

Multivitamin production in *Lactococcus lactis* using metabolic engineering

Wilbert Sybesma,^{a,1} Catherine Burgess,^{b,1} Marjo Starrenburg,^a Douwe van Sinderen,^b and Jeroen Hugenholtz^{a,*}

^a Department of Flavor, Nutrition and Natural Ingredients, Wageningen Centre for Food Sciences, NIZO food research, Kernhemseweg 2, P.O. Box 20, 6710 BA Ede, The Netherlands

^b Department of Microbiology and BioSciences Institute, National University of Ireland, Cork, Western Road, Cork, Ireland

Received 19 May 2003; accepted 3 November 2003

Abstract

The dairy starter bacterium *Lactococcus lactis* has the potential to synthesize both folate (vitamin B11) and riboflavin (vitamin B2). By directed mutagenesis followed by selection and metabolic engineering we have modified two complicated biosynthetic pathways in *L. lactis* resulting in simultaneous overproduction of both folate and riboflavin: Following exposure to the riboflavin analogue roseoflavin we have isolated a spontaneous mutant of *L. lactis* strain NZ9000 that was changed from a riboflavin consumer into a riboflavin producer. This mutant contained a single base change in the regulatory region upstream of the riboflavin biosynthetic genes. By the constitutive overproduction of GTP cyclohydrolase I in this riboflavin-producing strain, the production of folate was increased as well. Novel foods, enriched through fermentation using these multivitamin-producing starters, could compensate the B-vitamin-deficiencies that are common even in highly developed countries and could specifically be used in dietary foods for the large fraction of the Caucasian people (10–15%) with mutations in the methylene tetrahydrofolate reductase (MTHFR).

© 2003 Elsevier Inc. All rights reserved.

Keywords: Folate; Riboflavin; Metabolic engineering; *Lactococcus lactis*

1. 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 Vecchia et al., 2002; Kim, 1999; Choi and Mason, 2002), and mental disorders, such as psychiatric syndromes among elderly and decreased cognitive performance (Calvaresi and Bryan, 2001; Hultberg et al., 2001). The daily recommended intake of dietary folate for an adult is 400 µg, while for pregnant women it 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

*Corresponding author.

E-mail address: jeroen.hugenholtz@nizo.nl (J. Hugenholtz).

¹Equal contribution of both authors.

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. 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 child-bearing age in these highly developed countries.

In previous work we reported on increased folate production by metabolic engineering of the complicated folate biosynthesis pathway in 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 aimed at deregulation of the riboflavin biosynthetic pathway 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

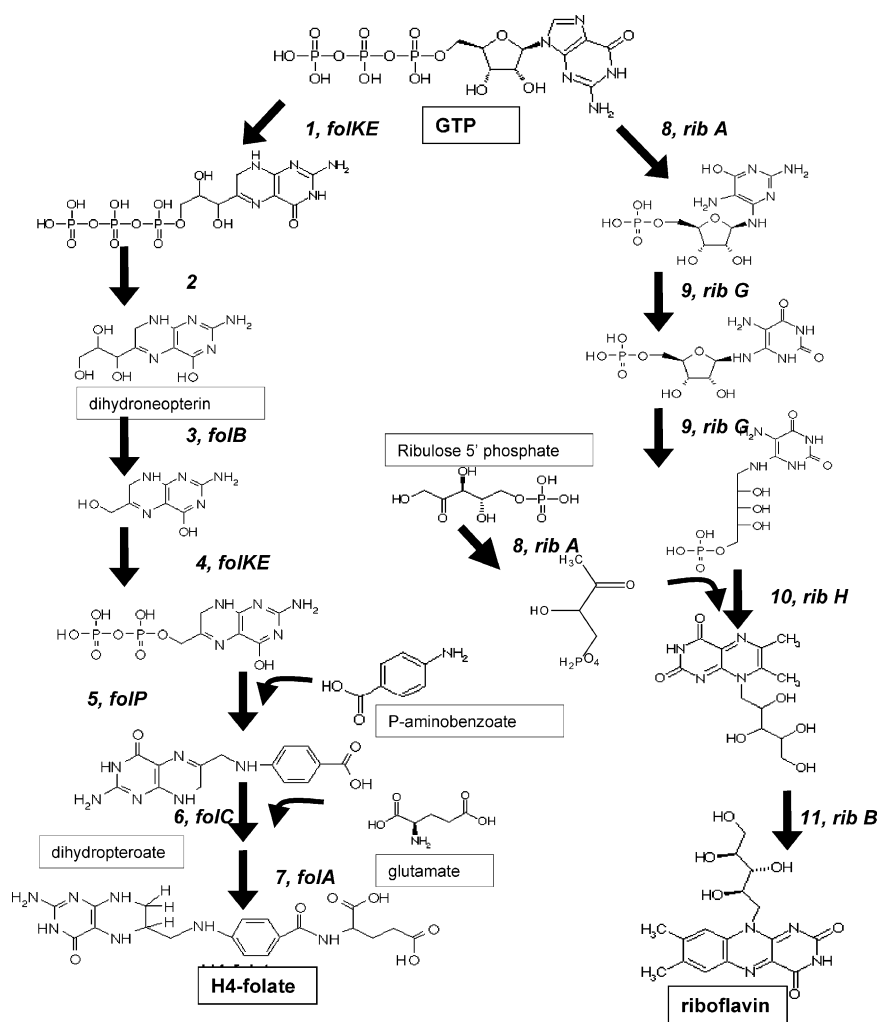


Fig. 1. Riboflavin and folate biosynthesis pathway. Indicated are the genes and enzymatic reaction steps: 1. GTP cyclohydrolase I (EC 3.5.4.16), 2. phosphatase reaction, 3. dihydroneopterin aldolase (EC 4.1.2.25), 4. 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.5.1.15), 5. dihydropteroylglutamate 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).

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). Our results describe an efficient approach to simultaneously increase the production of two essential vitamins in lactic acid bacteria.

2. Materials and methods

2.1. Bacterial strains, media, and culture conditions

All *L. lactis* 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 μ g/ml). Plasmids were generated and transformed to *L. lactis* as described below.

2.2. Isolation of roseoflavin resistant mutants

Spontaneous roseoflavin resistant *L. lactis* mutants were isolated by plating an undiluted *L. lactis* NZ9000 culture on CDM-agar containing 100 mg/L roseoflavin (Toronto Research Chemicals, Toronto, Canada). Potential roseoflavin resistant mutants were plated on CDM-agar 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.

2.3. 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).

2.4. 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-hydroxymethylidihydropteridine 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 μ l containing deoxyribonucleoside triphosphates (0.25–0.5 mM each), oligo nucleotides (50 pM) (*FolKE*2-F, 5'- ATACATGCATGCAAA-CAACTTATTTAAGCATGGG-3'; *FolKE*2-R, 5'- ATACATGCATGCGATTCTTGATTAAAGTTCTAAG-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 s (3 min in first cycle), annealing at 50°C for 30 s and elongation at 68°C for 1 min. 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.

2.5. 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 ($OD_{600\text{ nm}}$ approximately 2.5) by centrifugation (12,000g, 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 12,000g, 3 min, 4°C) 100 μ l 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 μ l 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 mass spectrometry using a VG Quattro II mass spectrometer (Micromass UK Ltd., Manchester, UK). 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 (250 × 3 mm ID, 3 μm) (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 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.

3. 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 obtain similar results with the food-grade lactic acid bacterium *L. lactis* mutants, *L. lactis* strain NZ9000 was exposed to roseoflavin. Next, spontaneous roseoflavin resistant variants were analyzed for riboflavin production (data not shown). *L. lactis* CB010 was chosen for further use in this study as the highest riboflavin-producing variant and this strain was transformed with pNZ7017 overexpressing the gene *folKE* that codes for the bifunctional protein 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase 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 HPLC, while in the wild type cultures no riboflavin could be detected, extracellularly (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

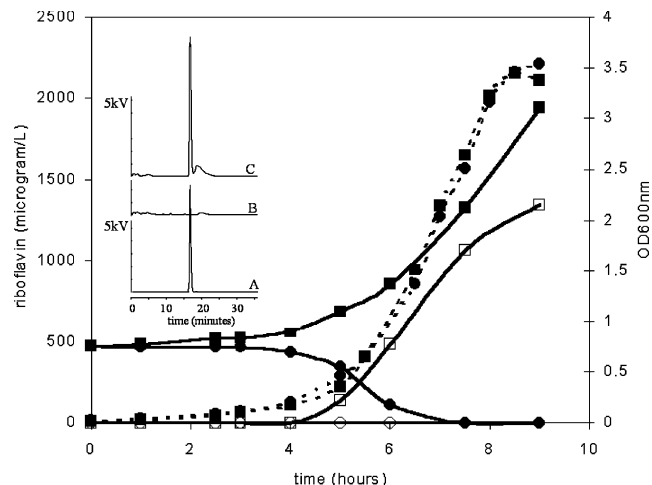


Fig. 2. Growth and extracellular riboflavin levels detected in growing strains of *L. lactis* NZ9000 harboring pNZ8048 (negative control), or strain CB010 harboring pNZ7017 (deregulated in riboflavin biosynthesis and overexpressing *folKE*). The spheres indicate strain NZ9000 and the squares indicate strain CB010. 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*). 1, Riboflavin peak at 17.5 min.

(Fig. 2). The inset of Fig. 2 shows the chromatographic separation of riboflavin of the culture supernatant of strain CB010 harboring pNZ7017 (C) or of the control strain (B).

In *B. subtilis*, roseoflavin-resistant mutants were shown to contain mutations in regulatory genes and/or in the up-stream regions of the *rib* operon. In order to see if the same is true for *L. lactis* these regions were sequenced in strain CB010. No mutations were found in *ribC*, but in the RFN element, a putative regulatory region upstream of the riboflavin biosynthesis operon (Gelfand et al., 1999), 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 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

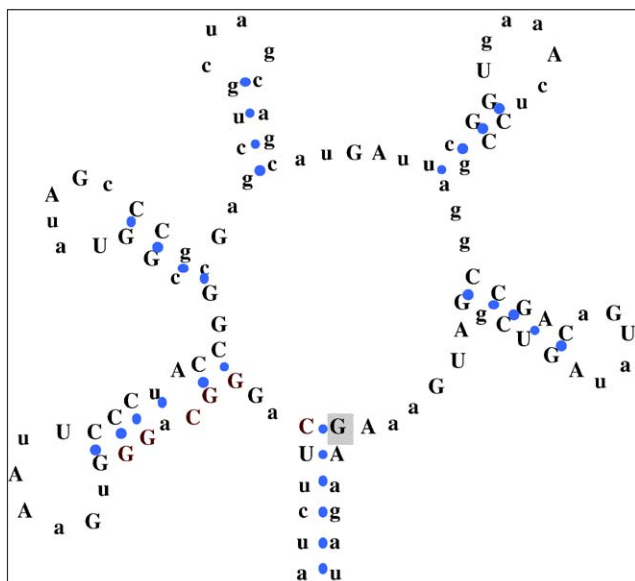


Fig. 3. *L. Lactis* NZ9000 RFN element. The capital letters indicate conserved bases between species and the dots illustrate base pairing. The gray box indicates where a guanine to cytosine substitution occurs in the roseoflavin resistant *L. lactis* CB010. The RNA secondary structure was predicted using Zuker's algorithm of free energy minimization (Lyngso et al., 1999) which is utilized in the Mfold program (<http://bioinfo.math.rpi.edu/~mfold/rna>).

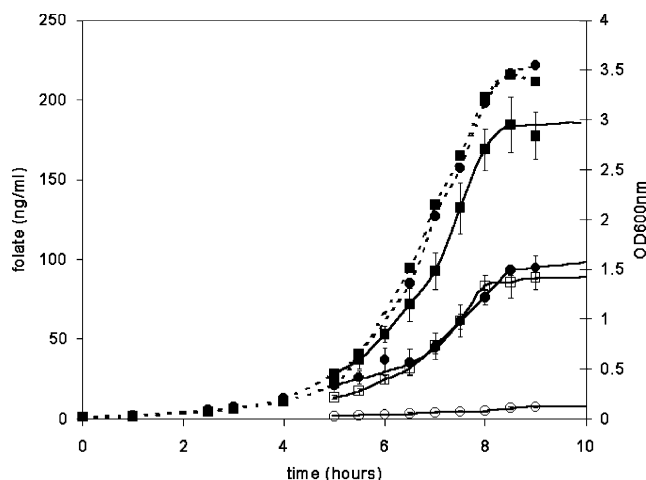


Fig. 4. Growth, intra-, and extracellular folate levels detected in growing strains of *L. lactis* NZ9000 harboring pNZ8048 (negative control), or strain CB010 harboring pNZ7017 (deregulated in riboflavin biosynthesis and overexpressing *folKE*). The spheres indicate *L. lactis* strain NZ9000 and the squares indicate *L. lactis* strain CB010. 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.

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). Likewise, 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).

4. Discussion

As far as we know, this work describes the first successful combination of directed mutagenesis and metabolic engineering in two different biosynthetic pathways. This strategy has resulted in simultaneous overproduction, by the dairy lactic acid bacterium *L. lactis*, of two metabolic end products, riboflavin and folate, which add to the health benefit of fermented foods. 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 riboflavin production 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 (Gelfand et al., 1999). 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 flavin 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 over-

expression 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 affect 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 two 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 anemia (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% and 15% of the Caucasian race are homozygous for the C677T 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's 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 previously. 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 directed mutagenesis followed by selection and metabolic engineering can be used for controlling, simultaneously, secondary metabolic pathways, such as the folate and riboflavin

biosynthetic pathways. The industrial application of the described multivitamin producing strain, *L. lactis* CB010 harboring pNZ7017, during the production of fermented foods could significantly contribute to the daily recommended intake for humans of 1.6 mg and 400 μ g for riboflavin and folate, respectively. This seems especially relevant for the health status of humans all over the world since vitamin deficiency is common among a large part of the Western population (Konings et al., 2001; O'Brien et al., 2001) or for sub-populations diagnosed with altered genotypes (Guenther et al., 1999). Natural fortification of foods, through fermentation, has major advantages over food fortification by the addition of chemically synthesized vitamins. The use of these fortified fermented foods is not limited by legislation, which sometimes prohibits the addition of (health) ingredients to foods, and (the vitamins in) the fermented foods should be more accessible to people in underdeveloped countries. However, in all cases of food fortification, one should keep an eye on possible adverse effects of over-intake of vitamins such as reported for folate by Stover and Garza (2002).

Acknowledgments

This research was supported by the EU project QLK1-CT-2000-01376.

References

- Abrams, S.A., 2002. Nutritional rickets: an old disease returns. *Nutr. Rev.* 60, 111–115.
- Akompong, T., Ghori, N., Haldar, K., 2000. In vitro activity of riboflavin against the human malaria parasite *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 44, 88–96.
- Boels, I.C., Kleerebezem, M., De Vos, W.M., 2003. Engineering of carbon distribution between glycolysis and sugar nucleotide biosynthesis in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 69, 1129–1135.
- Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S.D., Sorokin, A., 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res.* 11, 731–753.
- Boushey, C.J., Beresford, S.A., Omenn, G.S., Motulsky, A.G., 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *J. Am. Med. Assoc.* 274, 1049–1057.
- Calvaresi, E., Bryan, J., 2001. B vitamins, cognition, and aging: a review. *J. Gerontol. B. Psychol. Sci. Soc. Sci.* 56, 327–339.
- Choi, S.W., Mason, J.B., 2002. Folate status: effects on pathways of colorectal carcinogenesis. *J. Nutr.* 132, 2413–2418.
- Clifford, A.J., Heid, M.K., Peerson, J.M., Bills, N.D., 1991. Bioavailability of food folates and evaluation of food matrix effects with a rat bioassay. *J. Nutr.* 121, 445–453.
- Coquard, D., Huecas, M., Ott, M., van Dijk, J.M., van Loon, A.P., Hohmann, H.P., 1997. Molecular cloning and characterisation of the *ribC* gene from *Bacillus subtilis*: a point mutation in *ribC* results in riboflavin overproduction. *Mol. Gen. Genet.* 254, 81–84.

- De Vos, W.M., Vos, P., de Haard, H., Boerrigter, I., 1989. Cloning and expression of the *Lactococcus lactis* SK11 gene encoding an extracellular serine proteinase. *Gene* 85, 169–176.
- Fishman, S.M., Christian, P., West, K.P., 2000. The role of vitamins in the prevention and control of anaemia. *Public Health Nutr.* 3, 125–150.
- Gelfand, M.S., Mironov, A.A., Jomantas, J., Kozlov, Y.I., Perumov, D.A., 1999. A conserved RNA structure element involved in the regulation of bacterial riboflavin synthesis genes. *Trends Gene* 15, 439–442.
- George, L., Mills, J.L., Johansson, A.L., Nordmark, A., Olander, B., Granath, F., Cnattingius, S., 2002. Plasma folate levels and risk of spontaneous abortion. *J. Am. Med. Assoc.* 288, 1867–1873.
- Guenther, B.D., Sheppard, C.A., Tran, P., Rozen, R., Matthews, R.G., Ludwig, M.L., 1999. The structure and properties of methylenetetrahydrofolate reductase from *Escherichia coli* suggest how folate ameliorates human hyperhomocysteinemia. *Nat. Struct. Biol.* 6, 359–365.
- Hols, P., Kleerebezem, M., Schanck, A.N., Ferain, T., Hugenholtz, J., Delcour, J., de Vos, W.M., 1999. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. *Nat. Biotechnol.* 17, 588–592.
- Horne, D.W., Patterson, D., 1988. *Lactobacillus casei* microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clin. Chem.* 34, 2357–2359.
- Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., de Vos, W.M., Hols, P., 2000. *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Appl. Environ. Microbiol.* 66, 4112–4114.
- Hultberg, B., Isaksson, A., Nilsson, K., Gustafson, L., 2001. Markers for the functional availability of cobalamin/folate and their association with neuropsychiatric symptoms in the elderly. *Int. J. Geriatr. Psychiatr.* 16, 873–878.
- Hustad, S., Ueland, P.M., Vollset, S.E., Zhang, Y., Bjorke-Monsen, A.L., Schneede, J., 2000. Riboflavin as a determinant of plasma total homocysteine: effect modification by the methylenetetrahydrofolate reductase C677T polymorphism. *Clin. Chem.* 46, 1065–1071.
- Jacques, P.F., Kalmbach, R., Bagley, P.J., Russo, G.T., Rogers, G., Wilson, P.W., Rosenberg, I.H., Selhub, J., 2002. The relationship between riboflavin and plasma total homocysteine in the Framingham Offspring cohort is influenced by folate status and the C677T transition in the methylenetetrahydrofolate reductase gene. *J. Nutr.* 132, 283–288.
- Kil, Y.V., Mironov, V.N., Gorishin, I.Y., Kreneva, R.A., Perumov, D.A., 1992. Riboflavin operon of *Bacillus subtilis*: unusual symmetric arrangement of the regulatory region. *Mol. Gen. Genet.* 233, 483–486.
- Kim, Y.I., 1999. Folate and cancer prevention: a new medical application of folate beyond hyperhomocysteinemia and neural tube defects. *Nutr. Rev.* 57, 314–321.
- Klerk, M., Verhoef, P., Clarke, R., Blom, H.J., Kok, F.J., Schouten, E.G., 2002. MTHFR 677C->T polymorphism and risk of coronary heart disease: a meta-analysis. *J. Am. Med. Assoc.* 288, 2023–2031.
- Konings, E.J., Roomans, H.H., Dorant, E., Goldbohm, R.A., Saris, W.H., van den Brandt, P.A., 2001. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *Am. J. Clin. Nutr.* 73, 765–776.
- Krymchantowski, A.V., Bigal, M.E., Moreira, P.F., 2002. New and emerging prophylactic agents for migraine. *CNS Drugs* 16, 611–634.
- Kuipers, O.P., de Ruyter, P.G., Kleerebezem, M., de Vos, W.M., 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* 64, 15–21.
- La Vecchia, C., Negri, E., Pelucchi, C., Franceschi, S., 2002. Dietary folate and colorectal cancer. *Int. J. Cancer.* 102, 545–547.
- Lucock, M., 2000. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol. Genet. Metab.* 71, 121–138.
- Lyngso, R.B., Zuker, M., Pedersen, C.N., 1999. Fast evaluation of internal loops in RNA secondary structure prediction. *Bioinformatics* 15, 440–445.
- Mack, M., van Loon, A.P., Hohmann, H.P., 1998. Regulation of riboflavin biosynthesis in *Bacillus subtilis* is affected by the activity of the flavokinase/flavin adenine dinucleotide synthetase encoded by *ribC*. *J. Bacteriol.* 180, 950–955.
- McNulty, H., McKinley, M.C., Wilson, B., McPartlin, J., Strain, J.J., Weir, D.G., Scott, J.M., 2002. Impaired functioning of thermolabile methylenetetrahydrofolate reductase is dependent on riboflavin status: implications for riboflavin requirements. *Am. J. Clin. Nutr.* 76, 436–441.
- Melse-Boonstra, A., West, C.E., Katan, M.B., Kok F.J., Verhoef, P., 2003. Comparison of bioavailability of heptaglutamyl folic acid with monoglutamyl folic acid in healthy adults. *Am. J. Clin. Nutr.* (in press).
- O'Brien, M.M., Kiely, M., Harrington, K.E., Robson, P.J., Strain, J.J., Flynn, A., 2001. The efficacy and safety of nutritional supplement use in a representative sample of adults in the North/South Ireland food consumption survey. *Public Health Nutr.* 4, 1069–1079.
- Otto, R., ten Brink, B., Veldkamp, H., Konings, W.N., 1983. The relation between growth rate and electrochemical proton gradient of *Streptococcus cremoris*. *FEMS Microbiol. Lett.* 16, 69–74.
- Pero, J.G., Perkins, J.B., Sloma, A., 1991. Riboflavin overproducing strains of bacteria. EP0405370.
- Poolman, B., Konings, W.N., 1988. Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino acid transport. *J. Bacteriol.* 170, 700–707.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Stahmann, K.P., Revuelta, J.L., Seulerberger, H., 2000. Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. *Appl. Microbiol. Biotechnol.* 53, 509–516.
- Stover, P.J., Garza, C., 2002. Bringing individuality to public health recommendations. *J. Nutr.* 132, 2476S–2480S.
- Sybesma, W., Starrenburg, M., Mierau, I., Kleerebezem, M., De Vos, W.M., Hugenholtz, J., 2003. Increased production of folate by metabolic engineering of *Lactococcus lactis*. *Appl. Environ. Microbiol.* 69, 4542–4548.
- Van Guldener, C., Stehouwer, C.D., 2001. Homocysteine-lowering treatment: an overview. *Expert Opin. Pharmacother.* 2, 1449–1460.
- Vitreschak, A.G., Rodionov, D.A., Mironov, A., Gelfand, M.S., 2002. Regulation of riboflavin biosynthesis and transport genes in bacteria by transcriptional and translational attenuation. *Nucleic Acids Res.* 30, 3141–3151.
- Walter, J.H., Wraith, J.E., White, F.J., Bridge, C., Till, J., 1998. Strategies for the treatment of cystathionine beta-synthase deficiency: the experience of the Willink Biochemical Genetics Unit over the past 30 years. *Eur. J. Pediatr.* 157, S71–S76.
- Winkler, W.C., Cohen-Chalamish, S., Breaker, R.R., 2002. A mRNA structure that controls gene expression by binding FMN. *Proc. Natl. Acad. Sci.* 99, 15908–15913.
- Yamada, K., Chen, Z., Rozen, R., Matthews, R.G., 2001. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. *Proc. Natl. Acad. Sci.* 98, 14853–14858.