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Metabolic engineering of lactic acid bacteria for the production of nutraceuticals

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Abstract

Lactic acid bacteria display a relatively simple and well-described metabolism where the sugar source is converted mainly to lactic acid. Here we will shortly describe metabolic engineering strategies on the level of sugar metabolism, that lead to either the efficient re-routing of the lactococcal sugar metabolism to nutritional end-products other than lactic acid such as L-alanine, several low-calorie sugars and oligosaccharides or to enhancement of sugar metabolism for complete removal of (undesirable) sugars from food materials. Moreover, we will review current metabolic engineering approaches that aim at increasing the flux through complex biosynthetic pathways, leading to the production of the B-vitamins folate and riboflavin. An overview of these metabolic engineering activities can be found on the website of the Nutra Cells 5th Framework EU-project (www.nutracells.com). Finally, the impact of the developments in the area of genomics and corresponding high-throughput technologies on nutraceutical production will be discussed.

Lactic acid bacteria as cell-factories

Lactic acid bacteria (LAB) are industrially important microbes that are used all over the world in a large variety of industrial food fermentations. Their contribution in these fermentation processes primarily consists of the formation of lactic acid from the available carbon source resulting in a rapid acidification of the food raw-material, which is a critical parameter in the preservation of these products. However, besides their lactic acid forming capacity, LAB also have the ability to contribute to other product characteristics like flavour, texture and nutrition. Next to their most important application, which is undoubtedly in the dairy industry, LAB are also applied at an industrial scale in the fermentation of other food-raw materials like meat and vegetables.

Lactococcus lactis is by far the most extensively studied lactic acid bacterium, and over the last decades elegant and efficient genetic tools have been developed. 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. Especially, the nisin controlled expression (NICE) system for controlled heterologous and homologous gene expression in Gram-positive bacteria (de Ruyter et al. 1996; Kleerebezem et al. 1997; Kuipers et al. 1998) was proven to be very valuable and has been employed in most of the metabolic engineering strategies discussed here (Hols et al. 1999; Hugenholtz et al. 2000; Boels et al. 2001; Sybesma et al. 2002).

Initial metabolic engineering of *Lactococcus lactis* has focussed on rerouting of pyruvate metabol-

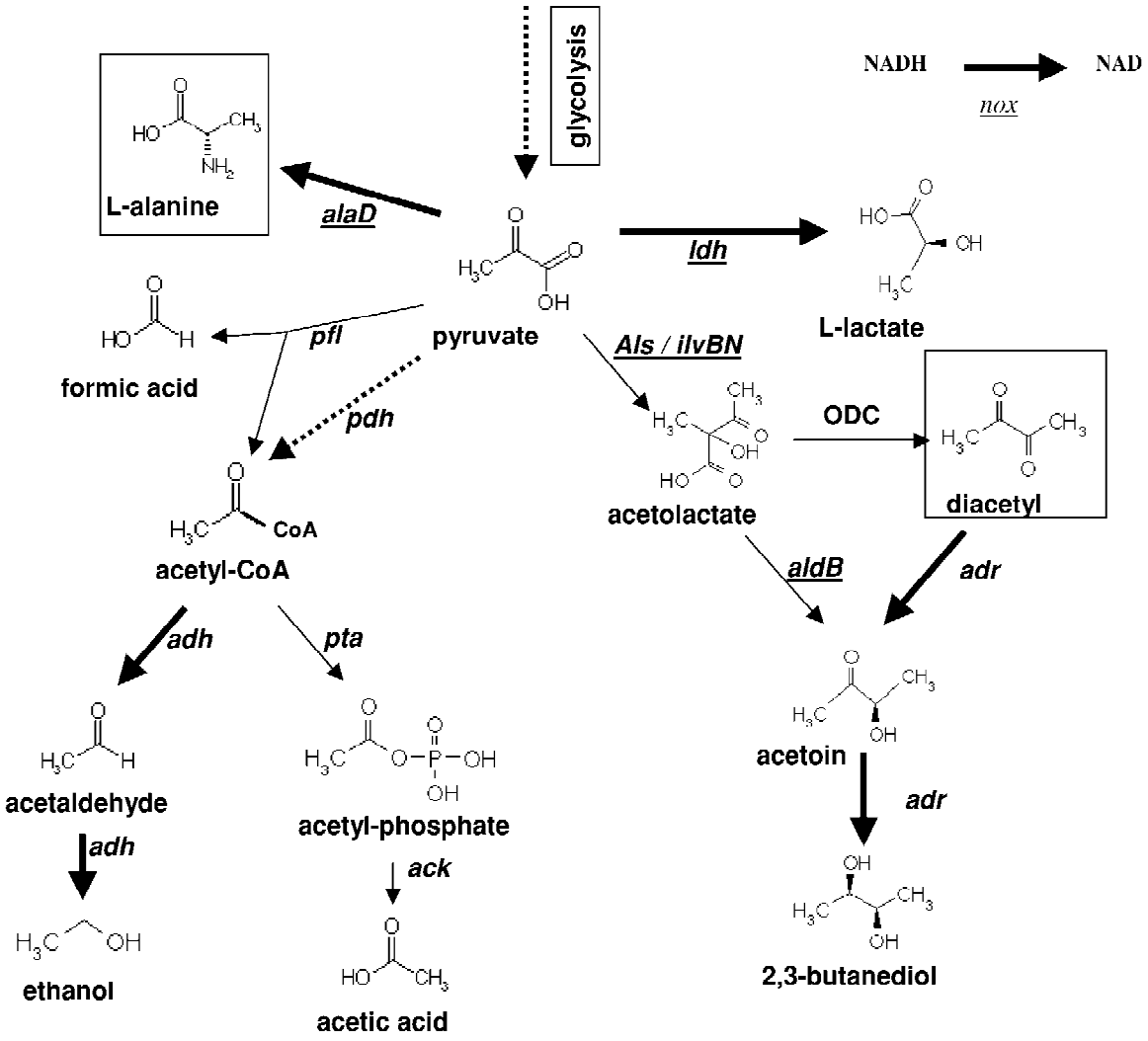

Figure 1. Schematic representation of pyruvate metabolism in *Lactococcus lactis*. NADH consuming (bold arrows) and producing (dotted arrows) and redox-neutral (normal arrows) conversions are indicated. The genes encoding the enzymes involved in individual conversions are indicated using abbreviations based on established genetic nomenclature. The heterologously introduced alanine dehydrogenase catalysed conversion (see text for further explanation) is added (top-left) and the conversion of acetolactate to diacetyl does not require an enzyme, but involves a chemical conversion (oxidative decarboxylation; ODC). Moreover, the reaction catalysed by the water-forming NADH-oxidase (see text for further explanation) is indicated. The metabolites that have been the target of metabolic engineering strategies (for more detail see text) are boxed, while the names of the genes that were targeted by genetic manipulations during these metabolic engineering procedures are underlined (for more detail see the text).

ism (Figure 1). Sugar metabolism was diverted away from lactate production towards production of α-acetolactate, the precursor of diacetyl, by either disruption of lactate dehydrogenase or overproduction of NADH oxidase. By combining this strategy with disruption of the *aldB* gene encoding for α-acetolactate decarboxylase, very effective diacetyl production from glucose and lactose was achieved. This example shows how a minor pathway in *Lactococcus lactis* could be boosted to become the major metabolic pathway, and how powerfull metabolic engineering can be in these, relatively simple, lactic acid bacteria. The extremely efficient production of L-alanine from sugar, was a subsequent achievement in metabolic engineering on the level of pyruvate metabolism. The (heterologous) *Bacillus sphaericus alaD* gene, encoding for alanine dehydrogenase enzyme, was cloned in *L. lactis*, using the NICE system (Hols et al. 1999). The alanine de-

hydrogenase enzyme converts pyruvate to L-alanine in the presence of ammonium (Figure 1). Upon introduction of this system in lactate dehydrogenase deficient lactococcal cells, a complete conversion of the pyruvate pool to alanine was obtained when cells were incubated under the appropriate, high ammonium, conditions (Hols et al. 1999). The alanine produced by these cells consisted of a mixture of both stereoisomers (D- and L-alanine) as a consequence of the endogenous alanine racemase activity of *L. lactis* (encoded by the *alr* gene). To obtain a stereo-specific L-alanine producing bioreactor, the *alr* gene was functionally disrupted in the lactate dehydrogenase deficient lactococcal strain. High level expression of alanine dehydrogenase in the resulting cells indeed led to stereo-specific L-alanine production as the only end-product of fermentation (Hols et al. 1999).

These two examples illustrate how the, basically simple, homofermentative metabolism of *Lactococcus lactis*, centred around lactate as sole fermentation product, can be redirected into efficient production of other, industrially important, (food) ingredients. In the remainder of this overview, we will present a number of examples where metabolic engineering of homofermentative lactic acid bacteria have led or will lead to efficient production of food ingredients (nutraceuticals) or food products with a beneficial effect for the consumer.

Nutraceuticals

The term 'Nutraceuticals', launched by Stephen DeFelici in the 1980s, defines a wide range of foods and food components with a claimed medical or health benefit (Pszczola 1992). An increasing number of food components are being labelled as such, often without proper scientific basis. In this overview, we will discuss the (increased) production of a range of metabolites produced by lactic acid bacteria, most of them definitely adding to the health status of (fermented) foods. Clear examples are the B-vitamins, riboflavin (B2), folate (B11) and cobalamin (B12). There is no doubt that these vitamins are essential in the human diet. The only discussion, especially for folate and cobalamin, centres on the protective role against a series of diseases, of increased dosage of these vitamins. Other examples of true nutraceuticals that can be produced by lactic acid bacteria are the low-calorie sweeteners. Production of alanine, a component with comparable sweetness as glucose and fructose was already discussed above. Production of sugars such as mannitol, sorbitol, and tagatose, as a result of metabolic engineering of lactic acid bacteria, will be described below. These sugars have similar sweetening power as sucrose and glucose, are all poorly degraded by the human body and, as such, can be considered low-calorie sugars. Oligosaccharides have a slightly weaker basis as true nutraceuticals. It is well established that some of these polysugars can actually stimulate growth of specific intestinal microorganisms, such as bifidobacteria, but a direct beneficial effect on the human health in more difficult to show. This holds even more true for food components such as antioxidants. Lactic acid bacteria are able to produce components such as glutathione and thioredoxin and overproduction is certainly feasible using metabolic engineering. However, the beneficial effect of increased intake of anti-oxidants on the human health is rather doubtful. In the final part of this overview, bacterial activities will be discussed that definitely contribute to the health benefit of food materials, but, as such, cannot really be considered nutraceuticals. We describe the use of the lactic acid bacteria to remove undesirable sugars present in the foods. Milk is a healthy and nutritious component of the human diet for only that part of the human population that is tolerant to the milk sugar, lactose. By removal of lactose (and galactose) from milk and liquid fermented dairy products, these food products would become suitable for the lactose-intolerant people. Strategies and actual examples will be shown where lactic acid bacteria are successfully used to achieve this goal. Similar solutions are considered for the raffinose and stachyose containing food materials, such as soybeans. Both these sugars are poorly metabolised by humans, and cause rapid growth of, often undesirable microorganisms, in the human intestine (Khane et al. 1994). Removal of these sugars from soy products would avoid intestinal problems after consumption.

Engineering of B-vitamin production

Folate

Folates are essential components in the human diet. They are involved, as cofactor, in many metabolic reactions, including the biosynthesis of nucleotides, the building blocks of DNA and RNA. The daily recommended intake for an adult is 200 μg. For pregnant women a double dose is recommended, since folates

are known to prevent neural-tube defect in newly borns (Wald et al. 1991). Moreover, folates are reported to protect against some forms of cancer (Ames 1999). In contrast, a low folate level in the diet is associated with high homocysteine levels in the blood and, consequently, with coronary diseases (Boushey et al. 1996; Brattstrom 1996). Folates are produced in different (green) plants (folium (latin)=leaf) and by some micro-organisms. Therefore, vegetables and dairy products are the main source of folates 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. Fermented milk products, especially yoghurt, are reported to contain even higher amounts of folate (Alm 1980).

The term 'folate' is a non-specific term referring to any folate compound with vitamin activity. In the general metabolism, folates act as acceptors or donors of C1-residues. Folate is isolated from natural sources in many different forms, in which the basic structure consists of a pteridine moiety, a *p*-aminobenzoic acid residue and one or more γ-linked L-glutamic acid residues. However, variation of the C1-residues (including methyl-, methenyl-, methylene-, formimino- or formyl-) substitutions at either the N-5 or N-10 position, combined with the variable lengths of the poly-γ-L-glutamate 'tail' (ranging from a single glutamyl residue to more than 10 residues) results in a large variety of chemical variants that are all clustered under the term 'folate' (Hamm-Alvarez et al. 1989). Finally, the pyrazine rings can be reduced to the so-called dihydro- and tetrahydro-form of folate. The polyglutamyl 'tail' is required for the intracellular retention of folate derivatives and plays a role in the binding efficiency of folate and substrates to enzymes involved in one-carbon transfer or inter-conversion of folic acid derivatives (McBurney & Whitmore 1974). Most mammals are not able to synthesise folates. Plants and more particular leafy vegetables and fruits and fermented dairy products are the main source of folates, but animal products like meat and milk also contain folate. Folates are taken up by mammals, including humans, in the small intestines. Only monoglutamyl-folate derivatives can directly be absorbed in the human gut. Polyglutamyl-folate derivatives with two or more glutamate residues have to be processed in the intestines by a mammalian enzyme deconjugase to release monoglutamyl-folate. The activity of the deconjugase enzymes is susceptible to inhibition by various constituents found in some foods (Rosenberg & Goldwin 1971; Bhandari & Gregory 1990; Seyoum & Selhub 1998). The level of monoglutamyl folates (MGF), and hence the bio-availability of folate, varies considerably between food products, i.e. egg yolk (72% MGF), cow liver (56% MGF), orange juice (21% MGF), cabbage (6% MGF), lima beans (5% MGF), and lettuce (less than 1% MGF, Seyoum & Selhub 1998).

Compared to vegetable sources, dairy and other fermented products have the advantage that at least part of the folate is stored intracellularly in (lactic acid) bacteria. As a result, the intracellularly stored folate – up to mM concentrations in the cytoplasm (Sybesma, personal communication) – may be better protected against the acid-peptic environment of the stomach, leading to a higher effective folate consumption.

Many fermented dairy products contain higher folate levels compared to raw milk. The explanation for this is that some of the starter bacteria used in dairy fermentation execute *de novo* biosynthesis of folates and secrete a surplus of these compounds into the growth medium. Examples are the yoghurt bacterium *Streptococcus thermophilus* and the cheese and butter(milk) bacterium *Lactococcus lactis*. This property of LAB offers the possibility to fortify fermented dairy products with folate by natural means, i.e. without the addition of food supplements. The natural diversity amongst dairy starter culture 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. Using this strategy, production of experimental yoghurt containing up to 150 μg/L folate has been reported (Smid et al. 2001). Besides selecting vitamin-producing lactic acid bacteria for inclusion in starter cultures, these lactic acid bacteria can also be supplied as probiotics or health-promoting additives in food or pharmaceutical products. These new applications of lactic acid bacteria will be boosted even more if folate production can be improved through optimisation of fermentation conditions or if LAB-variants (resulting from classical mutagenesis) with improved folate production capacities can be selected.

Total folate production in *L. lactis* strain MG1363 is approximately 100 ng/ml. Most of the folate (90%) is accumulated intracellularly, probably due to polyglutamylation of these intracellular folates. Only a small amount (10%) is released into the environment (Sybesma et al. 2002). Folate biosynthesis involves a multi-enzyme pathway, which is schematically presented in Figure 2. The genes involved in folate biosynthesis in *L. lactis* MG1363 have re-

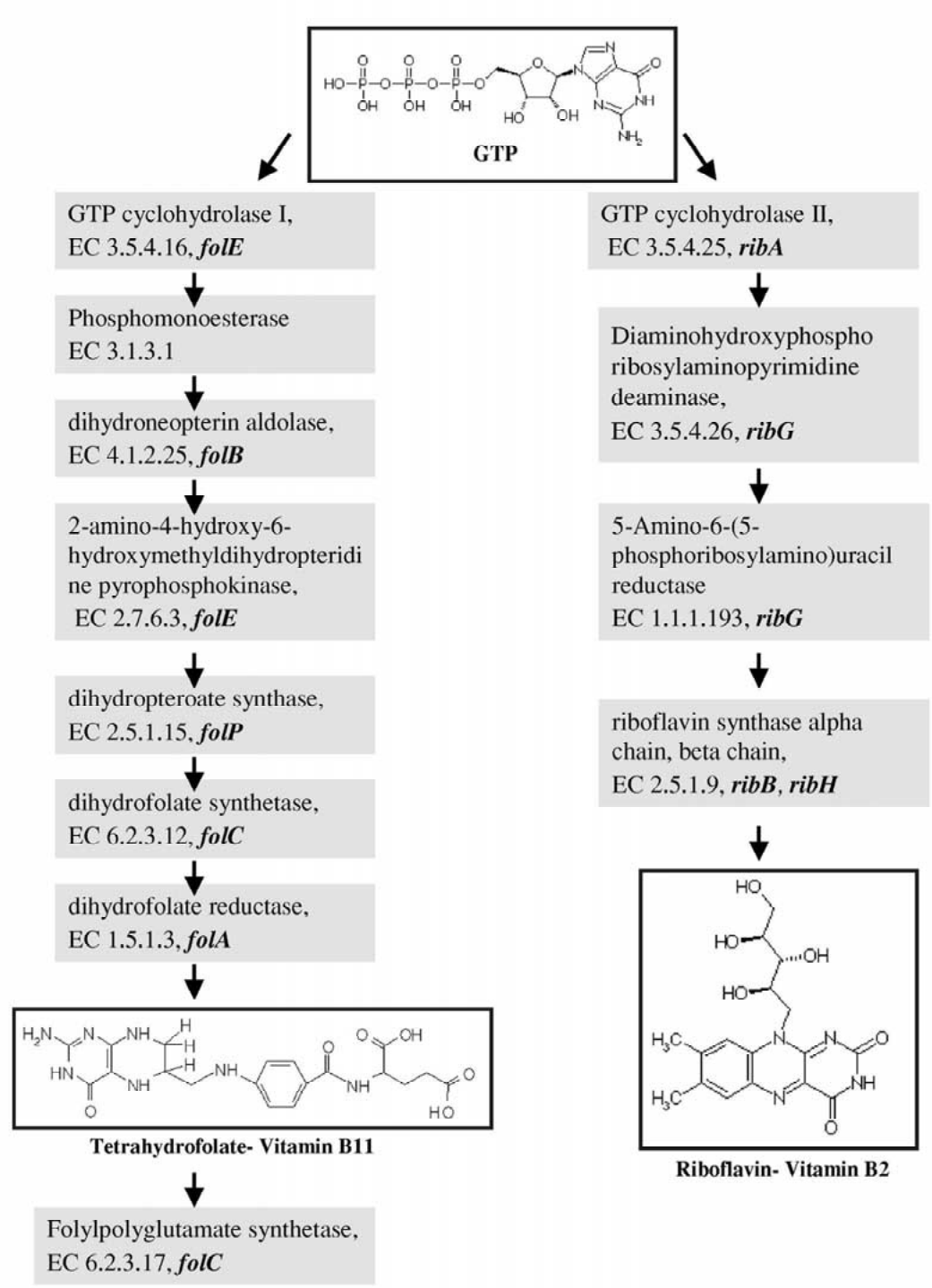

Figure 2. Biosynthetic pathway for folate (Vitamin B11) and for riboflavin (vitamin B2) (see text for more detail).

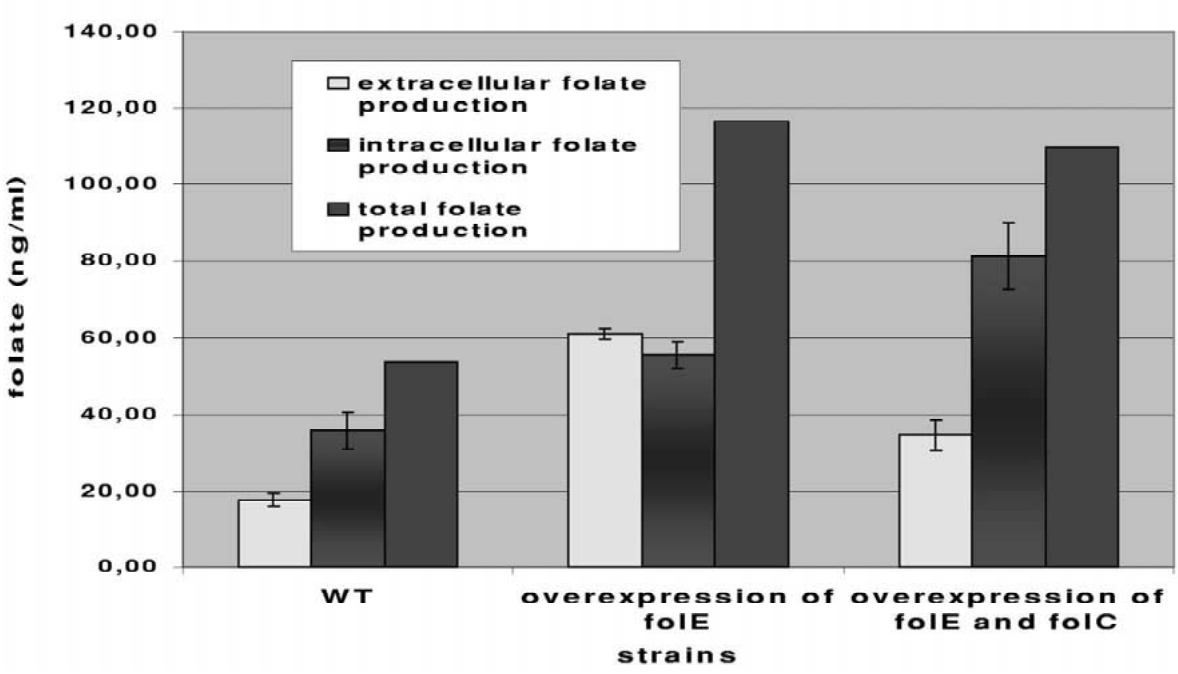

Figure 3. Extracellular, intracellular and total folate production by wild-type *Lactococcus lactis* overexpressing *folE (gch)* and *L. lactis* overexpressing both *folE* and *folC*. *L. lactis* was cultivated in chemically defined medium without folate.

cently been analysed. The availability of the folate biosynthesis genes allows efficient genetic engineering aiming at increasing the folate biosynthesis level in *L. lactis*. Several of the folate biosynthetic genes have been overexpressed individually, or in combination, in *L. lactis* strain NZ9000 using the NICE system. In Figure 3, a typical result is presented where the first enzyme in folate biosynthesis, GTP cyclohydrolase, is overproduced leading to a 3-fold increased production of folate. In addition, it results in altered ratios of monoglutamyl-folate over polyglutamyl-folate, resulting in higher release of the folate into the environment. The latter modulation of spatial distribution and glutamylation level of the folate produced by *L. lactis* would lead to improved uptake of this microbially produced vitamin in the human small intestine (see above), thereby generating improved folate bioavailability in fermented products produced with such strains. To further expand these bio-availability studies, *L. lactis* strains were constructed that express the γ-glutamyl hydrolase enzyme derived from rat or human origin (Yao et al. 1996). cDNA clones encoding these enzymes were cloned in the NICE system and introduced in *L. lactis* and it could be shown that both the human and rat enzymes could be expressed functionally in this organism. The γ-glutamyl hydrolase expression in growing *L. lactis* cultures, resulted in an inversion of folate spatial distribution, i.e. from mainly intracellular (ca. 90%) to extracellular accumulation (Sybesma et al. 2002). In the beginning of the stationary phase, the level of extracellularly produced folate is six times higher in the strain expressing γ-glutamyl hydrolase relative to the wild-type. These results demonstrate that the expression of human γ-glutamyl hydrolase in the folate producer *L. lactis* leads to intracellular deconjugation of polyglutamyl-folate to monoglutamyl-folate resulting in decreased retention of folate in the cell and, consequently, increased excretion of folate to the environment. It can be concluded that expression of heterologous γ-glutamyl hydrolase in *L. lactis* increases the extracellular monoglutamyl folate level, i.e. the level of bio-avalaible folate, thereby relieving the requirement for the intestinal hydrolase activity (Sybesma et al. 2002).

An important tool in the study of the (increased) bioavailability of folate, is a good methodology to separate and quantify the different folate molecules. HPLC-methods are now available to distinguish folates with different bound C1-moieties and with varying lengths of the polyglutamate-tail (Figure 4). Formyl-tetrahydrofolate and methylene-

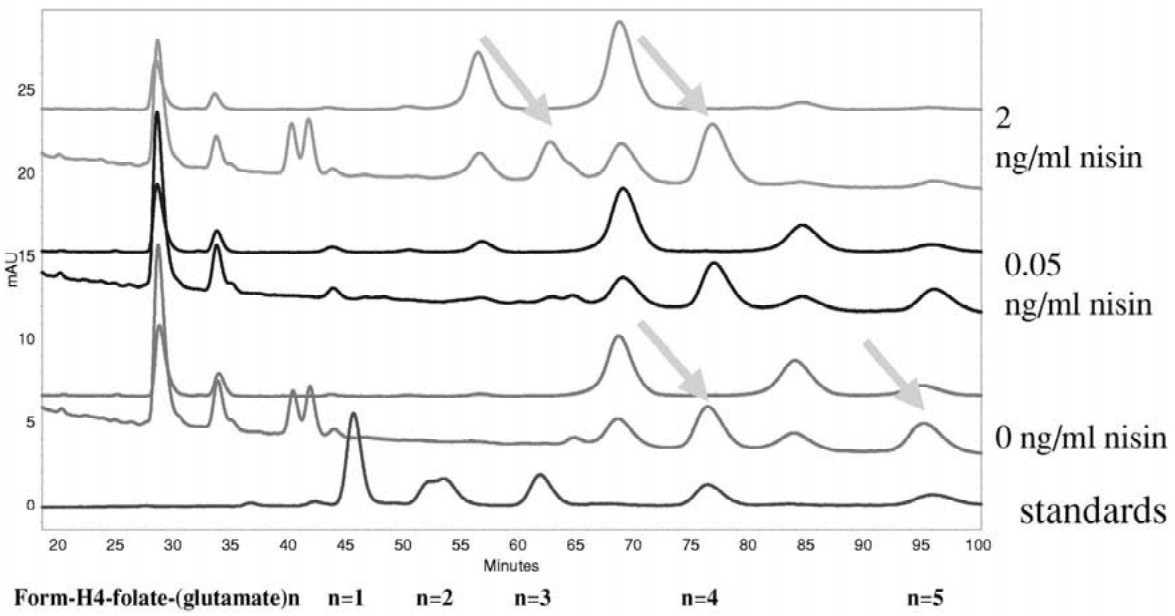

Figure 4. HPLC-analysis of intracellular folates produced by wild-type cells and cells producing increasing amounts of γ-glutamyl hydrolase. Analysis at 280 nm shows formyl-tetrahydrofolates with different polyglutamyl-tails and at 360 nm shows methenyl-tetrahydrofolates.

tetrahydrofolate represent the two major groups of folate in *Lactococcus lactis*. Preliminary results indicate that the major bound C1-moieties is not the same in all lactic acid bacteria, but that also the methyl-tetrahydrofolate form can be the major component as also found in many plant materials. The effect of the cloning strategies described above on the length of the polyglutamate-tail can be directly visualised via HPLC in for instance a clear change from predominantly tetra- and pentaglutamate folates into mostly monoglutamate-folates (Figure 4).

This work represents an important first step in the development of novel, functional foods with increased levels of bio-available folate. The identification of γ-glutamyl hydrolases from (food grade) bacterial origin may enhance the market introduction of a food grade micro-organism that has improved bio-available-folate production capacities. Moreover, metabolic engineering of the lactococcal folate biosynthesis pathway itself by changing expression levels of the relevant biosynthetic enzymes is expected to result in significant increases in folate production. These approaches could eventually be combined to generate starter bacteria that can be used for the production of novel fermented dairy products providing 100% instead of the current 15–20% of the human daily requirement for folate intake.

Other B-vitamins

Recent population studies performed all over the world have revealed that malnutrition is a frequently occurring phenomenon, also in the well-developed Western world. This is sometimes a direct result of medical treatment, but often also a result of social deprivation especially for the young and old. Malnutrition is specifically observed in deficiencies in B-vitamin content of the diet and/or the human blood. Besides deficiencies in folate, deficiencies in riboflavin (vitamin B2) and in cobalamin (vitamin B12) are most commonly observed. Riboflavin-deficiency can lead to various physiological effects such as liver- (Ross & Klein 1990) and skin-disorders (Lakshimi 1998), disturbed metabolism of the red blood cells (Hassan & Thurnham 1977) and, in general, reduced performance during physical exercise (Belko et al. 1983; Bates 1987). Some lactic acid bacteria, such as *Lactococcus lactis*, are able to produce and excrete small amounts of riboflavin into the surrounding medium. Based on this idea, fermented products could serve as valuable source of this vitamin for the human diet. The riboflavin is synthesised from the precursor (GTP) as is the case for folate (Figure 2), and the initial step in its biosynthesis involves a similar enzyme (GTP cyclohydrolase II) as present in the folate

biosynthetic pathway (see above). In *Bacillus subtilis*, this first reaction in riboflavin biosynthesis has been demonstrated to be rate limiting (Humbelin et al. 1999). The gene coding for this enzyme, *ribA*, has been brought to overexpression in *L. lactis* using the NICE-system. Similar as described for folate production, this metabolic engineering strategy resulted in a 3-fold overproduction of riboflavin. Further studies in this respect are focussed on obtaining natural mutants showing increased riboflavin-production by selecting for strains or variants that are resistant to analogues of purine and riboflavin, or their biosynthetic precursors, such as 8-azaguanine and decoyinine.

Another B-vitamin that is conspicuously low in many diets and repeatedly leads to deficiencies in humans, is vitamin B12 or cobalamin. This vitamin is produced by only a limited number of micro-organisms and, related to food fermentations, only very few food-grade micro-organisms are known to produce this vitamin. Up to now, no lactic acid bacteria have been described producing this essential co-factor in metabolism. In recent studies on *Lactobacillus reuteri*, some interesting observations were made on the production of a cobalamin-like substance, especially in the presence of glycerol which is converted in this organisms to propanediol (and to hydroxypropionaldehyde, reuterin) through a cobalamin dependent enzymatic step. Culture supernatant, containing this substance, was shown to complement vitamin B12-deficiency in auxotrophic *Lactobacillus delbrueckii* ATCC7830 and *Escherichia coli* 113 (*metE*-negative), indicating that we are dealing with a cobalamin or a related component. Molecular analysis of *Lactobacillus reuteri* has revealed the presence of at least five genes involved in the biosynthesis of vitamin B12. This is the first report of production of vitamin B12 by a lactic acid bacterium. Currently, studies are on the way to analyse the exact nature of the chromophore and to investigate the potential to produce more via metabolic engineering. The potential to produce a corrin-containing component such as cobalamin, should also open the way to produce other corrin-containing (bio)molecules such as cytochromes. Earlier studies have indicated that addition of heme, the precursor for cytochromes, to *Lactococcus lactis* cells leads to more efficient growth, presumably via the introduction of, normally lacking, an aerobic electron transport chain. Inducing production of cytochromes in lactic acid bacteria could be a whole new strategy to improve growth rates and growth yields of these important starter and probiotic bacteria.

Production of low-calorie sugars

Body-weight control is a major concern in our Western countries and obesity has been estimated to cost between 2 and 5% of the total health-care expenses of various developed countries. New food products containing low-calorie sugars and/or fat-replacers are in constant progression on the market in response to the consumer's request. We will discuss here the production by lactic acid bacteria of mannitol, sorbitol, tagatose and possibly some other low-calorie sugars through metabolic engineering.

Production of mannitol and sorbitol

Polyols, such as mannitol and sorbitol, are low-calorie sugars that could replace sucrose, lactose, glucose or fructose in food products as they display equivalent sweetness and taste (Dwivedi 1978; Debord et al. 1987). Mannitol can also serve as anti-oxidant in biological cells (Shen et al. 1997) as shown by increased survival of cells during freezing and/or drying in the presence of mannitol (Efiuvwevwere et al. 1999). Heterofermentative lactic acid bacteria such as *Leuconostoc mesenteroides* are known for their ability to produce mannitol in the fermentation of fructose (Soetaert et al. 1995). These bacteria convert part of the fructose for energy generation via the usual heterofermentative pathway while the other part of fructose is reduced directly to mannitol. In this way, high yield of mannitol is found, especially when fructose is supplied with f.i. glucose. Mannitol production, at low levels, has also been reported for homofermentative lactic acid bacteria under unusual conditions. In both *Lactobacillus plantarum* (Ferain et al. 1996) and *Lactococus lactis* (Neves et al. 2000), disruption of lactate dehydrogenase (LDH) resulted in production of a whole new range of metabolites, including mannitol. The mannitol is, most probably, produced by reduction of fructose-6-P to mannitol-1-P, which is subsequently dephosphorylated to yield mannitol (Figure 5). With this in mind, metabolic engineering strategies can be designed to induce significant mannitol production in these lactic acid bacteria. Overproduction of the mannitol-P dehydrogenase (MPDH) in a LDH-deficient *L. lactis* strain has already resulted in strong increase in, mostly, intracellular mannitol pro-

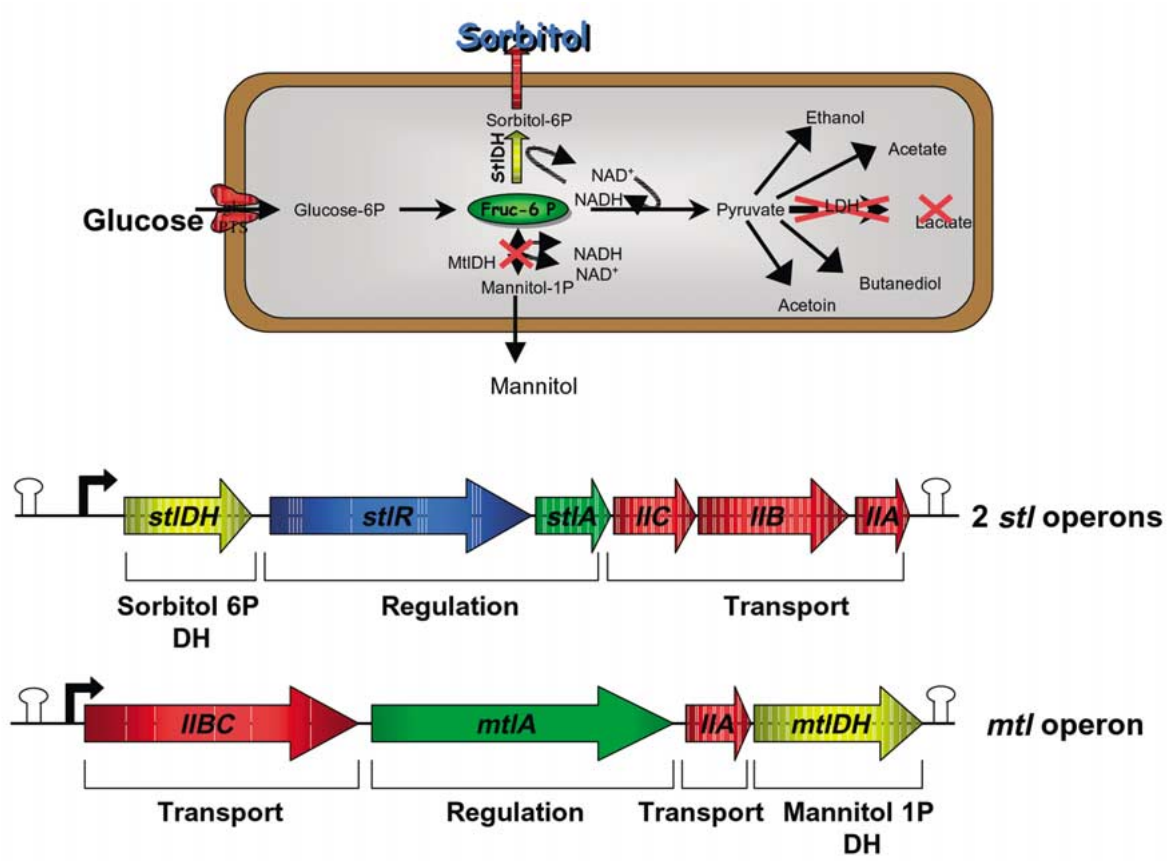

Figure 5. Schematic view of a *Lactobacillus plantarum* cell engineered for sorbitol production and organisation of sorbitol and mannitol genes.

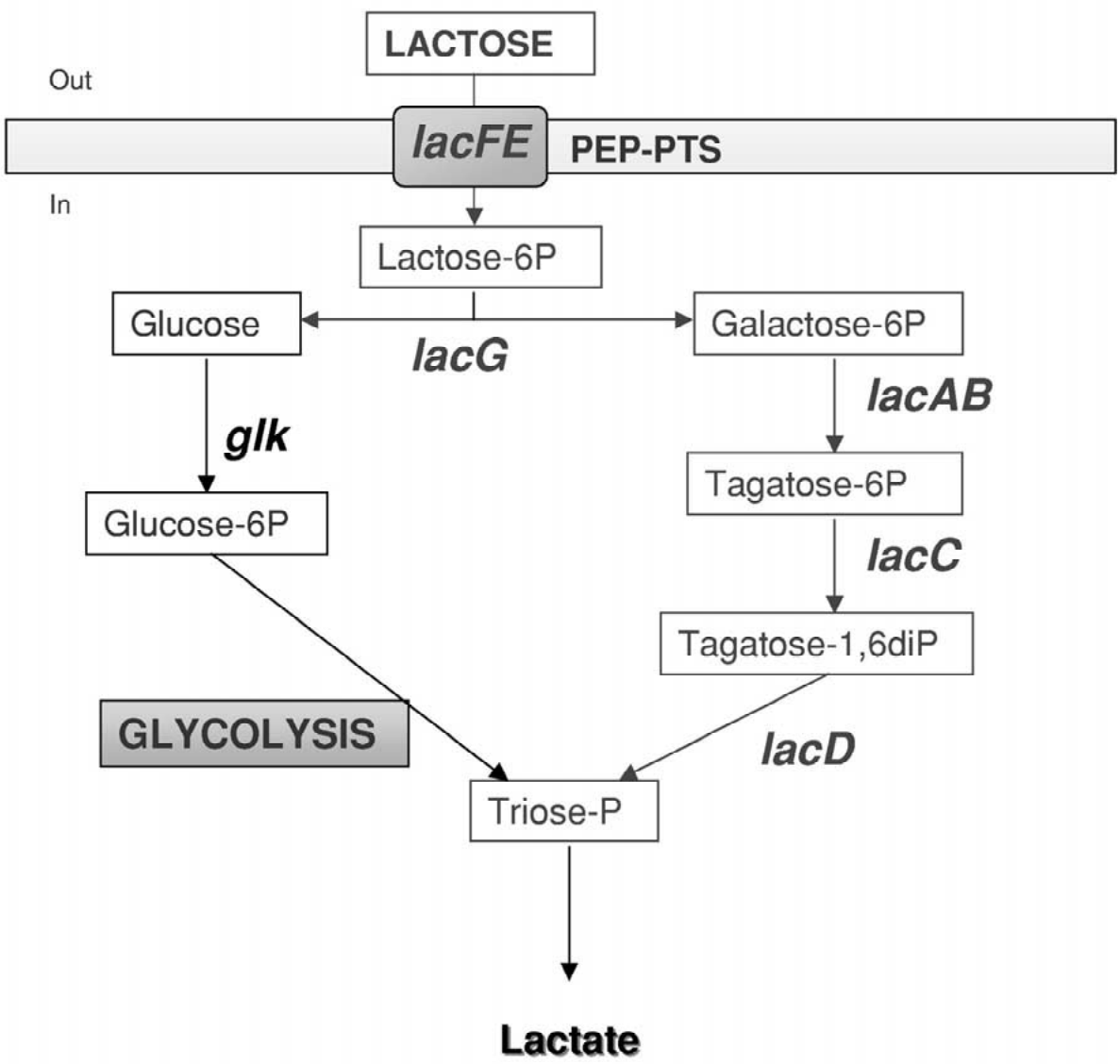

Figure 6. Pathway for lactose metabolism in *Lactococcus lactis* showing the tagatose-6-phosphate pathway encoded by the *lacABCD* genes (see text for more detail).

duction. Similar results were obtained when MPDH was overproduced in a strain with decreased phosphofructokinase (PFK) activity. Even higher production of mannitol by *Lactococcus lactis* can be expected if excretion of this polyol is facilitated, by introducing the mannitol-transporter present in *Leuconostoc mesenteroides*. Production of sorbitol can be induced in these bacteria in a similar way. By overexpression of the gene coding for sorbitol dehydrogenase, in combination with disruption of the MPDH and the LDH (Figure 5), considerable sorbitol production has been observed in *Lactobacillus plantarum* strains. Also in this case, even higher production of sorbitol is expected when the dephosphorylation and the transport (export) of sorbitol is enhanced.

Tagatose production

Tagatose is another carbohydrate that is considered as a potential sucrose replacement. It has almost equal sweetening power as sucrose – higher than similar components such as mannitol, sorbitol and erythritol – but much lower caloric value since it is poorly degraded by the human body (Zehner 1988). It has

recently been launched on the food market as low-calorie sugar, as prebiotic, and as anti-plaque agent by MD-foods (www.tagatose.dk). Their is no biological source available for extraction of tagatose so the current production process is by enzymatic conversion using arabinose as a substrate (Kim et al. 2001). For many practical reasons, a fermentation process involving intact microbial cells, is almost always the preferred process over an enzymatic conversion. A biotransformation process, from galactitol to tagatose, has recently been described using acetic acid bacteria (Manzoni et al. 2001). However, economy-wise this does not seem to be applicable since galactitol, although much more abundant and cheaper than tagatose, is still a relatively expensive substrate. The use of lactic acid bacteria could, very well, be an attractive alternative. There is only one metabolic pathway that features tagatose and that is in the degradation of lactose as found in lactic acid bacteria and a few other micro-organisms. The so-called tagatose-6-P pathway (van Rooijen et al. 1991) is responsible for breakdown of the galactose moiety during lactose metabolism by bacteria such as *Lactococcus lactis*. After hydrolysis of lactose-P, through the action of phospho-β-galactosidase, the glucose is degraded through glycolysis. The galactose-P moiety is converted into tagatose-6-P catalysed by galactose-P isomerase, then phosphorylated forming tagatose-diphosphate catalysed by tagatose-phosphate kinase and finally hydrolysed, by tagatose-diphosphate aldolase, to form the glycolytic intermediates dihydroxyaceton-P and glyceraldehyde-P (Figure 6). The genes encoding for this tagatose pathway – *lacABCD* – in *Lactococcus lactis* can be found on the lactose operon (Figure 6). Using *L. lactis* as micro-organism with its relatively simple carbon metabolism, its broad arsenal of genetic tools, including an efficient gene integration system and its very efficient system (NICE) for overproduction of enzymes, this lactic acid bacterium was chosen as potential cell factory for the production of tagatose. To induce accumulation of tagatose-derivatives in lactococcal cells, the initial chosen strategy was to disrupt the *lacC* and/or *lacD* genes resulting in production of either tagatose-6-P or tagatose-1,6-diphosphate. Disruption of *lacD* was accomplished via a two step recombination process, involving integration of an erythromycin-resistance plasmid containing only the *lacC* and *lacF* genes via single crossing-over, followed by removal of *lacD* (or reversion to the wild-type) in a second, spontaneous, recombination event ('double cross-over'). The double crossing-over seemed to occur in almost the expected 50% of the cases, so the desired mutant was obtained relatively easy. Growth rates of the *lacD*-negative mutant on glucose were identical to the wild-type, but growth on lactose was severely compromised in the engineered strain. This was already a good indication that an unusual physiological response was occurring in the construct. HPLC-analysis focussing on phosphorylated metabolic intermediates, revealed the accumulation of a unique metabolite in the constructed strain, in comparison with the wild-type where basically the glucose- and fructose-phosphates were visible. Spiking the samples with specially for this purpose synthesised tagatose-phosphate and tagatose-biphosphate, gave strong indication that the unique metabolite was indeed tagatose-1,6-diphosphate. So, *L. lactis* was successfully transformed in a tagatose (phosphate) producing cell-factory. However, for the use in practical (food) situations, strategies will have to developed to dephosphorylate the tagatose-diphosphate and excrete the final product, tagatose.

Production of polysaccharides

Exopolysaccharides (EPS)

The exopolysaccharide (EPS) molecules produced by different lactic acid bacteria display a wide variety of chemical and biophysical properties, including variations in sugar composition, sugar linkage, polymer length and polymer branching. These polymers have an important role in the rheology and texture properties of fermented food products, and thus are of interest for food applications as *in situ* produced, natural bio-thickeners. Moreover, they could also be applied as natural, food-grade additives, replacing presently applied stabilisers and thickeners that are produced by non food-grade bacteria (Sutherland 1998; Becker et al. 1998). Interestingly, it has been suggested that some polysaccharides produced by lactic acid bacteria have prebiotic (Gibson & Roberfroid 1995), immunostimulatory (Hosono et al. 1997), antitumoral (Kitazawa et al. 1991) or cholesterol-lowering activity (Nakajima et al. 1992a). Over the last decade, the production of exopolysaccharides (EPS) by lactic acid bacteria has been extensively studied (for recent review see de Vuyst & Degeest 1999), generating important advances in our knowledge of EPS-genetics, biological diversity and distribution, bio-

synthetic pathways, metabolic models, and physics. This knowledge can now be applied to rationally design metabolic engineering studies to modify EPS production and composition.

The production of EPS by *L. lactis* is associated with strains that are isolated from highly viscous fermented milk products. The specific *eps* genes are encoded on large plasmids that can be conjugally transferred from one lactococcal strain to the next, thereby introducing the EPS-producing capacity in the recipient strain (Vedamuthu & Neville 1986; von Wright & Tynkkynen 1987; van Kranenburg et al. 1997). One of the *eps* gene clusters of *L. lactis* that is characterised in most detail, is that of strain NIZO B40 (for a review see Kleerebezem et al. 1999). This strain produces a polymer with a regular repeating unit, →4)[α-L-Rha*p*-(1→2)][α-D-Gal*p*-1-PO_4-3]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→, that is structurally identical to that produced by strain SBT 0495 (Nakajima 1992b; van Kranenburg et al. 1997). The NIZO B40 strain harbours a 42,180 bp EPS-plasmid pNZ4000 (van Kranenburg et al., 2000), containing the 12 kb NIZO B40 *eps* gene cluster with 14 co-ordinately expressed genes, *epsRXABCDEFGHIJKL* (Figure 7). Based on sequence comparisons putative functions could be assigned to several *eps* genes, predicting their involvement in biosynthesis of the repeating unit oligosaccharides by the sequential addition of sugars to a membrane-anchored lipid carrier, and subsequent export and polymerisation of these lipid-linked oligosaccharides. Functional analysis of the *epsDEFG* genes, encoding glycosyltransferases, generated a model for the biosynthesis of the B40-EPS repeating unit (van Kranenburg et al. 1999a).

Oligosaccharides

Current studies on *L. lactis* NIZO B40 *eps* genetics focus on the elucidation of the individual role of the genes that are predicted to be involved in export (*epsK*), polymerisation (*epsI*) and chain length determination (*epsA*, *epsB*) (van Kranenburg et al. 1997) (Figure 7). These studies might eventually allow directed manipulation of these functions, which should result in the production of polysaccharides that have shorter (oligosaccharides) or longer chain lengths compared to the native B40-polymer. Moreover, the strong increase in the available *eps* gene cluster sequences from various organisms, including several lactic acid bacteria, has generated a wealth in genetic information including a large number of glycosyltransferase encoding genes. The genetic information combined with biochemical information regarding donor- and acceptor-specificity of individual glycosyltransferase enzymes, could be exploited in a glycosyltransferase combinatorial approach aiming at construction of the biosynthetic machinery for the production of oligo- and/or polysaccharides in *L. lactis* that have a predetermined and novel structure. The oligosaccharide structure and composition will ultimately determine its functionality as a nutraceutical. For instance, fructose-containing oligosaccharides are claimed to, specifically, stimulate the growth of Bifidobacteria in the human intestine (Gibson & Wang 1994), while phosphorylated oligosaccharides display the best fiber-like properties in the protection of intestinal mucosa against oxidative damage (Kawaguchi et al. 1999). The latter approach requires that the export and polymerisation machinery do not display a unique specificity for its corresponding, native polysaccharide. Interestingly in this respect is the observation that heterologous expression of the *Streptococcus thermophilus* Sfi6 *eps*-gene cluster in *L. lactis* resulted in the production of a polysaccharide containing an altered repeating unit compared to the polymer produced in its original host (Stingele et al. 1999). These results show that neither the glycosyltransferases, nor the export and polymerisation machinery encoded by this *eps* gene clusters exhibit absolute specificity for their native substrate and product, suggesting that the approach above could be successful. Moreover, a first example of such an approach is the possibility to functionally exchange glycosyltransferases with identical substrate specificity originating from different genera in Gram-positive bacteria (van Kranenburg et al. 1999b). Current and future *eps* engineering strategies should clarify the possibilities of these approaches towards the production of 'designer'-polysaccharides.

Improving sugar conversion

Lactose/galactose removal

Lactose is present in amounts of 4–4.5% (w/v) in cow milk. In liquid fermented dairy products, such as yoghurt or buttermilk, usually less than half of this is fermented to lactic acid. This makes all these liquid dairy products unsuitable for the lactose-intolerant population and deprives them of all the nutritious components that are present in milk such as pro-

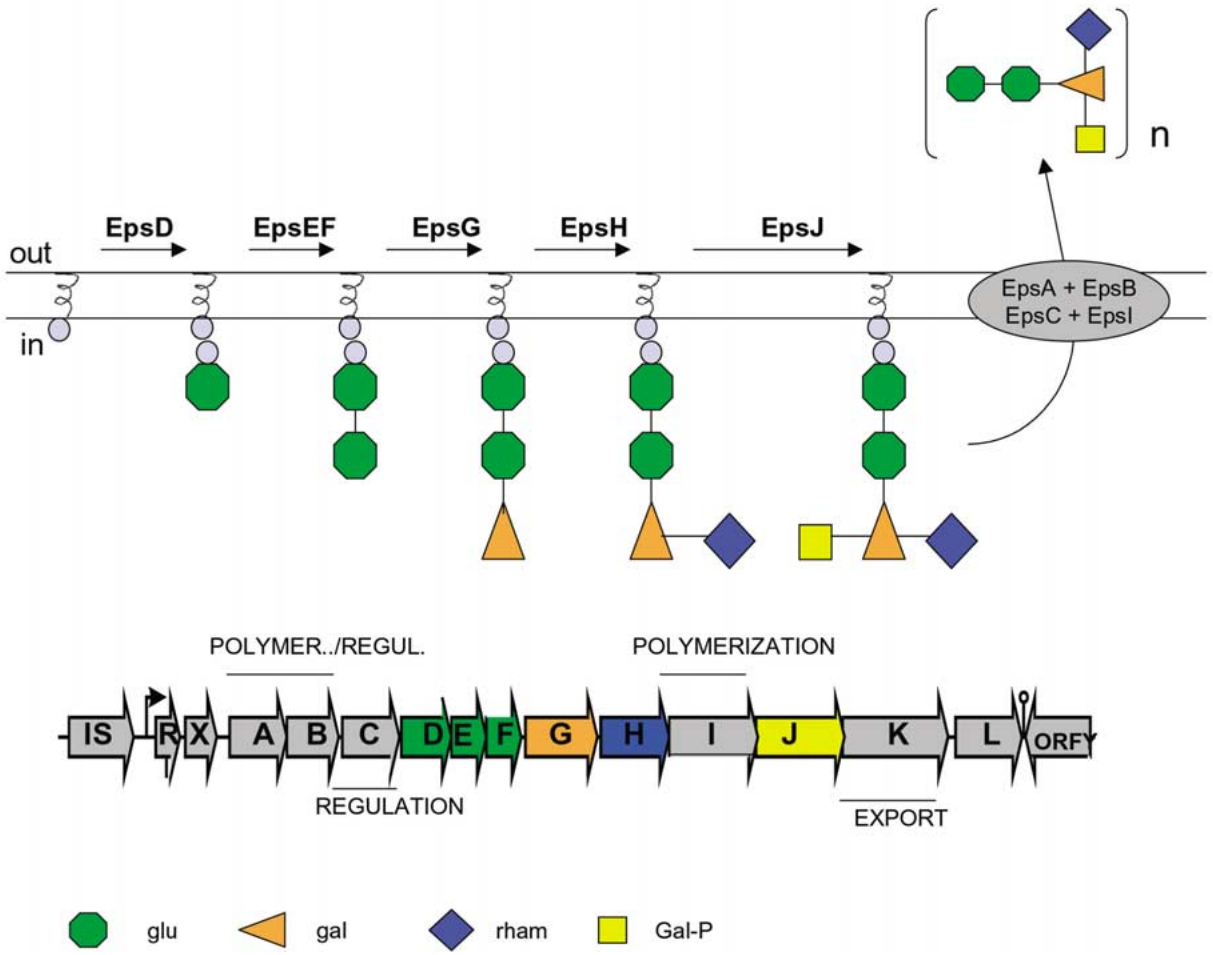

Figure 7. Schematic representation of the *L. lactis* NIZO B40 *eps* gene cluster and its flanking regions. The predicted individual roles of the *eps* genes in repeating unit biosynthesis (schematically indicated above the gene-cluster) and subsequent export, polymerisation and chain length determination are indicated; the role of *epsD*, *epsE*, *epsF* and *epsG* have been established, biochemically (see text for more detail).

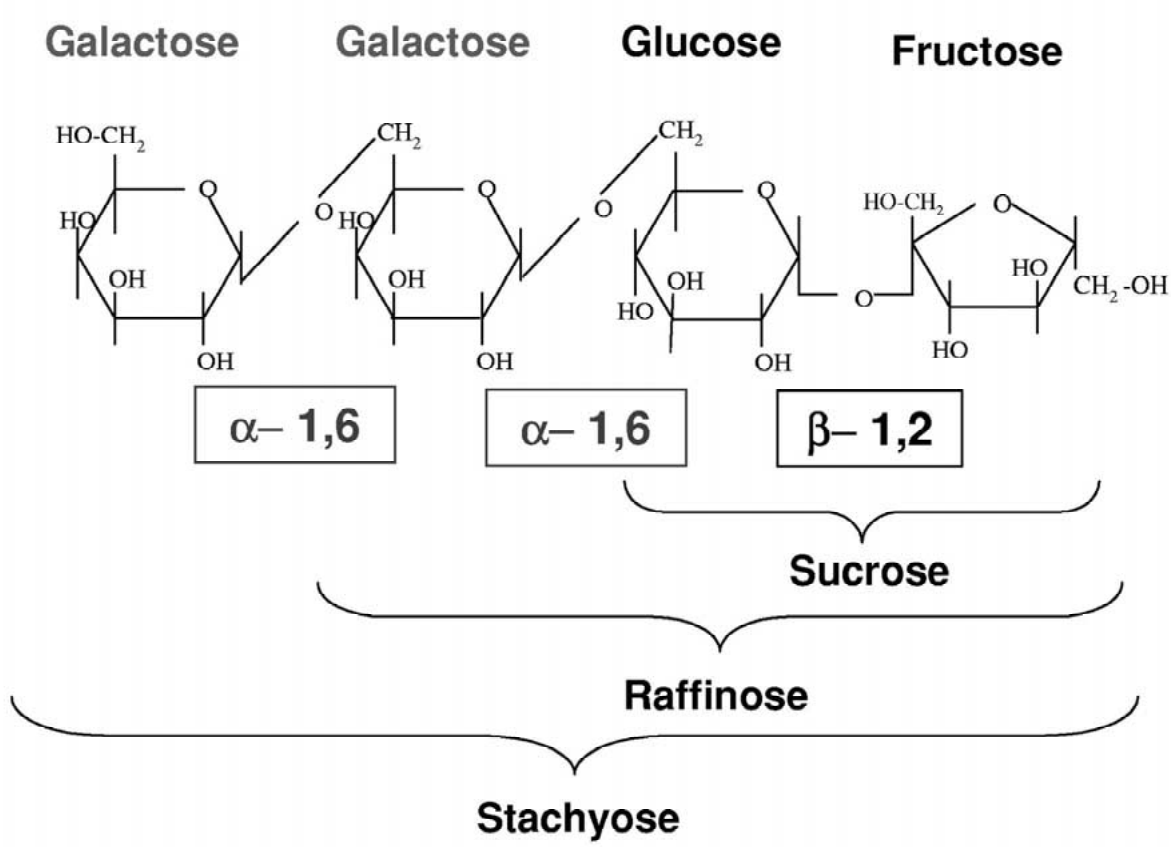

Figure 8. Structure of the (oligo)saccharides stachyose, raffinose and sucrose.

tein, minerals (calcium) and essential vitamins. A way to circumvent this problem is by developing processes to free these dairy products from lactose. This can be achieved by enzymatic treatment of milk with β-galactosidase or, more elegantly, by fermentation of milk with starter cultures having elevated β-galactosidase activities.

Another potential problem associated with the presence of lactose in milk, is the fact that, quite often, galactose is formed, and excreted in to the milk, during fermentation of lactose. This occurs readily in yoghurt where lactose is rapidly converted by both yoghurt bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, but only the glucose moiety appears to be metabolised. Galactose is excreted in almost stoichiometric amounts by the lactic acid bacteria (Veenhoff & Poolman 1999) resulting in accumulation of galactose upto concentrations of 1% and even higher. Besides being a poor sugar source for the human body, galactose presents another problem that too high consumption can lead to reduction of galactose to galactitol, especially when consumed together with alcohol. When galactitol accumulates in tissue it can lead to all kinds of physiological disorders such as cataract in the eye lens (Hirasuka et al. 1992).

For most lactic acid bacteria, galactose is a poor substrate. Growth is often retarded and galactose conversion often leads to production of other metabolites than lactic acid (Grossiord et al. 1998). Many yoghurt bacteria, especially the fast acidifying strains used as yoghurt starters, do not seem to utilise the galactose-moiety at all during yoghurt fermentation. Free galactose is accumulated intracellularly as a result of the absence of galactokinase activity in these strains. The strains use the excretion of galactose as actual driving force for the rapid uptake of lactose, in a one-to-one exchange reaction, allowing extremely fast growth of these bacteria on this sugar with doubling times that are even lower than 10 min. Molecular analysis of the galactose (and lactose) gene cluster in these strains of *Streptococcus thermophilus*, has shown that the gene for galactokinase is completely

intact, but that one or more point mutations have taken place leading to a 'silent' phenotype (Vaughan et al. 2001). These mutations can, sometimes, spontaneously revert, especially when the bacteria are cultured for prolonged times on galactose. The use of these revertants in yoghurt production could be a way to produce galactose-free yoghurt, although it does lead to reduced growth rate, and more serious for practical purposes, reduced acidifcation rates.

Removal of raffinose

Not all oligosaccharides are beneficial in the human and animal diet. Soy- and pulse-derived food products contain high levels of α-galactosides such as stachyose and raffinose (Figure 8).

Humans and other monogastric animals do not produce intestinal α-galactosidase, the enzyme responsible for the degradation of these oligosaccharides. Thus, α-galactosides pass into the lower gut where they are fermented by gas-producing bacteria. Consequently, consumption of soy and pulses induces intestinal disturbance such as flatulence in all monogastric animals (Khane et al. 1994). Removal of the α-galactosides, through enzymatic treatment of the soy milk, has already been described successfully (Maity & Paul 1991), but does not seem to be an economically feasible process. The use of lactic acid bacteria, for prefermentation or as probiotic, could well present the perfect solution for removing undesirable sugars from soy.

The ability to hydrolyse α-galactosides has already been identified in a number of lactic acid bacteria (Ahrne & Molin 1991; Garro et al. 1996). By applying metabolic engineering strategies, lactic acid bacteria will be constructed with high α-galactosidase activities as starters for removal of α-galactosides during soy fermentation and as possible probiotics to deliver α-galactosidase activity in the gut for prevention of flatulence. The first step in this strategy has been the cloning of the relevant gene (*melA*) coding for α-galactosidase from *Lactobacillus plantarum* (Silvestroni et al. 2002). The gene encodes a 738-amino acid protein (theoretical MW=84 kDa) and is associated with genes involved in raffinose and galactose metabolism (*rafP* for a putative raffinose permease, *galM* for galactose epimerase and *lacLM* for β-galactosidase). The *melA* gene could complement an α-Gal deficient *Escherichia coli* strain strongly suggesting that the *melA* gene is functional in *Lb. plantarum*. Additional evidence for functionality is provided by regulation studies showing that *melA* is actually induced 4-fold in the presence of the α-galactoside melibiose as growth substrate in comparison to glucose as growth substrate. For construction of starter and probiotic bacteria with high α-galactosidase activity, the *melA* is being cloned in *Lactococcus lactis* in three different constructions resulting in: (1) expression of the enzyme in the cytoplasm for maximum protection of enzyme activity, (2) expression as a secreted enzyme for maximum exposure to the sugar substrate, or (3) expression on the surface but anchored to the surface of the cell. The first construct would provide a suitable starter for α-galactoside-removal during soy fermentation, the second construct would serve best as cell-factory for α-galactosidase production to be used as means for (bio)chemical removal of stachyose and raffinose from soy- and pulse-material, while the third construct will be applied as probiotic to supply high α-galactosidase activity in the gut for assistance in α-galactoside degradation.

Impact of genomics and related technologies

We have presented a number of examples where activity of lactic acid bacteria have lead or can lead to healthier fermented food products or to the production of food ingredients with a clear health benefit. This has been achieved through altered expression of relevant homologous or heterologous genes. With the rise of (functional) genomics research, we can expect a tremendous boost in this type of development. The current list of publicly available complete genome sequences already exceeds 60 microbes as indicated on the Comprehensive Microbial Resource homepage of the TIGR-Website (www.tigr.org) and is exponentially growing. Currently, only one LAB genome – *Lactococcus lactis* – has been published (Bolotin et al. 2001), but several others are expected shortly (see other reports LAB 7). The genome is the 'blueprint' of an organism and tells us directly what is the potential of the organism to produce certain metabolites and presents us with the relevant genes that could be targeted for overexpression or disruption in order to reach increased functionality. In addition, by comparative genomics it has become relatively easy to determine why certain bacteria cannot produce specific nutraceuticals and how production of these specific components can be introduced. For control or improvement of production levels, however,

more is needed than only the genome sequence. Information is needed on how the flux through a specific pathway is controlled and how the activity of an individual pathway relates to other pathways. Kinetic, metabolic models have become available describing the dynamics of (pyruvate) metabolism in homofermentative lactic acid bacteria (Garrigues et al. 1997; Hoefnagel et al. 2002). However, these models seem, so far, only to be suitable for relatively simple metabolic pathways and are basically able to calculate, and simulate, the flux through the major metabolic pathways, but are much less accurate in describing pathways with much lower metabolic fluxes. The latter situation holds true, especially, for most biosynthetic pathways. The metabolic flux through these pathways is in most cases many orders of magnitude lower compared with the flux through catabolic pathways. Moreover, the enzymes involved generally have much lower activities and the metabolic intermediates and end-products are produced at much lower levels. In some cases this general problem can be solved by dissection of complicated (biosynthetic) pathways into several, simplified units and modelling each unit separately. However, in many cases this is just not possible. Even simple flux analysis can be an impossible task in metabolic networks involving loops and cycles. To analyse these networks, more advanced measurement techniques have been used successfully, such as the application of ^{13}C-NMR with metabolic substrates labelled at a specific position. Variation in the level of label-scrambling resulting from either enantiomer-specific or enantiomer-unspecific enzymes, will generate insight in control and distribution of different fluxes within a metabolic network (Rollin et al. 1995; Klapa et al. 1999; Park et al. 1999; Schmidt et al. 1999; de Graaf et al. 2000). However, it cannot provide insight in metabolic regulation, nor can it be used to predict fluxes under conditions that have not been tested. The optimistic vision that the knowledge of a (microbial) genome will automatically provide us with a comprehensive model of overall metabolism seems quite unrealistic for the reasons discussed above. Extensive experimentation on the physiology, enzymology and molecular biology of the organism is required before any practical modelling can be performed. The rapid development of genomics based high-throughput technologies, including transcriptomics, proteomics and especially metabolomics, might allow the effective analysis of the differential, global responses in micro-organisms upon the changing of environmental conditions or upon the engineering of specific enzyme activities.

With the genome sequence in hand of many industrial micro-organisms, traditional mutagenesis and natural selection of mutants has become a more directed and controlled activity and can now progress in parallel with the metabolic engineering approaches as described in this overview. It now seems possible to induce and select mutants that show f.i increased B-vitamin production, in a directed way without the use of recombinant DNA-techniques. The rise of high-throughput-screening facilities that, automatically and simultaneously, can sample and analyse thousands of samples is a powerful tool in this type of development. Many improved starter bacteria and cell factories for high nutraceutical production will be discovered by these natural selection strategies and have the huge advantage over engineered lactic acid bacteria that they can be implemented promptly in actual industrial fermentation. In addition, the molecular analysis of the selected mutants will provide very relevant information on how to effectively improve production of the desired nutraceutical. Many high-throughput molecular methods have been developed over the last few years to specifically pinpoint single nucleotide polymorphism (SNP) in these (natural) mutants, through direct Southern-blotting (Sokinenko 2001), through the use of whole-genome (Cutler et al. 2001) or pathway-specific DNA microarrays (Huber et al. 2002), or by specific mass-labelling techniques such as the Masscode System (Qiagen, Inc) that identify, by mass spectrometry, polymorphism directly in PCR-products (Kokoris et al. 2000).

Most examples of effective metabolic engineering in lactic acid bacteria discussed above deal with rerouting the major metabolic flux in these bacteria, i.e. the conversion of sugar into lactic acid. The results obtained include high production of metabolites that otherwise are not produced or produced in low amounts. For reasons discussed above, the engineering of more complex (biosynthetic) pathways and the analysis of the results of these engineering strategies is a much more complicated affair. Nevertheless, some promising results are already available for *Lactococcus lactis* in the case of folate and riboflavin biosynthesis. Future analyses of this and other complex pathways will provide us with valuable knowledge concerning the potential of LAB as cell factories for biologically active compounds like vitamins, low-calorie sugars or other nutraceuticals.

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References

Ahrne S & Molin G (1991) Spontaneous mutations changing the raffinose metabolism of *Lactobacillus plantarum*. Antonie van Leeuwenhoek 60: 87–93.

Alm L (1980) Effect of fermentation on B-vitamin content of milk in Sweden. J. Dairy Sci. 65: 353–359.

Ames BN (1999) Micronutrient deficiencies cause DNA damage and cancer. Food Sc. Agric. Chem. 1: 1–15.

Bates CJ (1987) Human riboflavin requirements and metabolic consequences of deficiency in man and animals. World Rev. Nutr. Diet 50: 215–265.

Becker A, Katzen F, Pühler A & Ielpie L (1998) Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Appl. Microbiol. Biotechnol. 50: 145–152.

Belko AZ, Obarzanek E, Kalkwarf HJ, Rotter MA, Bogusz S, Miller D, Haas JD & Roe DA (1983) Effects of exercise on riboflavin requirements of young women. Am. J. Clin. Nutr. 37: 509–517.

Bhandari SD & Gregory JF (1990) Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity. Am. J. Clin. Nutr. 51: 87–94.

Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD & Sorokin A (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 11: 731–753.

Boushey CJ, Beresford AA, Omenn GS & Moltulsky AG (1996) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J. Amer. Med. Assoc. 274: 1049–1057.

Brattstrom L (1996) Vitamins as homocysteine-lowering agents. J. Nutr. 126 (4 Suppl): 1276S–1280S.

Covert MW, Schilling CH, Famili I, Edwards JS, Goryanin II, Selkov E & Palsson BO (2001) Metabolic modeling of microbial strains *in silico*. Trends Biochem. Sci. 26: 179–186.

Cutler DJ, Zwick ME, Carrasquillo MM, Yohn CT, Tobin KP, Kashuk C, Mathews DJ, Shah NJ, Eichler EE, Warrington JA & Chakravarti A (2001) High-throughput variation detection and genotyping using microarrays. Genome Res 11: 1913–1925

Debord B, Lefebvre C, Guyot-Hermann AM, Hubert J, Bouche R & Guyot JC (1987) Study of different forms of mannitol: Comparative behaviour under compression. Drug Develop. Ind. Pharm. 13: 1533–1546.

De Graaf AA, Mahle M, Mollney M, Weichert W, Stahmann P & Sahm M (2000) Determination of full 13C isotopomer distributions for metabolic flux analysis using heteronuclear spin echo difference NMR spectroscopy. J. Biotechnol. 77: 25–35.

de Ruyter PGGA, Kuipers OP & de Vos WM (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62: 3662–3667.

de Vuyst L & Degeest B (1999) Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev. 21: 153–177.

de Vos WM (1996). Metabolic engineering of sugar catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 70: 223–242.

Dwivedi BK (1978) Low Calorie and Special Dietary Foods. CRC Press, Inc, West Palm Beach.

Efiuvwevwere BJO, Gorris LG, Smid EJ & Kets EPW (1999) Mannitol-enhanced survival of *Lactococcus lactis* subjected to drying. Appl. Microbiol. Biotechnol. 51: 100–104

Ferain T, Schanck AN & Delcour J (1996) 13C nuclear magnetic resonance analysis of glucose and citrate end products in an *ldhL-ldhD* double-knockout strain of *Lactobacillus plantarum*. J. Bacteriol. 178: 7311–7315

Garrigues C, Loubiere P, Lindley ND & Cocaign-Bousquet M (1997) Control of the shift from homolactic acid to mixed acid fermentation in *Lactococcus lactis*: predominant role of NADH/NAD^+ ratio. J. Bacteriol. 179: 5282–5287.

Garro MS, Giori GS, de Valdez GF & Oliver G (1996) Characterization of a-galactosidase from *Lactobacillus fermentum*. J. Appl. Bacteriol. 75: 485–488.

Gibson GR & Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 124: 1401–1412.

Gibson GR & Wang X (1994) Bifidogenic properties of different types of fructo-oligosaccharides. Food Microbiol. 11(6): 491–498.

Griffin WC & Lynch MJ (1972) Polyhydric alcohols. In Furia TE (ed.) CRC Handbook of Food Additives, Vol 1 (2nd edn.), CRC-Press, Cleveland, OH (pp 431–455).

Grobben GJ, Peters SWPG, Wisselink HW, Weusthuis RA, Hoefnagel MHN, Hugenholtz J & Eggink G (2001) Spontaneous formation of a mannitol-producing variant of *Leuconostoc pseudomesenteroides* grown in the presence of fructose. Appl. Environ. Microbiol. 67: 2867–2870.

Grossiord B, Vaughan EE, Luesink E & de Vos WM (1998a) Genetics of galactose utilisation via the Leloir pathway in lactic acid bacteria. Lait 78: 77–84.

Hamm-Alvarez S, Sancar A & Rajagopalan KV (1989) Role of enzyme-bound 5,10-methenyltetrahydropteroylpolyglut amate in catalysis by *Escherichia coli* DNA photolyase. J. Biol. Chem. 264: 9649–9656.

Hassan RM & Thurnham DJ (1977) Effects of riboflavin deficiency on the metabolism of the red blood cell. Ind. J. Vit. Nutr. Res. 47: 349–355.

Hirasuka Y & Li G (1992) Alcohol and eye diseases: a review of epidemiologic studies. J. Stud. Alcohol 62: 397–402.

Hoefnagel MHN, Starrenburg MJC, Martens DE, Hugenholtz J, Kleerebezem M, van Swam II, Bongers R, Westerhoff HV & Snoep JL (2002) Metabolic engineering of lactic acid bacteria, the combined approach: kinetic modelling, metabolic control and experimental analysis. Microbiology, 148: 1003–1013.

Hols P, Kleerebezem M, Schank AN, Ferain T, Hugenholtz J, Delcour J & de Vos WM (1999a) Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nat. Biotechnol. 17: 588–592.

Hosono A, Lee J, Ametani A, Natsume M, Hirayama M, Adachi T & Kaminogawa S (1997) Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci. Biotech. Biochem. 61: 312–316.

Huber M, Mundlein A, Dornstauder E, Schneeberger C, Tempfer CB, Mueller MW & Schmidt WM (2002) Accessing single nucleotide polymorhisms in genomic DNA by direct multiplex poly-

merase chain reaction amplification on nucleotide microarrays. Anal. Biochem. 303: 25–33.

Hugenholtz J, Hols P, Starrenburg MJC, de Vos, WM & Kleerebezem M (2000) Metabolic engineering of *Lactococcus lactis* leading to high diacetyl production. Appl. Environ. Microbiol. 66: 4112–4114.

Humbelin M, Griesser V, Keller T, Schurter W, Haiker M, Hohmann H-P, Ritz H, Richter G, Bacher A & van Loon APGM (1999) J. Ind. Microbiol. Biotechnol. 22: 1–7.

Kawaguchi M, Fujioka Y, Kishimoto M, Matsuma T & Ichikawa T (1999) Dietary fiber and phophorylated oligosaccharide protect small intestinal mucosa of rat from oxidative damages in vitro. J. Jap. Soc. Food Sci. Technol. 46: 487–490.

Khane SK, Jha K, Gandhi AP & Gupta MN (1994) Hydrolysis of flatulence-causing galactooligosaccharides by agarose-entrapped *Aspergillus oryzae* cells. Food Biochem. 51: 29–31.

Kim P, Yoon SH, Roh HJ & Choi JH (2001) High production of D-tagatose, a potential sugar substitute, using immobilized L-arabinose isomerase. Biotechnol. Prog. 17: 208–210.

Kitazawa H, Toba T, Itoh T, Kumano N, Adachi S & Yamaguchi T (1991) Antitumoral activity of slime-froming, encapsulated *Lactococus lactis* subsp. *cremoris* isolated from Scandinavian ropy sour milk, 'viili'. Animal Sci. Technol. 62: 277–283.

Klapa MI, Park SM, Sinskey AJ & Stephanopoulos G (1999) Metabolite and isotopomer balancing in the analysis of metabolic cycles: theory. Biotechnol. Bioeng. 62: 375–391.

Kleerebezem M, van Kranenburg R, Tuinier R, Boels IC, Zoon P, Looijesteijn E, Hugenholtz J & de Vos WM (1999) Exopolysaccharides produced by *Lactococcus lactis*: from genetic engineering to improved rheological properties? Antonie van Leeuwenhoek 76: 357–365.

Kleerebezem M, Beerthuyzen MM, Vaughan EE, de Vos WM & Kuipers OP (1997) Controlled gene expression systems for lactic acid bacteria: transferable nisin inducible expression cassettes for *Lactococcus, Leuconostoc* and *Lactobacillus* spp. Appl. Environ. Microbiol. 63: 4581–4584.

Kleerebezem M, Hols P & Hugenholtz P (2000) Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. Enzyme Microb. Technol. 26: 840–848.

Kokoris M, Dix K, Moynahan K, Mathis J, Erwin B, Grass P, Hines B & Duesterhoeft A (2000) High-throughput SNP genotyping with the Masscode system. Mol. Diagn. 5: 329–340.

Kuipers OP, de Ruyter PGGA, Kleerebezem M & de Vos WM (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotech. 64: 15–21.

Lakshini AV (1998) Riboflavin metabolism – relevance to human nutrition. Ind. J. Med Res. 108: 182–190.

Levander F, Andersson U & Radstrom P (2001) Physiological role of beta-phosphoglucomutase in *Lactococus lactis*. Appl. Environ. Microbiol. 67: 4556–4553.

Levander F & Rådström P (2001) Requirement for phosphoglucomutase in exopolysaccharide biosynthesis in glucose- and lactose-utilizing *Streptococcus thermophilus*. Appl. Environ. Microbiol. 67: 2734–2738.

Looijesteijn PJ, Boels IC, Kleerebezem M & Hugenholtz J (1999) Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. 65: 5003–5008.

Lopez de Felipe F, Starrenburg MJC & Hugenholtz J (1997) The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis*. FEMS Microbiol. Lett. 156: 15–19.

Lopez de Felipe F, Kleerebezem M, de Vos WM & Hugenholtz J (1998) Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled overexpression of NADH oxidase. J. Bacteriol. 180: 3804–3808.

Maity TK & Paul SC (1991) Low-oligosaccharide soy milk: application of a-galactosidase for hydrolysing soy-oligosaccharide. Ind. Dairyman 43: 443–448.

Manzoni M, Rollini M & Bergomi S (2001) Biotransformations of D-galactitol to tagatose by acetic acid bacteria. Proc. Biochem. 36: 971–977.

McBurney MW & Whitmore GF (1974) Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells. Cell 2: 173–182.

Nakajima H, Suzuki Y, Kaizu H & Hirota T (1992a) Cholesterol lowering activity of ropy fermented milk. J. Food Sci. 57: 1327–1329.

Nakajima H, Hirota T, Toba T, Itoh T & Adachi S (1992b) Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subspecies *cremoris* SBT0495. Carbohydr. Res. 224: 245–253.

Neta T, Tabata K & Hirasawa M (2000) Low-cariogenicity of trehalose as a substrate. J. Dent 28: 571–576.

Neves AR, Ramos A, Shearman C, Gasson MJ, Almeida JS & Santos H (2000) Metabolic characterization of *Lactococcus lactis* deficient in lactate dehydrogenase using in vivo 13C-NMR. Eur. J. Biochem. 267: 3859–3868.

Park SM, Klapa MI, Sinskey AJ & Stephanopoulos G (1999) Metabolite and isotopomer balancing in the analysis of metabolic cycles: applications. Biotechnol. Bioeng. 62: 392–401.

Pavan S, Hols P, Delcour J, Geoffroy MC, Grangette C, Kleerebezem M & Mercenier A (2000) Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study *in vivo* biological effects. Appl. Environ. Microbiol. 66: 4427–4432.

Pszczola DE (1992) The nutraceutical initiative: a proposal for economic and regulatory reform. Food Biotechnol. 46(4): 77–79.

Ramos A, Boels IC, de Vos WM & Santos H (2001) Relationship between glycolysis and exopolysaccharides biosynthesis in *Lactococcus lactis*. Appl. Environ. Microbiol. 67: 33–41.

Rollin C, Morgant V, Guyonvarch A & Guerquin-Kern JL (1995) 13C-NMR studies of *Cyanobacterium melassecola* metabolic pathways. Eur. J. Biochem. 227: 488–493.

Rooijen van RJ, van Schalkwijk S & de Vos WM (1991) Molecular cloning, characterization and nucleotide sequence of the tagatose-6-phosphate pathway gene cluster of the lactose operon of *Lactococcus lactis*. J. Biol. Chem. 266: 7176–7181.

Rosenberg IH & Godwin HA (1971) Inhibition of intestinal gamma-glutamyl carboxypeptidase by yeast nucleic acid: an explanation of variability in utilization of dietary polyglutamyl folate. J. Clin. Investig. 50: 78a.

Ross NS & Klein MR (1990) Riboflavin deficiency in cultured rat hepatoma cells: a model for studying the hepatic effects of riboflavin deficiency. In Vitro Cell Dev. Biol. 26: 280–287.

Schmidt K, Nielsen J & Villadsen J (1999) Quantitative analysis of metabolic fluxes in *Escherichia coli*, using two-dimensional NMR spectroscopy and complete isotopomer models. J. Biotechnol. 71: 175–189.

Seyoum E & Selhub J (1998) Properties of food folates determined by stability and susceptibility to intestinal pteroylpolyglutamate hydrolase action. J. Nutr. 128: 1956–1960.

Shen B, Jensen RG & Bohnert HJ (1997) Mannitol protects against oxidation by hydroxyl radicals. Plant Phys. 115: 527–532.

Silvestroni A, Sesma F, Savoy G, Connes C & Piard J-C (2002) Structure, organization, and expression of the melA struc-

tural gene for α-galactosidase in *Lactobacillus plantarum* ATCC 8014. Submitted for publication.

Smid EJ, Starrenburg MJC, Mierau I, Sybesma W & Hugenholtz J (2001) Increase of folate levels in fermented foods. Innovations in Food Technology Feb/Mar, 13–15.

Smirnoff N & Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057–1060.

Soetaert W, Buchholz K & Vandamme EJ (1995) Production of D-mannitol and D-lactic acid by fermentation with *Leuconostoc mesenteroides*. In: Agro Food Ind. High-Tech (pp 41–44).

Sokinenko EV (2001) Discovering the sweeping power of point mutations using a GIRAFF. Trends Microbiol. 9: 522–525.

Stingele F, Vincent SJF, Faber EJ, Newel JW, Kamerling JP & Neeser JR (1999) Introduction of the exopolysaccharide gene cluster from *Streptococcus thermophilus* Sfi6 into *Lactococcus lactis* MG1363: production and characterization of an altered polysaccharide. Mol. Microbiol. 32: 1287–1295.

Sutherland IW (1998) Novel and established applications of microbial polysaccharides. TIBTECH 16: 41–46.

Sybesma W, Hugenholtz J, Mierau I & Kleerebezem M (2001) Improved efficiency and reliability of RT-PCR using tag-extended RT primers and temperature gradient PCR. BioTechniques 31: 466–468.

Sybesma W, van den Born E, Starrenburg M, Mierau I, Kleerebezem M, de Vos WM & Hugenholtz J (2002) Increased production of bioavailable folate by engineering of *Lactococcus lactis*. Submitted for publication.

van Kranenburg R, Marugg JD, van Swam II, Willem NJ & de Vos WM (1997) Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. 24: 387–397.

van Kranenburg R, van Swam II, Marugg JD, Kleerebezem M & de Vos WM (1999a). Exopolysaccharide biosynthesis in *Lactococcus lactis* NIZO B40: functional analysis of the glycosyltransferase genes involved in synthesis of the polysaccharide backbone. J. Bacteriol. 181: 338–340.

van Kranenburg R, Vos HR, van Swam II, Kleerebezem M & de Vos WM (1999b) Functional analysis of glycosyltransferase genes from *Lactococcus lactis* and other Gram-positive cocci: complementation, expression, and diversity. J. Bacteriol. 181: 6347–6353.

van Kranenburg R, Kleerebezem M & de Vos WM (2000) Nucleotide sequence analysis of the lactococcal EPS plasmid pNZ4000. Plasmid 43: 130–136.

Vaughan EE, van den Bogaard PT, Catzeddu P, Kuipers OP & de Vos WM (2001) J. Bacteriol. 183: 1184–1194.

Vedamuthu ER & Neville JM (1986) Involvement of a plasmid in production of ropiness (mucoidness) in milk cultures by *Streptococcus cremoris* MS. Appl. Environ. Microbiol. 51: 1385–1386.

Veenhoff LM & Poolman B (1999) Substrate recognition at the cytoplasmic and extracellular binding site of the lactose transport protein of *Streptococcus thermophilus*. J. Biol. Chem. 274: 33244–33250.

Von Wright A & Tynkkynen S (1987) Construction of *Streptococcus lactis* subsp. *lactis* strains with a single plasmid associated with mucoid phenotype. Appl. Environ. Microbiol. 53: 1385–1386.

Wald N (1991) Prevention of neural tube defects. Results of the Medical Research Council vitamin study. Lancet. 338: 131–137.

Weinstock GM, Smajs D, Hardham J & Norris SJ (2000) From microbial genome sequence to application. Res. Microbiol. 151: 158.

Wren BW (2000) Microbial genome analysis: insights into virulence, host adaptation and evolution. Nat. Rev. Genet. 1: 30–39.

Yao R, Schneider E, Ryan TJ & Galivan J (1996) Human gamma glutamyl hydrolase: cloning and characterization of the enzyme expressed in vitro. Proc. Natl. Acad. Sci. U.S.A. 93: 10134–10138.

Zehner LR (1988) D-tagatose as a low-calorie carbohydrate sweetener and bulking agent. European Patent Application.