl-polyglutamylfolaten met 4,5, en 6 glutamaat residuen. De gemiddelde staartlengte werd sterk gereduceerd en de efflux naar de omgeving nam toe door de overexpressie van *folKE*. De overexpressie van *folC* leidde tot polyglutamyfolaten met meer dan 12 gekoppelde glutamaat residuen en een volledige ophoping van het folaat in de cel. Een totaal ander beeld werd verkregen door de expressie van een menselijk gen in *L. lactis* dat codeert voor gamma glutamylhydrolase en dat verantwoordelijk is voor het verwijderen van de glutamaatstaart van folaat. Dit leidde tot de productie van monoglutamylfolaat en een volledige efflux van het folaat naar de omgeving. Het controleren van de staartlengte van folaat kan worden gebruikt voor het efficiënt toedienen aan mensen van folaat via bijvoorbeeld melkzuurbacteriën of probiotica met een hoge concentratie intracellulair folaat. Daarentegen zal de productie van monoglutamylfolaten de concentratie van folaten in gefermenteerde producten kunnen verhogen waaruit de melkzuurbacteriën zelf zijn verwijderd. (Hoofdstuk 4 en 5).

Biobeschikbaarheid

Een ander belangrijk kenmerk van monoglutamylfolaat heeft te maken met biobeschikbaarheid. Polyglutamylfolaat is de vorm van folaat die voornamelijk in de natuur voorkomt. Maar folaat wordt geabsorbeerd in de menselijke darm als monoglutamylfolaat nadat het poly- in monoglutamylfolaat is omgezet door het darmenzym carboxypeptidase II. De activiteit van dit enzym kan geremd worden door sommige bestanddelen die in een dieet aanwezig kunnen zijn. De biobeschikbaarheid van monoglutamyl folaat kan daarom als hoger beschouwd worden, omdat deze vorm niet meer ontdaan dient te worden van de polyglutamylstaart. (Hoofdstuk 6).

De biobeschikbaarheid van folaat met een verschillende glutamaatstaartlengte dat geproduceerd is in genetisch gemodificeerde *L. lactis* stammen is getoetst in een dierproef. Een aantal ratten dat op een folaatvrij dieet was gezet, werd vervolgens gevoed met een dieet waaraan de betreffende melkzuurbacteriën waren toegevoegd. Na een maand kon duidelijk worden aangetoond dat het folaatgehalte in een aantal organen van

deze ratten verhoogd was ten opzichte van een controlegroep. Dit was het eerste experiment dat de folaatbiobeschikbaarheid bepaalde van genetisch gemodificeerde melkzuurbacteriën. De uitkomst biedt perspectieven voor een vervolgexperiment met menselijke vrijwilligers. (Hoofdstuk 11).

Transformatie van een folaat-consumerende bacterie in een folaat-producerende bacterie

De klonering van een plasmide, dat het volledige folaatgencluster uit het genoom van *L. lactis* bevat, in de folaat auxotrofe melkzuurbacterie *Lactobacillus gasseri*, een probioticum, leidde tot folaatproductie in deze stam. Deze eigenschap was niet aanwezig in de wild-type stam. Dit concept zou kunnen worden toegepast in de voedingsmiddelenindustrie voor de productie van bijvoorbeeld yoghurt, die ontstaat uit een melkfermentatie met de folaat-producerende *Streptococcus thermophilus* en de folaat-consumerende *Lactobacillus bulgaricus*. De introductie van een folaat biosynthese route in deze laatste stam zal het resultaat hebben dat er meer folaat in de uiteindelijke yoghurt terecht komt (Hoofdstuk 8).

Multivitamine-productie

L. lactis heeft niet alleen de mogelijkheid om folaat te produceren maar ook de B-vitamine riboflavine. Een spontane mutant van de melkzuurbacterie met een gedereguleerde riboflavine biosynthese was geïsoleerd na blootstelling aan een riboflavine analoog, roseoflavine. Deze stam produceerde riboflavine, in tegenstelling tot de wild-type stam die deze vitamine normaal consumeerde (werk van Kaye Burgess, UCC, Cork, Ierland). De constitutieve expressie van het folaatgen *folKE* in deze geïsoleerde bacteriestam via de klonering van een multicopy plasmide, leidde er vervolgens toe dat deze stam zowel extra riboflavine als extra folaat produceerde. De nieuw ontwikkelde stam zal kunnen bijdragen aan het verhogen van de consumptie van B-vitaminen via gefermenteerde voedingsmiddelen. Naast de grote groep mensen die een te kort aan folaat en andere vitaminen hebben, bestaat er ook een groep mensen die leidt aan een bescheiden genetische afwijking en daardoor een grotere behoefte heeft aan folaat en riboflavine: zo'n 10-15% van het Kaukasische ras is homozygoot voor de zogenaamde C6777T transitie in het gen dat codeert voor methyleentetrahydrofolaatreductase enzym. Dit heeft als gevolg dat deze groep een verhoogd risico loopt op een hoog homocysteïne gehalte in het bloed. Door extra inname van folaat en riboflavine kan dit enzym weer naar behoren functioneren en de concentratie homocysteïne kan worden gecontroleerd. De ontwikkeling van een stam met verhoogde productiecapaciteit van zowel folaat als riboflavine kan bijdragen aan de ontwikkeling van nutrigenomics, door het ontwikkelen van

gefermenteerde voedingsmiddelen met deze stam voor mensen die gezien hun genetisch profiel een hogere behoefte hebben aan bepaalde vitaminen (Hoofdstuk 7).

Verdere benoeming van het folaatgencluster

Experimenteel onderzoek en zogenaamd *in silico* onderzoek, waarbij van bio-informatica gebruik wordt gemaakt, heeft het mogelijk gemaakt om een functie te koppelen aan het voorheen onbekende gen *ylgG* dat aanwezig is in het folaat gencluster. De inactivering van dit gen leidde namelijk tot een drastische verlaging van de folaatproductiecapaciteit en bovendien vertoonde het enzym een homologie met een nucleotidetrifosfaat-fosfatase enzym. Dit is een enzym dat verantwoordelijk kan zijn voor een reactie in de folaatbiosyntheseroute waar nog geen gen voor was gevonden. Gezien bovenstaande observatieen wordt voorgesteld het gen *ylgG* te annoteren als *folQ* dat codeert voor dihydroneopterinetrifosfaat-fosfatase. Nauwkeurige analyse van de rest van het folaat gencluster leidde er toe dat een nieuw gen werd gevonden, *orfX*, dat codeert voor een klein eiwit bestaande uit 56 aminozuren. Het eiwit bestaat waarschijnlijk uit twee transmembraan-helixen en heeft een homologie van 80% met een onbekend eiwit in een aantal streptococcen, waar dit gen ook gesitueerd is tussen *folA* en *clpX*. Verder onderzoek is nodig om de functie van dit gen en het bijbehorend eiwit op te helderen. (Hoofdstuk 10).

Het gebruik van genetisch gemodificeerde melkzuurbacteriën

Vandaag de dag wordt er door de voedingsmiddelenindustrie nog geen gebruik gemaakt van de voordelen die in dit proefschrift staan beschreven om de concentratie van natuurlijke folaten in het dieet van mensen te verhogen en zodoende deficiëntieziekten te beperken. Dit wordt vooral veroorzaakt door de bezwaren van een (kleine) groep mensen die zich gekeerd hebben tegen het gebruik van zogenaamde GGOs (genetisch gemodificeerde organismen) in voeding. In het algemeen wordt er echter geen onderscheid gemaakt tussen GGOs van plantaardige, dierlijke, of microbiële oorsprong, terwijl er geen sprake is van een gelijkluidend risicoprofiel. Bovendien stelt de huidige wetgeving geen beperking aan het gebruik van organismen die verbeterd zijn via klassieke veredeling of random mutagenese. Men kan zich hierbij afvragen of voedingsmiddelen verkregen met behulp van dit soort random gemutageniseerde organismen veiliger zijn dan voedingsmiddelen verkregen via GGOs. Een grondige evaluatie van de door sommigen vermeende risico's van het gebruik van genetisch gemodificeerde melkzuurbacteriën in vergelijking met traditioneel gebruikte melkzuurbacteriën die al een lange en veilige geschiedenis hebben in de voedselbereiding, zal kunnen leiden tot een betere voedselveiligheidsprocedure. Hiermee zal het veilig gebruik van voedingsmiddelen gefermenteerd door traditionele melkzuurbacteriën of genetisch gemodificeerde

melkzuurbacteriën gegarandeerd kunnen worden en zal de acceptatie van dit soort nieuwe voedingsmiddelen, zolang als dat deze voordelen bieden aan de consument, vereenvoudigd worden. (Hoofdstuk 2).

Curriculum Vitae

Wilbert Feike Henricus Sybesma werd geboren op 6 april 1971 in Breda. In 1989 behaalde hij het diploma ongedeeld gymnasium aan het Stedelijk Gymnasium Breda. In hetzelfde jaar begon hij met de Studies Moleculaire Wetenschappen en Bioprocestechnologie aan de toenmalige Landbouwuniversiteit Wageningen. Afstudeervakken werden gedaan bij de vakgroepen Microbiologie, Biochemie, Fytopathologie en bij het Institute of Technology for Chemistry and Biology (ITQB), Oeiras, Portugal. In augustus 1994 werd voor beide studies het ingenieursdiploma behaald. In september 1994 begon hij met een postuniversitaire opleiding in het Internationaal en Europees Recht aan de Vrije Universiteit Brussel. Een jaar later werd met onderscheiding het diploma behaald. In 1996 en 1997 was hij werkzaam aan de Technische Universiteit Delft en droeg bij aan het onderzoek naar de stereospecifieke reductie van 3-oxo esters door gist. Van 1997 tot 1999 was hij werkzaam als wetenschappelijk medewerker bij het plantenbiotechnologiebedrijf Keygene N.V. in Wageningen. Van juli 1999 tot juli 2003 verrichtte hij een promotie-onderzoek bij NIZO Food Research in Ede voor de vakgroep Microbiologie van Wageningen Universiteit en in opdracht van het Wageningen Centre for Food Sciences. Het promotie-onderzoek, dat in dit proefschrift wordt beschreven, stond onder begeleiding van Prof. Dr. Willem M. de Vos en Dr. Jeroen Hugenholtz.

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