Metabolic engineering of lactic acid bacteria: overview of the approaches and results of pathway rerouting involved in food fermentations

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Lactic acid bacteria such as *Lactococcus lactis* are the microorganisms of choice for performing metabolic engineering in relation to food fermentation. These bacteria are used extensively in food fermentations, they have a simple and therefore controllable metabolism and the molecular genetics of these food bacteria is well-developed. There have been recent successes in metabolic engineering in these lactic acid bacteria, including examples of changes in both primary metabolism (diacetyl and alanine) and secondary metabolism (exopolysaccharides and flavour).

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Abbreviations

ALDB	α-acetolactate decarboxylase
EPS	exopolysaccharide
LDH	lactate dehydrogenase
NOX	NADH oxidase
PAM	phosphoglucomutase

Introduction

Microbial fermentation usually plays a very specific role in food fermentation. The breakdown of sugar and protein results in the production of a large array of organic compounds, most of them contributing to the flavour, preservation and outer appearance of the food product. The dominant microorganism in the specific food fermentation determines the oucome of the fermentation process: for example, a *Saccharomyces cerevisiae* fermentation will lead to an alcoholic end product; *Lactobacillus plantarum* as the dominant microorganism will give highly acidified products, containing mostly lactic acid; and *Propionibacterium* will give a typical nutty and sweet taste to a fermented product by the production of propionic acid and acetic acid. In this review, attention will be focused on lactic acid bacteria, because of their global and massive usage in food fermentations and their suitability for metabolic engineering. Homofermentative lactic acid bacteria have a relatively simple metabolism completely focussed on the rapid conversion of sugar to lactic acid. The general habitat of lactic acid bacteria are nutritious, high-sugar-containing environments. Under these conditions, the lactic acid bacteria have developed a typical metabolism that allows (very) rapid sugar conversion and is devoid of most biosynthetic activities. Under normal food fermentation conditions, the main product of metabolism is lactic acid, but other products are formed as by-products, such as acetic acid, acetaldehyde, ethanol, and diacetyl, all contributing to the specific flavour of fermented products. The main function of this sugar metabolism is to generate the energy necessary for rapid growth and for maintenance of intracellular pH during acidification of the environment.

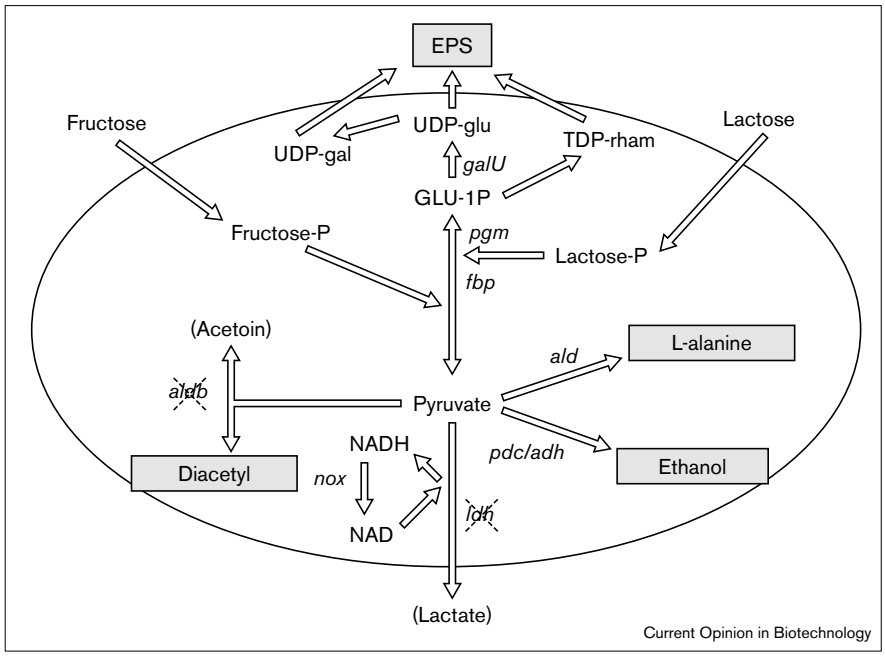
The biosynthetic capacity of these food microorganisms is very limited. The building blocks for growth generally originate from hydrolysis of food protein. The bacteria posess an elaborate proteolytic system centred around the complete breakdown of protein fragments into free amino acids. The amino acids are subsequently taken up and used for cell-protein synthesis or for modification reactions in the biosynthesis of other nitrogen compounds, such as vitamins and nucleotides.

There is almost no overlap between the energy (carbon) metabolism and the biosynthesis (nitrogen) metabolism in lactic acid bacteria. This makes them ideal as targets for metabolic engineering. Either metabolism can be changed dramatically without influencing the other as long as energy generation or biosynthesis of cell material is undisturbed. Several recent examples of succesfull engineering will be presented in this review with comparison to similar attempts in other, non-food, bacteria, such as *Bacillus subtilis* and *Escherichia coli*. The examples will be mostly on the level of main carbon metabolism, leading to ethanol, diacetyl, and alanine formation instead of lactic acid. In addition, a few examples and possibilities will be presented on the level of biosynthesis (nitrogen metabolism), such as proteolytic products, exopolysaccharides and vitamins.

Energy (carbon) metabolism

Homofermentative lactate metabolism is typified by high metabolic rates and not by maximal efficiency of energy conservation. These high rates are achieved mainly by the (very) high activity of lactate dehydrogenase (LDH). The reaction catalysed by this enzyme relieves the cells of accumulated reducing equivalents (NADH) which are formed during glycolysis and can cause direct inhibition of several key metabolic reactions, such as the pyruvate dehydrogenase rection producing acetyl-CoA, which is essential for lipid biosynthesis. The result of these high activities is population dominance in very nutritious environments because of the huge competitive advantage over other sugar-utilising microorganisms and the rapid acidification inactivating any other microorganism able to grow on other growth substrates. With metabolism in the lactic acid bacteria so focused on lactic acid production, it has turned out to be suprisingly easy to redirect metabolic flux towards production of other metabolites (Figure 1).

Figure 1



Overview of succesful metabolic engineering on the level of central carbon metabolism in homofermentative lactic acid bacteria. ALD, L-alanine dehydrogenase; ALDB, α-acetolactate decarboxylase; FBP, fructose biphosphatase; gal, galactose; GALU, UDP-glucose pyrophosphorylase; glu, glucose; LDH, lactate dehydrogenase; NOX, NADH oxidase; P, phosphate; PGM, phosphoglucomutase; rham, rhamnose.

Lactate dehydrogenase inactivation

It had been known for quite some time that under unusual conditions other metabolites can be formed by lactic acid bacteria. Acetic acid, formic acid, acetoin and acetaldehyde, and diacetyl can be found as a result of fermentation by lactic acid bacteria. In particular, under conditions of reduced sugar metabolism, for example in the case of a poor sugar substrate such as galactose [1] and sugar-limited conditions [2], or high aeration [3,4], high production of alternative end-products has been observed. These are all conditions that induce low LDH activity or low levels of the reducing cofactor NADH. These observations have generated the idea that diliberate inhibition or even complete disruption of LDH could lead to complete redirection of metabolic flux. This has indeed been observed in several lactic acid bacteria. In *Lactococcus lactis*, the *ldh*-gene was disrupted by plasmid integration [5]. The resulting LDH-negative strain proved to have a mixed acid fermentation under anaerobic conditions, producing acetic acid, formic acid, acetoin and ethanol, and a near homoacetoin fermentation under aerobic conditions [5]. Similar results were observed with a LDH-deficient *Lactobacillus plantarum*, an engineered strain that was more complicated to create, compared to *L. lactis*, because this lactic acid bacteria contains two quite different LDH-enzymes specific for L- or D-lactate, respectively [6]. Interestingly, an LDH-negative variant could not be obtained in *Streptococcus mutans*; the LDH mutation in *S. mutans* is lethal as this organism has no alternative method for regeneration of NADH [7].

The ethanol production in both LDH-negative lactic acid bacteria was greatly improved by additionally cloning in these bacteria the ethanol genes originating from the efficient ethanol-producing bacterium *Zymomonas mobilis*. Cloning of these genes, *pdc* and *adh*, coding for alcohol dehydrogenase and pyruvate decarboxylase, respectively, in *L. lactis* and *Lb. plantarum* with inactivated LDH resulted in ethanol production of more than 400 mM (or almost 2% w/v) in these bacteria [6,8]. This is still low compared to the production by yeast, but using the same approach for more ethanol-tolerant lactic acid bacteria, such as *Lactobacillus* strains isolated from port wine [9], much higher production of ethanol can be expected, possibly competing with the ability of yeast. Interestingly, when these *pdc* and, especially, *adh* genes were cloned in *S. mutans*, LDH-disruption was not lethal anymore, showing the vital importance of redox balancing (NADH-oxidation via LDH or alcohol dehydrogenase) in these lactic acid bacteria [10].

NADH oxidation

An alternative approach with a similar result to LDH disruption has turned out to be the direct oxidation of NADH by oxygen. As mentioned above, strong aeration of lactic acid bacteria also results in alternative metabolite production, such as acetic acid and acetoin [4,11]. By physiologically influencing, in *L. lactis*, the activity of the NADH oxidase by adjusting pH and the saturation level of oxygen, even stronger effects of oxygen on metabolism (i.e. less production of lactate and increased production of acetate, acetoin and diacetyl) were observed [12]. Using this knowledge, an effective metabolic engineering strategy was developed, aimed at overexpression of NADH oxidase (NOX). The *nox* gene of *S. mutans* was cloned under control of the nisine-inducible *nisA* promotor (NICE; [13]) in *L. lactis* and, by addition of sub-inhibitory amounts of nisin as inducer, very high (>1000-fold) overexpression of

NOX was accomplished [14••]. The metabolic analysis of these constructed strains showed very similar result to the LDH-negative constructs, with high production of acetoin and acetic acid under aerobic conditions [5].

Diacetyl production

The increased production of acetoin and acetic acid as a result of metabolic engineering is of minor impotance to the food industry. The butter aroma compound diacetyl, however, is chemically very similar to acetoin and is also produced, in very low amounts, during lactic fermentations. The conversion pathway leading to acetoin production involves the formation of an unstable intermediate, α-acetolactate. This intermediate is generally enzymatically converted to acetoin, but in rare cases, such as mutants lacking the enzyme α-acetolactate decarboxylase (ALDB), α-acetolactate can accumulate and can be chemically converted, in the presence of oxygen, to diacetyl [15]. This is the mechanism that generates diacetyl in dairy products. By combining the knowledge on redirecting metabolic flux and the knowledge on the mechanism of diacetyl production, a very effective strategy to enhance diacetyl production by *L. lactis* via metabolic engineering could be designed. Either disruption of LDH or overexpression of NOX in combination with disruption of ALDB would result in a high diacetyl production. Combination of two gene disruptions by plasmid integration is technically extremely complicated, so the strategy of NOX-overexpression and simultaneous ALDB-disruption was chosen. This turned out to be a very effective strategy, resulting in conversion of more than 50% of the available sugar into the desired aroma compound, diacetyl [16]. This presents a powerfull example of lactic acid bacteria as effective cell factories for the production of food components.

Alanine producton

Another example of complete metabolic rerouting in homofermentative lactic acid bacteria was recently presented by Hols *et al.* [17]. They managed to overexpress the alanine dehydrogenase from *Bacillus sphaericus* in *L. lactis*, using the previously mentioned NICE system, resulting in ammonium-dependent production of alanine (from sugar) instead of lactic acid. When the cloning was done in an LDH-negative mutant, the results were spectacular. More than 99.5% of glucose (or lactose) was observed to be converted into alanine, provided that the ammonium concentration remained above the K_M of the enzyme for this substrate (28 mM). By deletion of the alanine racemase activity in these bacteria, complete stereo-specific production of L-alanine was conceived. This process of high alanine production by a food-grade bacterium is interesting because it can act as a taste-enhancer and sweetener in fermented foods. It also illustrates the potential of metabolic engineering for the production of stereospecific organic compounds.

Production of exopolysaccharides

Although exopolysaccharide (EPS) production is a result of a biosynthetic pathway, and not an energy generating pathway, it is closely linked to the general glycolysis reactions. In one of the reviews in this issue, Richard van Kranenburg *et al.* (pp 498–504) will discuss the possibilities and the accomplishments in changing the composition of the polysaccharides produced by lactic acid bacteria. Here, we will briefly describe attempts to produce higher amounts of these structural components in fermented foods. The sugar moieties in the polysaccharide repeating unit are supplied by sequential addition reactions of 'activated' sugar–nucleotide building blocks by specific glycosyltranferases. These sugar–nucleotides, such as UDP–glucose, UDP–galactose and TDP–rhamnose, are all synthesized from glucose-1-phosphate as a general precursor. The conversion of the glycolytic intermediate glucose-6-phosphate to glucose-1-phosphate, catalysed by the enzyme phosphoglucomutase (PGM), and the synthesis of UDP–glucose from glucose-1-phosphate, catalysed by GalU, could very well be controlling points in EPS production. Preliminary studies in our laboratory have already shown that overexpression of either the *pgm* or the *galU* gene results in increased accumulation of the UDP–glucose and UDP–galactose, respectively, in cells of *L. lactis* [18]. Interestingly, EPS production by *L. lactis* is much lower when growing on fructose than on glucose or lactose as the energy source [19]. This could be a result of the (very) low activity of fructose biphosphatase, which is essential for growth (and EPS-production) on fructose but not on the other sugars. Overexpression of the *fbp* gene in *L. lactis*, via the NICE-system, led to increased intracellular levels of nucleotide sugars, accelerated growth and higher levels of EPS during growth on fructose [20]. This is the first example of increased EPS production via metabolic engineering on the level of central carbon metabolism in lactic acid bacteria.

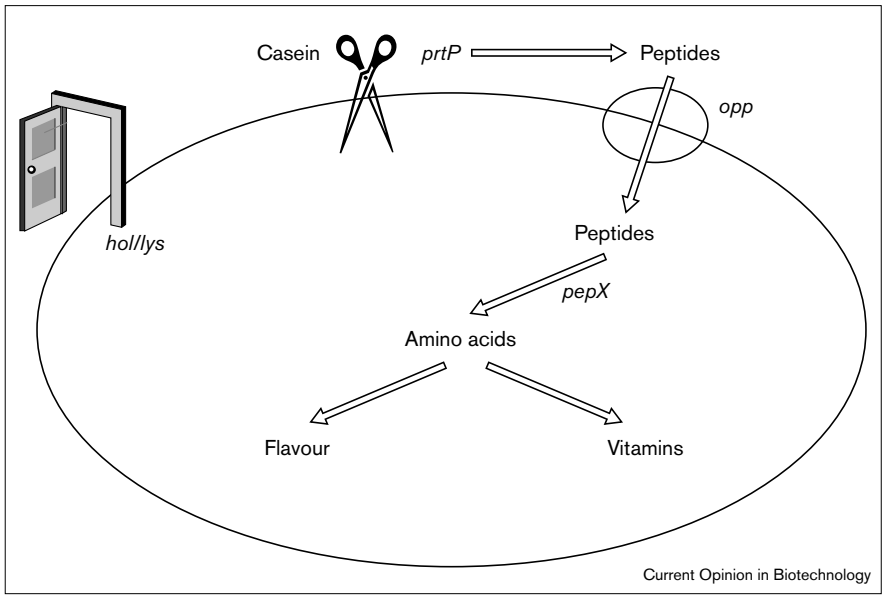
Nitrogen metabolism

Lactic acid bacteria that form the inherent bacteria flora in fermented foods usually have an intricate machinery for breakdown of (food) protein. This has been most extensively studied in the dairy lactic acid bacteria such as *L. lactis* and several *Lactobacillus* sp. (for overviews see [21–23]). This proteolysis provides the lactic acid bacteria with the essential free amino acids for growth and, as a result, these bacteria have a very limited capacity for the biosynthesis of amino acids. Some remnants of these reactions remain in specific strains, most evident as amino acid converting reactions resulting in the generation of flavour components, for example, methanethiol as the product of methione metabolism. Some metabolic engineering on the level of increased proteolysis and/or flavour production has been undertaken in lactic acid bacteria (Figure 2).

Because most of the fermentable substrates are rich in vitamins, nucleotides and minerals, the resident lactic acid bacteria generally have a limited biosynthetic capacity for these compounds. *Lactobacillus* is especially known for its inability to synthesize vitamins, such as folic acid, vitamin B_{12}, pathotenic acid, and so on. In fact, these bacteria are used in the biological assays for these vitamins because the growth of these bacteria is strictly dependent

Figure 2

Overview of metabolic engineering on the level of proteolysis and biosynthesis of nitrogen compounds. *hol/lys*, the holin and lysin from bacteriophage origin; OPP, oligopeptide transporter; pepX, different peptidases; PrtP, cell-wall proteinase.



on the presence of (small amounts of) these compounds. Still, some lactic acid bacteria are able to produce vitamins and, using other bacteria as examples, it is interesting to speculate how production by these lactic acid bacteria can be enhanced.

Proteolysis

Proteolysis is an essential process for growth of lactic acid bacteria in milk. Already several years ago, increased growth rate of *L. lactis* in milk was observed upon overproduction of the cell-wall-associated proteinase PrtP [24]. It was also reported that in the whole process of protein breakdown to peptides and subsequently to free amino acids, the uptake of larger peptides from the external medium to the inside of the cell, dictated by the oligopeptide transporter (OPP), was a crucial step in growth of *L. lactis* in milk [25]. This was evident from the inability of OPP-negative mutants to grow on culture medium with casein as the sole source of amino acids.

The complete breakdown of the oligopeptides to single amino acids, in lactic acid bacteria, is a result of the simultaneous action of a whole set of intracellular peptidases. These peptidases have overlapping substrate specificity and none of them are individually essential for growth on these peptides. This was elegantly shown by the work of Mierau *et al.* [26] where the genes coding for the intracellular peptidases were disrupted individually and in combinations of two, three, four and five different peptidase-disruptions. Only when three or more peptidases were disrupted simultaneously, was growth of *L. lactis* in milk clearly effected.

The proteolysis and subsequent amino acid conversion by lactic acid bacteria is an essential process in flavour formation in cheese during the ripening process. Metabolic engineering of lactic acid bacteria, on the level of proteolysis, has been attempted in numerous occasions to improve flavour development in cheese. The most promising results, however, have not been gained by increased activity of the enzymes involved, but by increased release of some relevant enzymes into the culture medium. By directly controlling lysis of the lactic acid bacteria, resulting in release of intracellular peptidases and/or amino acid converting enzymes, increased flavour formation has been observed [27]. The most effective example of accelerating the cheese ripening process by metabolic engineering, so far, has been the nisin-induced expression of bacteriophage lysin and holin in *L. lactis*, resulting in complete lysis of the cells [28••], complete release of peptidases and of other enzymes and a sharp increase in production of free amino acids and flavour compounds in cheese.

Vitamin production

As mentioned before, lactic acid bacteria have a very limited biosynthetic capability for the production of vitamins; however, there are certain exceptions. The yogurt bacterium *Streptococcus thermophilus* has been observed to produce folic acid [29] which, in fact, stimulates the growth of the other yogurt bacterium, *Lactobacillus bulgaricus* [30]. *L. lactis* also produces substantial amounts of folic acid during fermentation (MJC Starrenburg, J Hugenholtz, unpublished data). Many of the genes coding for the pathway of folic acid biosynthesis have been identified in the genome of this bacterium. Also, genes for riboflavin (vitamin B_2) and biotin (vitamin B_6) biosynthesis have been identified in *L. lactis* [31]. This would make it possible to engineer the production of these vitamins in these food-grade bacteria, just as recently reported for *B. subtilis* [32••]. Vitamin production processes by lactic acid bacteria would have huge advantages over the current used processes (by *Bacillus* or

Pseudomonas) as they could also be implemented for *in situ* production processes, such as food fermentations.

Conclusions

Lactic acid bacteria have already proven to be ideal hosts for metabolic engineering. Efficient production of diacetyl, alanine, and ethanol, resulting from sugar metabolism, has been engineered in different lactic acid bacteria. Most of these feats were directly succesful because, in contrast to yeasts and fungi, the genetics of the lactic acid bacterium is simple and there is little or no concern about multiplicity of genes. This is a tremendous advantage, especially when a specific enzyme activity needs to be disrupted for the best result.

The efficacy of metabolic engineering of lactic acid bacteria for the increased production of biosynthetic metabolites is yet to be demonstrated. On the level of amino acid conversion to flavour components, some successes have already been achieved; however, in the production of vitamins, such as folic acid and riboflavin, work has just started. The near future will tell us if the lactic acid bacteria are also the ideal cell factory for the production of these nutraceuticals.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Thomas TD, Turner KW, Crow VL: **Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways, products, and regulation.** *J Bacteriol* 1980, **144**:672-682.
2. Thomas TD, Ellwood DC, Longyear VMC: **Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures.** *J Bacteriol* 1979, **138**:109-117.
3. Starrenburg MJC, Hugenholtz J: **Citrate fermentation by *Lactococcus* and *Leuconostoc* spp.** *Appl Environ Microbiol* 1991, **57**:3535-3540.
4. Condon S: **Responses of lactic acid bacteria to oxygen.** *FEMS Microbiol Rev* 1987, **46**:269-280.
5. Platteeuw C, Hugenholtz J, Starrenburg M, van Alen-Boerrigter I, de Vos W: **Metabolic engineering of *Lactococcus lactis*: influence of the overproduction of α-acetolactate synthase in strains deficient in lactate dehydrogenase as a function of culture conditions.** *Appl Environ Microbiol* 1995, **61**:3967-3971.
6. Ferain T, Schanck AN, Hugenholtz J, Hols P, de Vos WM, Delcour J: **Redistribution metabolique chez une souche de *Lactobacillus plantarum* deficiente en lactate deshydrogenase.** *Lait* 1998, **78**:107-116. [Title translation: Metabolic redistribution of a lactate dehydrogenase-deficient strain of *Lactobacillus plantarum*.]
7. Chen A, Hillman JD, Duncan M: **L-(+)-lactate dehydrogenase deficiency is lethal in *Streptococcus mutans.*** *J Bacteriol* 1994, **176**:1542-1545.
8. de Vos WM: **Metabolic engineering of sugar metabolism in lactic acid bacteria.** *Antonie van Leeuwenhoek* 1996, **70**:223-242.
9. Couto JA, Hogg TA: **Diversity of ethanol-tolerant lactobacilli isolated fron Douro fortified wine: clustering and identification by numerical analysis of electrophoretic protein profiles.** *J Appl Bacteriol* 1994, **76**:487-491.
10. Hillman JD, Chen A, Snoep JL: **Genetic and physiological analysis of the lethal effect of L-(+)-lactate dehydrogenase deficiency in *Streptococcus mutans*: complementation by alcohol dehydrogenase from *Zymomonas mobilis.*** *Infect Immun* 1996, **64**:4319-4323.
11. Hillman JD, Andrews SW, Dzuback AL: **Acetoin production by wild-type strains and a lactate dehydrogenase-deficient mutant of *Streptococcus mutans.*** *Infect Immun* 1987, **55**:1399-1402.
12. Lopez de Felipe F, Starrenburg MJC, Hugenholtz J: **The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis.*** *FEMS Microbiol Lett* 1997, **156**:15-19.
13. de Ruyter PGGA, Kuipers OP, de Vos WM: **Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin.** *Appl Environ Microbiol* 1996, **62**:3662-3667.
14. •• Lopez de Felipe F, Kleerebezem M, de Vos WM, Hugenholtz J: **Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase.** *J Bacteriol* 1998, **180**:3804-3808.

This is the first example of metabolic engineering by changing enzyme activities on the level of w-factor regeneration.

15. Hugenholtz J, Starrenburg MJC: **Diacetyl production by different strains of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp.** *Appl Environ Microbiol* 1992, **38**:17-22.
16. Kleerebezem M, Hols P, Hugenholtz J: **Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering.** *Enzyme Microb Technol* 1999, in press.
17. Hols P, Kleerebezem M, Schank AN, Ferain T, Hugenholtz J, Delcour J, de Vos WM: **Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering.** *Nat Biotechnol* 1999, **17**:588-592.
18. Kleerebezem M, van Kranenburg R, Tuinier R, Boels IC, Zoon P, Looijesteijn E, Hugenholtz J, de Vos WM: **Exopolysaccharides produced by *Lactococcus lactis:* from genetic engineering to improved rheological properties?** *Antonie van Leeuwenhoek* 1999, in press.
19. Looijesteijn PJ, Hugenholtz J: **Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimisation of its biosynthesis.** *J Fermentat Bioeng* 1999, in press
20. Looijesteijn PJ, Boels IC, Hugenholtz J: **Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the source of sugar.** *Appl Environ Microbiol* 1999, in press.
21. Kok J, Venema G: **Genetic manipulation of the peptidolytic system in lactic acid bacteria.** *Int Dairy J* 1995, **5**:737-756.
22. Kunji ERS, Mierau I, Hagting A, Poolman B, Konings WN: **The proteolytic systems of lactic acid bacteria.** *Antonie van Leeuwenhoek* 1996, **70**:187-221.
23. Siezen RJ: **Multi-domain, cell-envelope proteinases of lactic acid bacteria.** *Antonie van Leeuwenhoek* 1999, in press.
24. Bruinenberg PG, Vos P, de Vos WM: **Proteinase overproduction in *Lactococcus lactis* strains: regulation and effect on growth and acidification in milk.** *Appl Environ Microbiol* 1992, **58**:78-84.
25. Kunji ERS, Hagting A, de Vries CJ, Juillard V, Haandrikman AJ, Poolman B, Konings WN: **Transport of B-casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis.*** *J Biol Chem* 1995, **270**:1569-1574.
26. Mierau I, Kunji ERS, Leenhouts KJ, Hellendoorn MA, Haandrikman AJ, Poolman B, Konings WN, Venema G, Kok J: **Multiple-peptidase mutants of *Lactococcus lactis* are severely impaired in their ability to grow in milk.** *J Bacteriol* 1996, **178**:2794-2803.
27. Meijer WC, van de Bunt B, Twigt M, de Jonge B, Smit G, Hugenholtz J: **Lysis of *Lactococcus lactis* subsp. *cremoris* SK110 ant its nisin-immune transconjugant in relation to flavor development in cheese.** *Appl Environ Microbiol* 1998, **64**:1950-1953.
28. •• de Ruyter PGGA, Kuipers OP, Meijer WC, de Vos WM: **Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening.** *Nat Biotechnol* 1998, **15**:976-979.

This is a very elegant and powerful example of how the tightly controlled NICE expression system in *L. lactis* can be used for industrial application.

29. Friend BA, Fiedler JM, Shahani KM: **Influence of culture selection on the flavor, antimicrobial activity, beta-galactosidase and B-vitamins of yogurt.** *Milchwissenschaft* 1983, **38**:133-136.

30. Rao DR, Reddy AV, Pulusani SR, Cornwell PE: **Biosynthesis and utilization of folic acid and vitamin B12 by lactic cultures in skim milk.** *J Dairy Sci* 1984, **67**:1169-1174.

31. Bolotin A, Mauger S, Malarme K, Sorokin A, Ehrlich DS: ***Lactococcus lactis* IL1403 diagnostic genomics.** In *Proceedings of the 5th ASM Symposium on the Genetics of Streptococci, Enterococci and Lactococci: 1998 April 26–29; Vichy, France.* ASM*;* 1998:10-11.

32. •• Sauer U, Cameron DC, Bailey JE: **Metabolic capacity of *Bacillus subtilis* for the production of purine nucleosides, riboflavin, and folic acid.** *Biotechnol Bioeng* 1998, **59**:227-238.
This paper opens the way for use of *B. subtilis*, and other microorganisms, as a cell factory for the production of vitamins and nucleosides.