Regulatory and Adaptive Responses of *Lactococcus lactis* in situ

Herwig Bachmann

Thesis Comittee

Thesis supervisor

Prof. dr. Michiel Kleerebezem
Professor of Bacterial Metagenomics
Wageningen University

Thesis co-supervisor

Dr. Johan van Hylckama Vlieg
Principal Scientist
NIZO food Research, Ede, The Netherlands and Danone Research, Palaiseau Cedex, France

Other Members

Prof.dr. Tjakko Abee, Wageningen University, The Netherlands
Dr. Gérard Denariaz, Danone Research, Palaiseau Cedex, France
Prof.dr. Oscar Kuipers, University of Groningen, The Netherlands
Prof.dr. Bas Teusink, VU University Amsterdam, The Netherlands

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Abstract

Lactococcus lactis is one of the main bacteria used for the production of dairy products like buttermilk and semi-hard cheeses. During the fermentation of dairy products *L. lactis* influences product properties like flavor, texture and shelf life. As a consequence of its industrial importance the physiology and molecular biology of *L. lactis* have been well characterized. However, the vast majority of studies were carried out with pure cultures in a laboratory medium. One of the current challenges in microbiology is to understand the behavior of an organism in its natural or application environment. In this thesis the importance of environmental conditions during experimental procedures was established by the determination of the regulatory diversity of five lactococcal enzymes that contribute to the formation of key flavor compounds in cheese. In addition, the focus was on studying regulatory and adaptive responses of *L. lactis* in the dairy environment. For this purpose several tools were developed or optimized, including a high throughput cheese making protocol that allows the simultaneous production of approximately 600 individual cheeses. In order to identify genes that are specifically expressed in cheese a Recombinase-based In Vivo Expression Technology (R-IVET) system was improved and adapted for *L. lactis.* Subsequently, it was used to identify genes that are induced during the manufacturing and ripening of cheese. In a different approach adaptive evolutionary responses were investigated by experimental adaptation of a *L. lactis* plant isolate to growth in milk. Three independently evolved mutants were characterized phenotypically and genotypically by whole genome re-sequencing. Results of the regulatory (R-IVET) analysis as well as the results of the adaptive evolution experiment established the importance of amino acid metabolism and transport for growth in milk and cheese. The experimentally adapted *L. lactis* mutants revealed a deterministic component in the adaptive process and it demonstrated that niche adaptation can be reproduced by experimental evolution. Finally, an important cooperative trait present in lactococci in milk is the expression of an extracellular protease that degrades milk proteins into utilizable peptides, which also become available to protease-negative strains in a mixed culture. It was established that high local substrate concentrations around protease positive strains can explain the stabilization of this trait in cultures consisting of both, protease positive and protease negative strains.

The obtained results have underlined the importance of high throughput screening in a product environment when mining biodiversity. They have increased our insight in the in situ responses of lactococci in milk and cheese and may contribute to the improvement of industrial processes, such as stabilization of population composition of mixed dairy starter cultures.

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Chapter 1

General Introduction

Abstract

The investigation of a microbial organism in its natural or application environment is one of the key challenges in current microbiology. The approaches to understand environmental adaptation and in situ behavior are numerous, ranging from the understanding of the evolutionary processes involved to the unraveling of gene-regulatory circuits that play a role in microbial adaptation. Experimental procedures that allow the molecular analysis of microbial responses in complex environmental samples are crucial for such studies and dedicated strategies are often required. Recent examples of such approaches will be briefly reviewed below, focusing on the industrially important dairy starter culture bacterium *Lactococcus lactis*.

Introduction

The production of fermented drinks and foods is probably one of the first examples where humans unknowingly but actively controlled bacterial fermentations. The consumption of fermented food and drinks reaches as far back as 5000 BC and historical evidence suggests the consumption of fermented milk products as early as 3000 BC (5). It was in 1684 that Antonie van Leeuwenhoek discovered microorganisms and only in the mid 19th century Louis Pasteur related microorganisms to the fermentation process (52). Lactic acid bacteria (LAB) occur naturally on plant material and upon storage under anaerobic conditions these lactic acid bacteria usually become dominant and ferment the substrate in a process predominated by the conversion of sugars to lactic acid. Typical plant-derived foods fermented by LAB are sauerkraut, olives and pickles, but LAB are also dominantly present in animal feed fermentations like silage. *Lactococcus lactis* is a LAB that does not only occur on plant materials but is also frequently isolated from the dairy environment. Throughout the last century, our understanding of food fermentation increased tremendously, which facilitated the development of well-controlled fermentation processes of food materials with consistent product properties. The two main determinants for the manufacturing of fermented food products of consistent quality are the use of raw materials with low microbial contaminations (hence the pasteurization of the raw materials) as well as the use of specific (mixed strain) starter cultures. The production of product-specific dairy starter cultures has therefore turned into an important branch of the dairy-ingredient industry and it was estimated that the global annual production of starter cultures amounts to approximately 10^9 liters (2). These starter cultures bring many beneficial properties of the fermented food product, including increased shelf-life, improved microbial safety and specific organoleptic characteristics (86).

Due to the economic importance there is a large interest in the expansion of our understanding of dairy fermentations. In this respect, the emergence of genomics has been an important breakthrough and a variety of dairy related bacterial genomes have been fully sequenced (Table 1). Functional genomics like transcriptome and proteome analysis were performed with many of these strains under variable conditions (22, 25, 72, 103). During cheese manufacturing, the bacterial cells involved encounter various types of stress that include rapid temperature changes, acidification, carbon starvation, changes in osmolarity and oxidative stress. The lactococcal response to the majority of these types of stress has been investigated in pure cultures grown under well-defined conditions in typical laboratory media (98). Such approaches have the advantage of allowing a clear dissection of research questions and it allows targeted experimental interventions that are likely to generate unambiguous answers. Indisputably, these approaches have delivered valuable insights into detailed molecular mechanisms of lactic acid bacteria. However, an intrinsic drawback of these approaches is their simplification of the impact of application conditions on the research question addressed. They may therefore fail to elucidate important aspects of environmental adaptation to complex conditions as encountered in the process of e.g. cheese production.

The abovementioned drawback has been recognized for some time (16, 71) but despite extensive efforts, the field of in situ microbiology is still in its infancy. The approaches to tackle bacterial behavior in situ are numerous and will be briefly reviewed in the sections below. Although the basic principles apply to the whole field of microbiology the focus will be on the industrial fermentation of dairy products and the lactic acid bacteria related to it and in particular the model organism *L. lactis*.

Table1: Microbial genome sequences relevant to dairy fermentations. Reproduced from Siezen et al. (76) with permission from the publisher

Species and strain	subsp./strain	GenBank code	size (kb)	GC%	origin/use	publication	Reference
Lactococcus lactis	*lactis*/IL1403	NC_002662	2,365	35	cheese	published	(9)
	cremoris/SK11	NC_008527	2,641	35	cheese	published	(54)
	cremoris/MG1363	NC_009004	2,530	35	cheese	published	(105)
Streptococcus thermophilus	CNRZ1066	NC_006449	1,796	39	yoghurt, cheese	published	(8)
	LMG18311	NC_006448	1,797	39	yoghurt, cheese	published	(8)
	LMD9	NC_008532	1,864	39	yoghurt, cheese	published	(54)
	11 strains					unpublished	Danielsen & Rasmussen, 2008
Lactobacillus delbrueckii	*bulgaricus*/ATCC11842	NC_008054	1,865	49	yoghurt	published	(97)
	bulgaricus/ATCC-BAA365	NC_008529	1,857	49	yoghurt	published	(54)
Lactobacillus helveticus	DPC4571	NC_010080	2,081	38	cheese	published	(15)
	CNRZ32		~2,280	37	cheese	unpublished	J.Broadbent et al. Utah State University, USA
	R0052					unpublished	J.Broadbent et al. Utah State University, USA
	CM4		2,028			unpublished	Calpis; Kitasato University, Japan
Lactobacillus casei	BL23	NC_010999	3,079		cheese	unpublished	J Deutscher et al, Centre National de la Recherche Scientifique, France
	ATCC334	NC_008526	2,924	46	cheese	published	(54)
	casei/ATTC393		~3,000			unpublished	M. Hatori, Univ of Tokyo, Japan
Leuconostoc mesenteroides	*mesenteroides*/ ATTC8293	NC_008531	2,075	37	olives	published	DOE Joint Genome Institute, USA
Brevibacterium linens	BL2	AAGP01000000	4,366	63	cheese	unpublished	DOE Joint Genome Institute, USA
Propionibacterium freudenreichii	*shermanii*/CIP103027		~2,500	67		unpublished	(23)
	ATCC6207		~2,640	67	cheese	unpublished	DSM and Friesland Foods, the Netherlands

Evolution and adaptive responses

The continuous evolution of microbes is driven by several mechanisms. Spontaneous mutations occur at certain frequencies in microbes, but it is also described that mutator strains with higher mutation frequencies can have an advantage in rapidly changing environments (73). In this process, base substitutions, small insertions, and/or deletions are generated. At the population level mutation rates have to be balanced, allowing microbes to adapt to new situations on one hand but preventing the accumulation of excessive amounts of deleterious mutations on the other hand. Mutation rates themselves can therefore evolve (73). Horizontal gene transfer (HGT) is an important mechanism (31, 94) that allows much more drastic phenotypic changes due to the acquisition of genes encoding specific functions. HGT undoubtedly plays an important role in shaping the genomes of microbes and it was estimated that the genomes of *E. coli* K12 and *Synechocystis* PCC6803 contain more than 12% and 16% of foreign DNA, respectively (62). HGT mechanisms are various and include important roles of plasmids or transposable elements, bacteriophages as well as processes like natural competence (87, 94). Among the genes that are often described to be transferred by HGT are antibiotic resistance genes, pathogenicity related genes, but also whole operons coding for certain metabolic traits (62). Comparative analysis of lactic acid bacteria revealed that next to gene acquisition via HGT these genomes are often characterized by extensive genome decay (Fig. 1). The accumulation of mutations results in high numbers of pseudogenes, while extensive gene loss may also occur. These changes are often associated with the adaptation to nutritionally rich environments (54). The analysis of synonymous and non-synonymous substitution rates (dN/dS ratio) between closely related *Lactobacillales* reveals 3-7 times higher values than dN/dS ratios of related *Proteobacteria* (54).

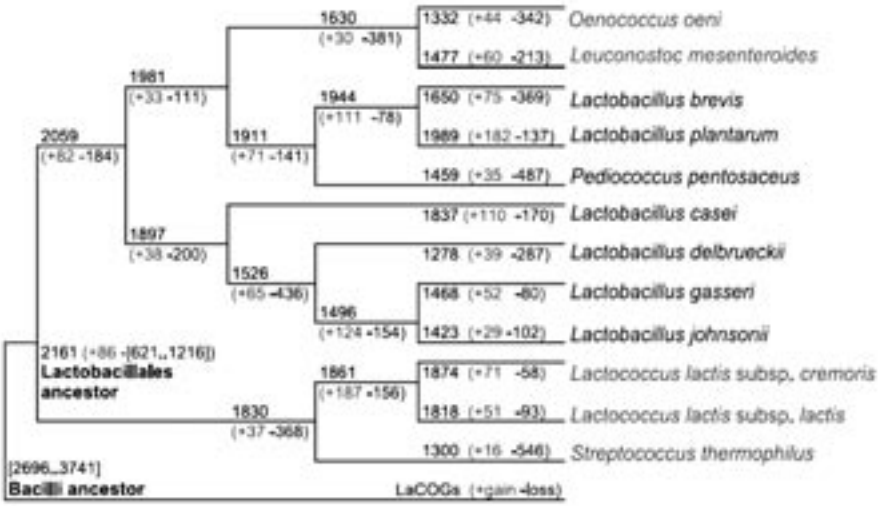

Figure 1: Reconstruction of gene content evolution in *Lactobacillales*. For each species and each internal node of the tree, the inferred number of *Lactobacillales*-specific clusters of orthologous genes (LaCOGs) present, and the numbers of LaCOGs lost (prefix "-") and gained (prefix "+") along the branch leading to the given node (species) are indicated. Reproduced from Makarova et al. (54) with permission from the publisher.

This observation is generally interpreted as a result of the strong evolutionary pressure on *Lactobacillales* as compared to *Proteobacteria*, which is described to be a consequence of the large effective population size and/or higher mutation rates of the Lactobacillales species (54). Illustrative for LAB genome decay are the observations that 10 % of the genes of two dairy isolates of *Streptococcus thermophilus* (8) and 12% of the genes of *L. bulgaricus* (97) are pseudogenes, which is attributed to the recent adaptations of those two species to the dairy environment. *S. thermophilus* and *L. bulgaricus* are the two species used for the production of yoghurt and their co-evolution in this environment led to a degree of specialization that severely decreased their fitness in milk if not grown in each others presence (75). Next to these clear examples of genome decay the *Lactobacillales* with larger genome size like *L. plantarum* and *L. casei* were found to loose and gain functional genes at similar rates (Fig. 1)(54, 55, 77).

To better understand adaptation of an organism to a new environment, experimental evolution is increasingly being employed (13). Such experiments are usually based on the serial propagation of a microbial culture in a test-tube followed by the characterization of adapted isolates (26). A well-known example is the experimental evolution of *E. coli* for more than 30.000 generations, which revealed the appearance of mutations throughout the evolutionary experiment (7, 66). For instance, after 31500 generations mutants appeared that were able to utilize citrate under oxic conditions (7). These mutants were shown to occur much more frequently from populations that had already evolved for 20000 generations, indicating that potentiating mutations were already present in these populations. A different example is the prolonged propagation of a *L. lactis* lactate dehydrogenase (*ldh*) deficient mutant, which resulted in the relief of growth retardation under anaerobic conditions by the activation of an alternative lactate dehydrogenase gene (*ldhB*) (10). This activation was shown to be a deterministic event, as in the majority of the analyzed revertants the mobilization of insertion sequence IS981 was responsible for the activation of *ldhB*. The effect of IS-mediated mutations in *L. lactis* IL1403 was also investigated during adaptation to various growth and starvation conditions (20). Adapted mutants with an increased fitness as compared to the ancestral strain carried deletions generated through recombinations between existing and/or new copies of IS981 demonstrating the importance of IS elements in genetic adaptation of lactococci. Recent advances in DNA sequencing technologies will clearly assist the genomic analysis of evolved strains, and thereby increase the attractiveness of adaptive evolution experiments approaches since acquired phenotypic changes that lead to improved fitness can readily be correlated to genetic adaptations by re-sequencing of the adapted strains (38). Understanding strain-dependent adaptations to environmental niches and/or application conditions and identification of the processes underlying these adaptations should allow the identification of molecular mechanisms and cognate molecular markers for niche specific properties and performance.

Biodiversity and the mining of industrially relevant phenotypes

Bacterial diversity is huge (29) and the vast majority of this diversity is not encompassed or represented in the currently investigated strains. For instance more than 1000 phylotypes were so far identified in the gastro intestinal tract (102) and the estimates for the number of distinct genomes per gram of pristine soil range from approximately 10^4 to 10^6 (29, 102). Novel

technologies like metagenomic approaches accelerated the investigation of biodiversity and they continuously reveal unexpected richness in different microbial communities (27). One of the challenges that accompany the exploration of microbial biodiversity is the phylogenetic classification of the organisms. The definition of a species is currently based on various phenotypic descriptions and on DNA-DNA similarity that should be >70% which roughly corresponds to >98.7% similarity on 16s rRNA sequence level (1). However, the current species concept is not providing a satisfying phylogenetic classification in several ways and, therefore, is heavily debated (1).

A relatively new method of describing the intra-species diversity is the pan-genome concept (58). The pan-genome is the sum of the core-genome of a species and the dispensable genes found in individual strains belonging to this species. The pan-genome can exceed the size of an individual genome by orders of magnitude (58), indicating that the diversity within a single species can be very high. Providing that a representative pan-genome is available the variability within one species can be investigated using comparative genome hybridization (CGH) (6). In view of the diversity-richness it is highly plausible that many industrially interesting phenotypes have remained unexplored to date, presenting the challenge to mine this diversity for the benefit of improving industrial application of microbes. A more classical approach of exploring biodiversity and finding new phenotypes is the screening of strain collections containing representative strains derived from various origins of isolation for the existence of e.g. specific enzyme activities. Such screens are often performed with high throughput protocols under laboratory conditions (11) or in media that aim to resemble a product environment as closely as possible (18). However, it was reported that the production of lactococcal volatile metabolites showed strain dependent differences when cultured in either milk or cheese-paste (3), indicating regulatory differences between closely related strains. These observations indicate that assessing phenotypic biodiversity and/or screening for industrially relevant phenotypes is clearly determined by the screening conditions employed, indicating that carefully designed screening conditions are likely to accelerate the identification of relevant novel phenotypic traits among strain collections.

Systems behaviour

The vast amounts of data produced with genome sequencing, whole genome transcriptome, proteome and metabolome analyses require dedicated data management and analysis tools that facilitate the storage, processing and comprehensive interpretation of the information generated. In view of these developments the application of (global) metabolic models is becoming increasingly important not only for rational improvement of the performance of industrial microorganisms (82, 89) but also for the understanding of the molecular fundaments that control and determine complex and multifactorial bacterial properties. A genome-scale metabolic model of *L. plantarum* was constructed and validated by comparing predictions of nutrient requirements with experimental data (90). The model proved its value further for the interpretation of growth on a complex medium (93) and formed the basis of subsequent metablolic models of other lactic acid bacteria (64). Recently the *L. plantarum* model was successfully used to predict the optimization of growth efficiency if glycerol is used as a carbon source (91, 92).

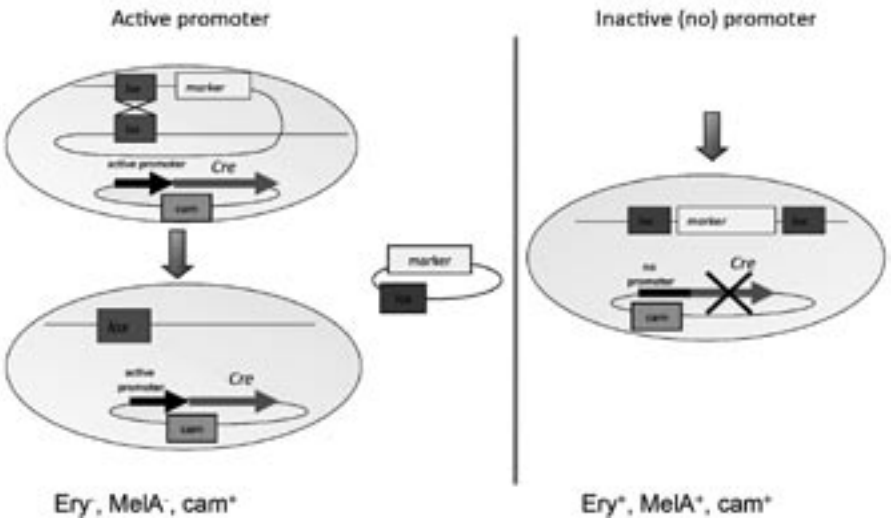

Figure 2: Basic principle of Recombinase-based In Vivo Expression Technology (R-IVET). A selectable marker gene is located between two recombination sites (*loxP*) on the chromosome. A plasmid library containing fusion constructs of random DNA fragments from the organism of interest upstream of the recombinase gene *cre* is introduced in this strain. If the random DNA fragment upstream of *cre* shows promoter activity the recombinase will be expressed leading to the recombination of the two recombination sites (*loxP*). This leads to the irreversible excision of the marker cassette from the chromosome and to a detectably different phenotype (left panel). If the DNA fragment upstream of the *cre* gene shows no promoter activity no changes will occur (right panel). Prior to an in situ experiment the library is propagated under e.g. laboratory conditions and strains harboring sequences active under these circumstances (e.g. household genes) are eliminated from the library by selecting for strains maintaining the marker cassette

To further improve our understanding of microbial behavior, mathematical modeling of microbiological systems is increasingly applied in various areas. The emergence of technologies to study single cells such as flow cytometry, time-lapse microscopy and microbial chip technologies established the concept of bacterial heterogeneity in clonal populations (41, 88, 100, 101). The practical relevance of such subpopulations is for instance the increased tolerance to acid challenges as shown for *L. plantarum* (41). One of the mechanisms underlying heterogeneity is the stochastic expression of genes. Such stochasticity is caused by fluctuations in transcription and translation and has major implications on gene regulatory networks and their robustness, and it can be advantageous in changing environments (42). The described mechanisms are usually complex and mathematical models have significantly contributed to the description and understanding of their biological implications (42, 50).

Microbial population dynamics is another area that increasingly employs mathematical models to understand the (sometimes) complex dynamics. Frequently, the ecological theories are tested by studying population dynamics in model systems where microbes compete for nutritional resources (32, 34), or have mutually beneficial interactions (14), or in model systems

where microbes outcompete each other by the production of antimicrobial (secondary) metabolites (43). A number of such examples were described with (game) theoretical models that were subsequently experimentally verified, which allowed the detailed description of such mixed cultures (34, 43, 44, 65). Dairy starter cultures are typically mixed cultures (75) and the various strains and species are not necessarily in a stable equilibrium. An industrially relevant as well as biologically intriguing example of such instability is the interaction between proteolytic and non-proteolytic *L. lactis* strains. As *L. lactis* has some amino acid auxotrophies it relies on extracellular peptides/amino acids as a nitrogen source. In milk an extracellular protease degrades the milk protein casein to utilizable peptides. Spontaneous mutants that do not express the protease can invade the population and lead to the extinction of the proteolytic positive strains. The result is that a well growing proteolytic positive culture turns into a poorly growing proteolytic negative culture. Because of the practical relevance to the dairy industry this phenomenon has been described as early as 1931 (36) and numerous studies followed, trying to explain this counter-intuitive behavior (40, 57, 63). A recent study in *Bacillus subtilis* established high expression of an extracellular protease only in a subpopulation, which might increase the stability of such a trait (100), but it is hard to envisage how this can explain its evolution. The evolution of cooperation is frequently investigated by evolutionary and theoretical biologists ((106) and references therein) and the behavior of the proteolytic trait in lactoccci presents an interesting and industrially relevant example for such analysis. Other microbes that express extracellular substrate degrading enzymes are likely to display similar cooperative behavior, indicating the relevance of the described example to other systems like sucrose or raffinose degradation in yeasts employing the extracellular enzymes invertase and α-galactosidase, respectively (34, 49).

Functional genomics and the dairy environment

In contrast to the above-described adaptive responses that deal with mutational changes occurring over longer periods, functional genomics tools are often used to investigate regulatory responses that deal with relatively short-term changes of e.g. gene expression levels after changing environments. The full genome sequences of 20 bacterial strains of dairy related lactic acid bacteria are determined (October 2008, (76)), comprising several strains of *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus* and *Pediococcus freudenreichii* as well as well as a single strain of *Leuconostoc mesenteroides* and one strain of *Brevibacterium linens* (Table 1). A common feature of functional genomics approaches is that they include elaborate procedures for sample preparation and purification that are poorly compatible to the complex matrices in environmental samples or samples from biotechnological processes. However, understanding of bacterial behavior under environmental conditions will ultimately be required to control, predict and improve their performance during these processes. Fluctuating and diverse microbial interactions as well as changing physico-chemical conditions can be anticipated to result in strongly variable bacterial characteristics (16, 72) that affect the process outcome. Depending on the nature of the sample one could alternatively try to first isolate bacteria followed by the envisioned functional genomics approach (30, 95). However, this process might lead to a cellular response during isolation and render data that are not representative for the physiological state

of a cell in situ (60). Next to the abundant use of functional genomics technologies in a laboratory environment ((45) and references therein), few studies are carried out under circumstances that resemble in situ conditions more realistically like the transcriptome analysis of *Lactobacillus helveticus* and *Lactococcus lactis* after growth in milk (70, 81) or proteome analysis of *Lactococcus lactis* during growth in milk (30). However, there is increasing interest for studying various lactic acid bacteria in the dairy environment as illustrated by the proceedings of the most recent symposium on lactic acid bacteria (47), which contained numerous abstracts and reports on this topic (76).

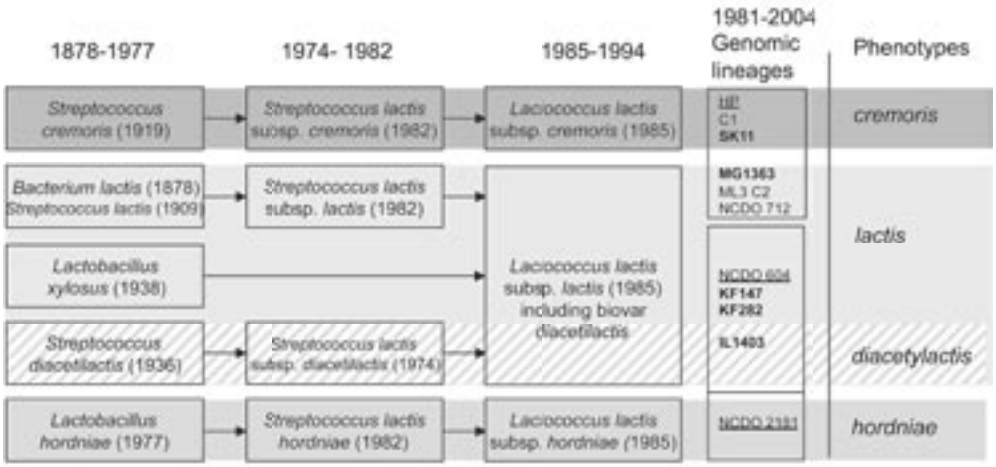

Figure 3: The taxonomy of *L. lactis* depicted from a historical perspective (67) starting with the description of *Bacterium lactis* by Lister in 1878. During the following century *Bacterium lactis* was reclassified as *Streptococcus lactis* and *Lactobacillus xylosus*. In 1985 a comprehensive reclassification was published by Schleifer et al. (74), which introduced the species *Lactococcus lactis* consisting of a combination of (parts of) the strains earlier defined as Streptococci and Lactobacillus. Within *L. lactis*, subspecies *lactis* is discriminated from *cremoris* by phenotype through its ability to produce ammonia from arginine, tolerance to high temperature and NaCl concentrations, and maltose fermentation. The *L. lactis* biovar *diacetylactis* is recognized by the ability to ferment citrate. Numerous studies involving DNA–DNA hybridization, 16S rRNA sequence, gene sequence and PCR fingerprint analyses show that two genomic lineages exists ((61, 68)and references therein), and these do not exactly match the phenotypic groups. One genomic lineage almost exclusively includes strains of the subspecies *lactis* including biovar *diacetylatis*. The second lineage is represented by strains of the subspecies *cremoris*, but also contains several strains with a subspecies *lactis* phenotype. The positions of frequently used model strains are indicated including type strains (underlined) and strains for which genome sequence data are available (in bold). Reproduced from van Hylckama Vlieg et al. (99) with permission from the publisher.

The measurement of bacterial metabolites is a promising and highly relevant approach for the investigation of functionality of dairy starter cultures. Many volatile bacterial metabolites act as potent flavor compounds and based on their volatility they can be sampled from the headspace of a sealed vial (84) without elaborate sample preparation. For *L. lactis* it was shown that within this species, strains vary significantly in their metabolic capabilities, especially concerning secondary metabolite production (83, 85). Notably, some of the variable secondary metabolites are key flavor compounds in dairy products and it was shown that the screening

environment and growth conditions strongly influence metabolite production in a strain dependent manner (4). The metabolomic analysis can be carried out in high throughput systems (84), however if the objective is to screen starter cultures in complex application habitats, like a cheese product environment appropriate high throughput protocols are still lacking. It is due to this lack of cost effective screening possibilities in a system with a high predictive value that industrial culture collections remain poorly characterized in terms of their true application potential to date.

For the investigation of the transcriptional response in situ, a number of strategies have been developed. Signature tagged mutagenesis (STM) investigates the survival of random insertion mutants which are individually tagged, allowing the identification of genes involved in the survival of an organism in a particular environment (37). Selective capture of transcribed sequences (SCOTS) allows the isolation of transcribed sequences from complex environmental samples (33) and differential fluorescence induction (DFI) uses fluorescence-activated cell sorting (FACS) to enrich active promoter sequences that are transcriptionally coupled to a fluorescent reporter protein (96). The potential applications of these and similar technologies that aim to investigate bacterial in situ responses have been reviewed elsewhere and will not be further addressed here (17, 35, 53, 71).

One technology that has been frequently applied to identify promoters that are specifically induced in situ was designated In Vivo Expression Technology (IVET) (59, 71). It is based on the transcriptional fusion of random DNA fragments to a selectable marker gene. A variation of the IVET approach is the Recombinase-based In Vivo Expression Technology (R-IVET), which uses a recombinase as the primary reporter (Fig. 2). Upon expression of the recombinase a chromosomally localized selective marker that is flanked by two recombination sites will be excised, leading to an irreversible phenotypic change that can be readily detected (71). Initially, IVET systems have been used primarily to investigate the bacterial response of human pathogens in animal models with one recent study of a *Vibrio cholerae* R-IVET system ingested by human volunteers (51). However over the past years it has been applied to many organisms besides the human pathogens, including soil bacteria (80), plant associated bacteria (28), human commensals (12, 104) and bacteria involved in food fermentation (19, 39). These studies have led to the identification of a variable number of genes that might be of importance for the performance of the investigated strain in a particular ecological niche, which in several cases could be confirmed by the evaluation of specific knock-out mutant strains. Despite all the advantages of IVET assays clear shortcomings are that only up-regulated genes can be detected and that the validation of identified target sequences requires subsequent, often very laborious, strategies (56) (79).

Next to the fundamental character of understanding bacteria in their natural/application habitat, the industrial relevance of such approaches was recently demonstrated. Perfoming a transcriptome analysis of samples directly obtained from an industrial fermentor revealed a purine limitation during the later phases of growth in a batch culture. The addition of extra purine sources increased the bacterial yield by more than 150%, thereby, increasing the cost-effectivenes of the production process (46). However, transcriptome analyses as in the case of the starter culture production are not readily applicable in more complex samples like cheese and for those cases the genetic screening methods described above provide a useful alternative.

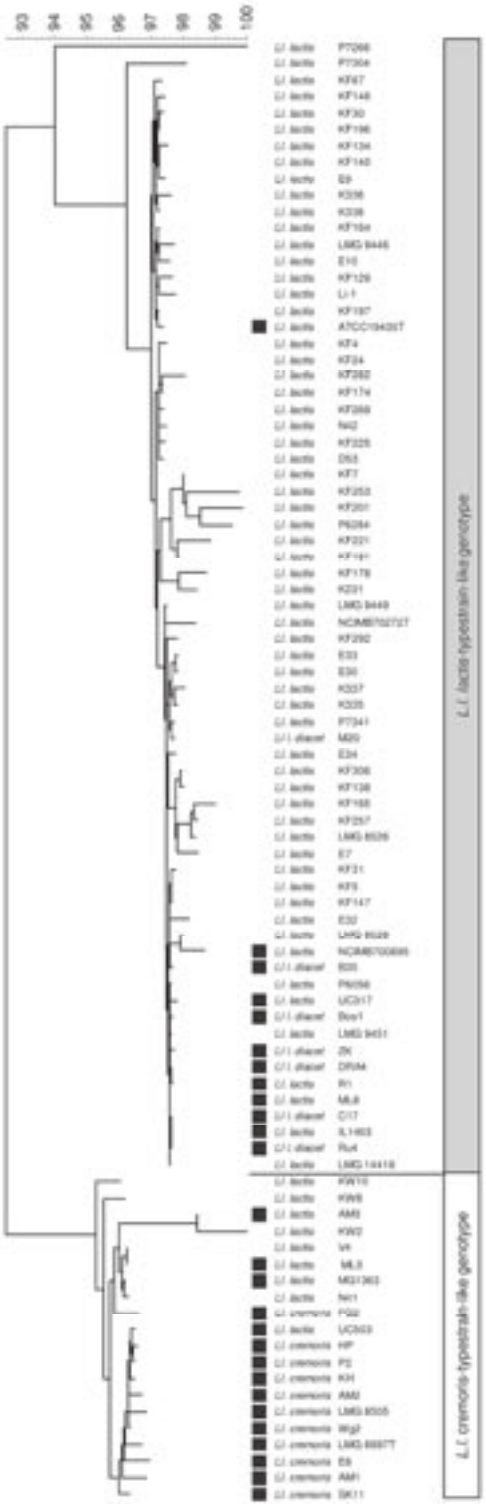

Figure 4: Comparative diversity analysis of 89 *L. lactis* isolates. Neighbor-joining cluster analysis of a five-locus MLSA (multi locus sequence analysis) based on a composite data set of DNA sequences of ~0.4 kb of the intragenic regions of housekeeping genes *atpA* and *rpoA* and flavor-related genes *bcaT, pepN*, and *pepXP* and the single partial pheS sequence. Bootstrap percentages (≥50) after 500 simulations are shown for the single sequence analyses. Strains with the *L. lactis* subsp. *lactis* typestrainlike genotype [SSU rRNA gene sequence and $(GTG)_5$-PCR fingerprint type] are represented with gray bars. Strains with an *L. lactis* subsp. *cremoris* typestrain-like genotype [SSU rRNA gene sequence and $(GTG)_5$ PCR fingerprint type] are represented with white bars. Isolates obtained from dairy substrates are indicated (■). The results of the cluster analysis allow the discrimination of two major genomic lineages designated the *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* typestrain- like genotypes, respectively and indicate a phylogenetic relationship based on strain origin. Adapted from Rademaker et al. (69) with permission from the publisher.

Lactococcus lactis

Lactococcus lactis is a gram-positive organism with a low percent GC content (105). It is used for the fermentation of various food products and is generally regarded as save (GRAS). The species *L. lactis* comprises the three subspecies *lactis*, *cremoris* and *hornidae*, including the subspecies *lactis* biovar *diacetylactis*. The nomenclature of *L. lactis* changed several times and the recent characterizations based one genotyping revealed some discrepancies between the genotype/phenotype classifications of some strains (Fig. 3). The fully sequenced strain MG1363 (105) is for instance classified as a subsp. *cremoris* genotype but displays a subsp. *lactis* phenotype. *L. lactis*, is mainly isolated from either the plant or the dairy environment and dairy isolates are believed to have evolved from plant isolates (99). A detailed phylogenetic analysis of lactococci showed that most subsp. *cremoris* strains are isolated from the dairy environment and cluster closely together with subsp. *lactis* strains isolated from the same environment (Fig. 4), which seems indicative for evolutionary adaptation to this niche (69). The notion that dairy isolates evolved from plant isolates is mainly based on examples of so called genome erosion, where pathways, often involved in amino acid and carbohydrate metabolism, are either mutated or deleted in dairy isolates (21, 54, 78). Such loss of function events result in a higher number of amino acid and carbohydrate auxotrophies in dairy isolates (4) which is interpreted as a reflection of the adaptation to this nutritionaly rich environment (54). However, to thrive on milk proteins a sophisticated proteolytic system is required to facilitate the degradation of milk proteins into peptides, which are subsequently taken up by the cell through dedicated transporters. The internalized peptides are degraded into utilizable amino acids by intracellular peptidases (24, 48). Therefore, the acquisition of such a proteolytic system is commonly regarded as an adaptation to the dairy environment (77).

Due to its commercial importance in the fermentation industry, the physiology of *L. lactis* has been studied in great detail (45, 98) and the species has become one of the paradigm LAB for dairy research. Currently there are 3 fully sequenced lactococcal isolates from dairy origin (Table 1) and one draft genome sequence of plant isolates (78) publicly available. The sequences of the plant isolates revealed numerous genes not found in the available sequences of the dairy isolates MG1363, IL1403 or SK11. They comprise mainly genes involved in the metabolism of carbohydrates found in plants material like xylan, arabinan, glucans, and fructans as well as enzymes required for the conversion of plant cell wall degradation products α-galactosides, β-glucosides, arabinose, xylose, galacturonate, glucuronate and gluconate (78). A recent diversity analysis of more than 100 lactococci showed that the sequenced strains represent only a fraction of the diversity of *L lactis* (69), suggesting that a large part of the genomic and functional diversity in the species remains to be explored.

Summary

The investigation of a microbe in its natural or application environment can be studied with different approaches. The adaptive responses caused by mutational changes can lead towards adjusted or improved performance including an increased overall fitness within a certain niche. Regulatory responses can similarly improve the performance in a new environment but overall fitness is likely to be better adjusted in an evolutionarily adapted strain. Molecular changes accompanying either of the mentioned responses can be explored by either functional genomics or by characterizing experimentally evolved strains, which, through the developments in sequencing technologies, became an increasingly interesting option only in recent years. Furthermore, the sequencing of metagenomes from various environmental niches as well as the application of targeted screening platforms will generate additional information that provides further depth to our insights in biological diversity. Clearly the biological relevance of experimental data and the predictive value of screens is enlarged when these experiments are performed in the natural environment or model systems that closely resemble those natural environments. Experimental procedures are often difficult to perform on complex samples but alternative methods to assess e.g. the transcriptional response have been developed and applied to a broad range of organisms e.g. R-IVET, STM, DFI and SCOTS technologies. The described approaches to understand in situ behavior are complementary to functional genomics approaches that work with pure cultures in a well defined environment and it will be through their integration that microbial behavior in a complex environmental system can ultimately be understood.
For the specific case of the use of microbes in industrial fermentation of dairy products the challenges primarily lie in the identification of starter bacteria with new functional properties like improving flavor and texture, prolonging shelf life or shortening ripening periods. Furthermore, the application of such cultures in a product environment as well as the understanding and controlling of regulatory responses ideally without the use of genetic modification are desired. *L. lactis* as one of the main species in many dairy starter cultures is relatively well characterized, genetically accessible and several genome sequences are available. These attributes make it a suitable organism to study in situ behavior, which should not only yield more insight into the adaptive responses and in situ physiology of this bacterium but also deliver relevant information to improve industrial fermentation processes.

Outline of this thesis

This thesis describes adaptive and regulatory responses of *L. lactis* in situ. It demonstrates on the simple example of enzyme activity measurements in different environments why in situ microbiology forms such an important challenge in current microbiology, followed by the development of some novel methods that allow accurate and time-resolved in situ activity measurements. The transcriptional response of *L. lactis* was investigated in different laboratory conditions as well as in the complex cheese matrix, using a sophisticated promoter trap system. In addition, the adaptive changes occurring in a *L. lactis* plant isolate when

transferred to milk were analyzed. Finally, the biotechnologically relevant cooperative trait occurring during growth of proteolytic positive and proteolytic negative lactococci in milk is described by modeling population dynamics and identifying localized peptide availability as a key contributor to the result.

Chapter 2
describes specific enzyme activities measured in 84 strains of *L. lactis* grown either in a nutritionally rich or a nutritionally poor medium. The results reveal little correlation between the activities after growth in the two environments indicating a high regulatory diversity among these strains and highlighting the importance of choosing appropriate screening conditions.

Chapter 3
describes the development of a high throughput cheese manufacturing protocol, allowing the simultaneous production of 600 cheeses with individual process specifications. The predictive value is exemplified with strains engineered to over-express flavor related enzymes and it is extensively benchmarked against industrially manufactured cheese. This method allows screening in a product environment and one of its applications will be demonstrated in chapter 6.

Chapter 4
describes the improvement of the detection of the bacterial luciferase (*luxAB*) as a promoter activity reporter in *L. lactis* during stationary phase. This study establishes that even in the rich medium M17 riboflavin is limiting luminescence activities when cells go into stationary phase and only its addition allowed the reliable luminescence measurements in nutritionally poor medium as described in chapter 5.

Chapter 5
describes the development of an improved Recombinant In Vivo Expression Technology (R-IVET) assay for *L. lactis*. The system allows rapid clone selection and subsequent validation of the identified promoters by in vitro and in situ comparison of gene expression. Genes induced in nutritionally poor medium were identified and the inducing conditions were specified at a molecular level for some of these genes.

Chapter 6
employs the R-IVET system as described in chapter 5, to determine gene expression throughout the cheese manufacturing and ripening process. In combination with the high throughput cheese manufacturing protocol gene expression in cheese could be monitored real-time for up to 200 hours. The results revealed the induction of numerous CodY regulated genes during cheese fermentation as well as the up-regulation of genes belonging to several other functional categories.

Chapter 7
describes the experimental evolution of a *L. lactis* plant isolate grown in milk for 1000 generations. Three adapted strains were characterized by full genome re-sequencing and by phenotypic characterizations. The results revealed several (partly deterministic) mutations related to amino acid metabolism and transport. This study supports the generally believed hypothesis that dairy isolates evolved from plant isolates and we could show that niche adaptations to environmental ecosystems can be reproduced by experimental evolution.

Chapter 8
describes the population dynamics of cooperating proteolytic positive and cheating proteolytic negative lactococci if grown together in milk. Population modeling and experimental data established localized peptide availability as a determining factor of the population dynamics.

Chapter 9
gives a brief summary of the results described in this thesis and includes some concluding remarks and future perspectives.

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Chapter

2

Regulatory phenotyping reveals important diversity within the species *Lactococcus lactis*

Herwig Bachmann, Marjo J.C. Starrenburg, Annereinou Dijkstra, Douwe Molenaar, Michiel Kleerebezem, Jan L.W. Rademaker, and Johan E.T. van Hylckama Vlieg

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Abstract

The diversity in regulatory phenotypes among a collection of 84 *Lactococcus lactis* strains isolated from dairy and non-dairy origin was explored. The specific activities of five enzymes were assessed in cell free extract of all strains grown in two different media, a nutritionally rich broth and a relatively poor chemical defined medium. The five investigated enzymes, BcaT, branched chain aminotransferase; PepN, aminopeptidase N; PepX, X-prolyl dipeptidyl peptidase; HicDH, Alpha-hydroxyisocaproic acid dehydrogenase and esterase, are involved in nitrogen and fatty acid metabolism and catalyze key steps in the production of important dairy flavour compounds. The investigated cultures comprise 75 *L. lactis* subsp. *lactis* (of which 7 biovar *diacetylactis*) and 9 *L. lactis* subsp. *cremoris*. All *L. lactis* subsp. *cremoris*, and 22 *L. lactis* subsp. *lactis* (of which 6 biovar *diacetylactis*) cultures originated from a dairy environment. All other cultures originated from (fermented) plant materials and were isolated at different geographic locations. Correlation analysis of specific enzyme activities revealed significantly different regulatory phenotypes for dairy and non-dairy isolates. The enzyme activities in the two investigated media were in general poorly correlated and reveal a high regulatory diversity within this collection of closely related strains. To the best of our knowledge, these results represent the most extensive diversity analysis of regulatory phenotypes within a single bacterial species, to date. The presented findings underline the importance of the availability of screening procedures for e.g. industrially relevant enzyme activities in models closely mimicking application conditions. Moreover, they corroborate the notion that regulatory changes are important drivers of evolution.

Introduction

Lactococcus lactis is an important component of many dairy starter cultures and has been studied extensively in the context of its function in these industrial processes. However, lactococci are also frequently isolated from (fermented) plant material, but these strains have been described in much less molecular detail (23). In dairy products *L. lactis* cultures are responsible for various enzymatic conversions, which affect the organoleptic characteristics of the fermented product. For instance the caseinolytic activity of lactococcal proteases and peptidases has a major influence on cheese texture (13) and secondary bacterial metabolites are key contributors to cheese taste and flavour (20). Enzyme activities are strain specific and simply by changing the starter culture composition one can alter product properties (20, 25). It is therefore attractive to screen culture collections and natural isolates for the activity of specific enzymes that contribute to specific flavour formation capacity during cheese production. Amino acids are the precursors of a large variety of flavour compounds and consequently, the bacterial amino acid metabolism has been studied extensively (24, 27). Some lactic acid bacteria are able to degrade milk proteins with a complex proteolytic system, which includes a number of endo-peptidases (13). These peptidases are thought to influence cheese flavour especially after cell lysis and their release into the cheese matrix (20). Branched chain amiotransferase (BcaT) and aromatic aminotransferase (AraT) catalyze the conversion of amino acids to the corresponding α-keto acids, which are the precursors for a number of aroma compounds (26-28). These α-keto acids are for example a substrate for hydroxy-isocaproic-acid dehydrogenases (HicDH), which reduce them to the corresponding hydroxy acid (27). Another reaction related to aroma compounds is the conversion of carboxylic acids to (thio)esters by esterases (20).

Bacterial gene expression is often tightly regulated and for several genes we have detailed knowledge about the regulatory mechanisms involved. However, this detailed knowledge of regulatory characteristics of specific enzymes is predominantly unravelled for a relatively small number of strains. An example of a well-characterized regulatory system is the pleiotropic regulator CodY, which regulates mainly the expression of genes involved in nitrogen metabolism such as amino acid biosynthesis and transport and genes of the proteolytic system (7, 12). The *codY* binding site is reported to be well conserved upstream of genes involved in branched chain amino acid metabolism throughout bacterial species belonging to the firmicutes (12). Nevertheless, the relationship of the conservation of regulatory elements like CodY and the sequence conservation at a protein level with the actual enzyme activities in individual strains of a specific species or subspecies remains to be established. Previously, we have performed an extensive genotypic and phenotypic analysis of 102 lactococci from either the dairy environment or plant material (19). For the detailed analysis of phylogenetic relationships the partial sequence of 6 genes were determined for 89 strains and it revealed 363 polymorphic sites on a total DNA length of 1970 bases. The ratio of synonymous to non-synonymous sites (dN/dS) was <0.08 for the partial sequences of *pepN*, *bcaT* and *pepX* respectively. This clearly indicates that at the protein sequence level these strains are very closely related to each other. In the present study we selected 84 *L. lactis* strains from the previously described collection (19) and determined the specific activities of 5 different enzymes with a renowned impact on flavour formation (PepN, PepXP, BcaT, HicDH and esterase). Of the 84

investigated strains the majority (75 strains) belonged to the subspecies *L. lactis*, while 7 of these belonged to the biovar *diacetylactis*, and 9 strains belonged to *L. lactis* ssp. *cremoris*. All strains of *L. lactis* ssp. *cremoris*, 6 strains of the *L. lactis* ssp. *lactis* biovar *diacetylactis*, and 16 strains of the *L. lactis* ssp. *lactis* were isolated from the dairy environment. All other strains were isolated from (fermented) plant materials originating from different geographic locations. To assess the extent to which the regulatory mechanisms are conserved between these closely related strains the enzyme activities of cells grown in different media were compared. We define the environment-dependent, strain specific variations of enzyme activities as the regulatory phenotype. Since specific enzyme activities were compared throughout this paper it should be noted that we have used the term regulation in the broad sense, as a cumulative effect of important mechanisms like transcription and translation or any other cellular factor that would influence specific enzyme activities of the bacterial culture. The results demonstrate distinguished differences between dairy and non-dairy isolates and remarkably little correlation between specific enzyme activities measured in the different media.

Table 1: Minimum and maximum enzyme activities (nmol/min/mg protein) of 84 strains measured after growth in either in GM17 or CDM. An estimate of the diversity of enzyme activities is given as the ratio of maximum over minimum values. The environment-to-environment prediction error of activities below the 10th or above the 90th percentile is given. Some measurements were below the detection limit (nd).

	BcaT	HicD	PepN	PepXP	Esterase
min. GM17	58,7	39,5	2,4	20,7	7
min. CDM	nd	39,6	42,2	15,8	2,8
max. GM17	1033,5	9826,1	289,7	694,5	42,9
max. CDM	436,8	4648	202,4	250,5	20
max/min - GM17	17,6	249	121,1	33,5	6,1
max/min - CDM	nd	117,4	4,8	15,9	7,1
prediction error above 90th percentile	100	55,6	77,8	66,7	44,4
prediction error below 10th percentile	92	92	92	93,3	93,3

Materials and methods

Bacterial isolates and media

A total of 84 *Lactococcus lactis* strains, which is a subset of an earlier described diversity study (19) was used for the presented experiments. The subset was obtained from the NIZO culture collection (NIZO food research, Ede, The Netherlands); see also Figure 4 (NJ tree). Throughout this paper we use the taxonomic classification as used by Rademaker *et al.* (19). The strains were grown at 30°C, either in M17 broth (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 0.5% glucose (w/v) (GM17) or in chemically defined medium (CDM) (17) supplemented with 0.5% glucose.

Enzyme activity measurements

For enzyme activity assays cells were grown overnight in 2 ml GM17 or CDM, in 96-well plates, in quadruplicate. Each plate contained a sample of strain ML3 (=NIZO643) as reference and non-

inoculated media as negative control. Cultures were centrifuged and washed with 2 ml of 50 mM sodium phosphate buffer pH 7.2. Subsequently, cells were disrupted using a minibeadbeater 96 Cell Disrupter (Merlin Diagnostic Systems, Breda, The Netherlands) with 300 µl of 0.1 mm Zirconium beads (Merlin Diagnostic Systems) and 1 ml of 50 mM sodium phosphate buffer pH 7.2. Crude extracts were prepared by four cycles of 30 s bead beating interspaced by 2 min cooling periods on ice. The resulting lysate was centrifuged (10 min, 830 g, 4°C) to remove cell-debris and the cell free extract obtained was used to determine enzyme activities as an average of four independent cultures. The standard deviations in these experiments were on average 10% (PepN), 10% (PepXP), 18% (esterase), 46% (BcaT) and 49% (HicDH). Protein concentrations were determined with a BCA protein assay (Pierce, Rockford, Ill., USA). In all cases specific activities were calculated and expressed as nanomoles of substrate converted per milligram of protein per minute. The reference strain ML3 was present in all 96-well plates and its enzyme activities showed variations from 11% to 26% in the separate experiments. All activities were normalized to strain ML3 to minimize the influence of experimental variation before activities were subjected to statistical analysis.

Esterase activities were determined by online monitoring of the release of *p*-nitrophenol from the substrate *p*-nitrophenyl butyrate in reaction mixtures consisting of 100 µl cell free extract and 145 µl of 50 mM sodium phosphate buffer (pH 7.5). The assay was started by adding 5 µl of substrate solution (10 µl *p*-nitrophenol-butyrate in 478 µl dimethylsulfoxide). The initial rates of release of *p*-nitrophenyl from the ester substrate at 30°C were quantified by measuring the increase in absorbance at 410 nm and specific activities were calculated.

Analogously, aminopeptidase N (PepN) activities were determined in a 96 well format by online monitoring of the release of *p*-nitroanilide from the substrate lys-*p*-nitroanilide dihydrobromide (FA Bachem, Bubendorf, Switzerland) at 30°C by measuring absorbance at 410 nm as described previously (8).

X-prolyl-dipeptidyl aminopeptidase (PepXP) activities were determined by online monitoring of the release of *p*-nitroanilide from the substrate H-Ala-Pro-*p*-nitroanilide by measuring the increase in absorbance at 410 nm. Reaction mixtures consisted of 250 µl of a solution of 0.256 mg ml^{-1} H-Ala-Pro-*p*-nitroanilide in 50 mM sodium phosphate buffer pH 7.2. Assays were carried out at 30 °C, started by adding 50 µl cell free extract and specific activities were calculated.

Alpha-hydroxyisocaproic acid dehydrogenase (HicDH) and branched-chain aminotransferase (BcaT) activities were determined as described earlier (3). It is important to note that BcaT activity was measured with leucine as substrate. Out of 84 BcaT measurements in CDM 24 showed no detectable activity. This data was omitted from the analysis.

Data analysis

The average values of 4 measurements were calculated for each condition and used to compute Spearman rank correlation coefficients (R). Classical decision trees were generated using the "ctree" function (22) as implemented in R (18).

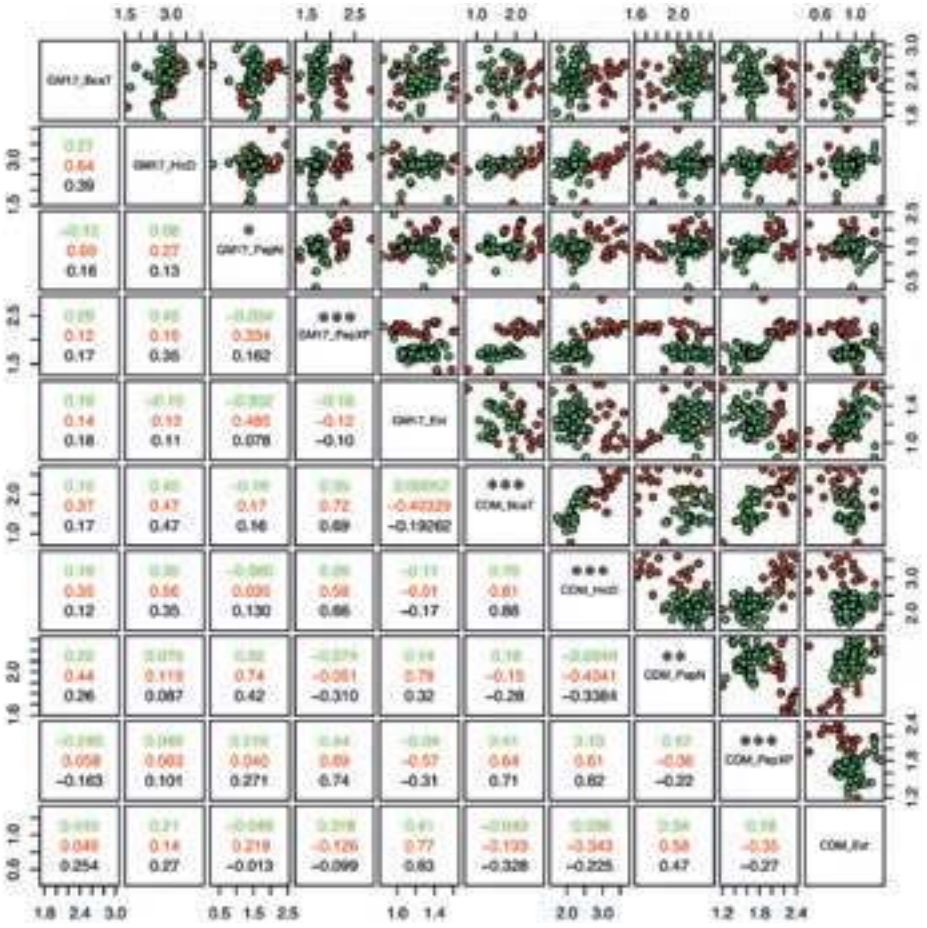

Figure 1: Correlation matrix and pair plots of all enzyme measurements in the two different environments. Dairy isolates are shown in red, non-dairy isolates are shown in green. Spearman rank correlation coefficients (R) are given for each group in the corresponding colour of the lower panels. The correlation coefficient for the total data is given in black. Each dot-plot displays the activities of the horizontally and vertically projected pair of enzymes indicated in the diagonal. The axes pair plots have a logarithmic scale. Significance levels of a two-tailed t-test comparing dairy and non-dairy isolates are given above the description in the diagonal panel (* p<0.05 ,** p<0.01, *** p<0.001). Dairy isolates can be clearly distinguished form non-dairy isolates, but correlations between measurements in the different media are relatively poor.

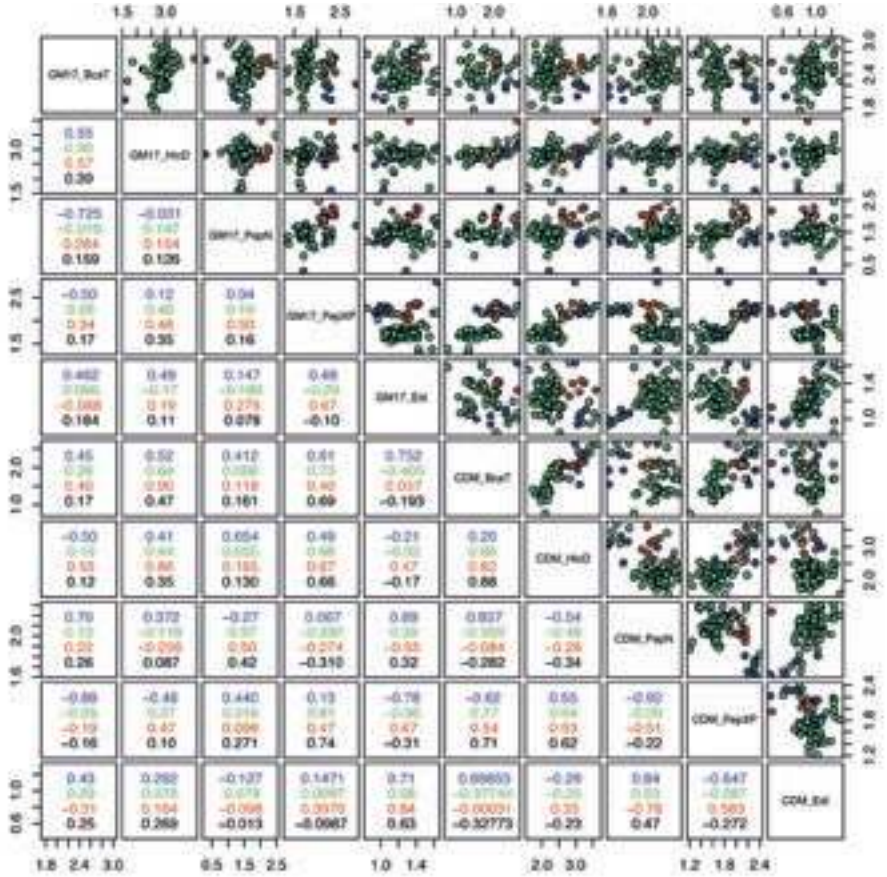

Figure 2: Correlation matrix and pair plots as in Figure 1 but strains are sorted by taxonomic position - *L. lactis* subsp. *lactis* (green) biovar *diacetylactis* (blue) and *L. lactis* subsp. *cremoris* (red). Analysis based on the taxonomic position of strains shows increased correlation only for some enzyme activities but overall correlations between measurements in different environments remain poor.

RESULTS

Enzyme phenotype screening

The analysis of 84 lactococci revealed that the highest specific activities observed for each of the five analysed enzymes are comparable with previous reports for *L. lactis* strains (1, 9, 21, 26). The variation of enzyme activities between the different isolates was considerable, with the largest variation observed for HicDH (249 fold) and relatively small variation for esterase (Table 1). Strain-to-strain variations were similar if measured in either GM17 or CDM with the exception of PepN. PepN activities varied approximately

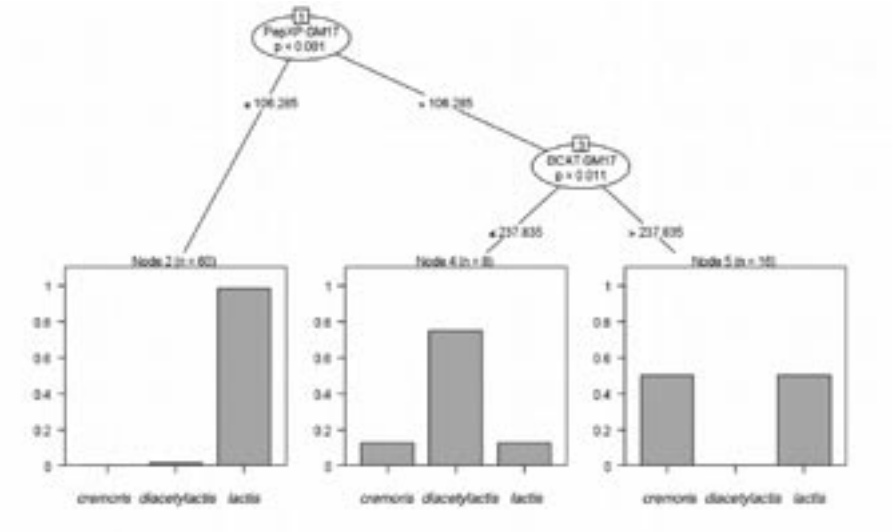

Figure 3: Decision tree to predict phylogenetic classification based on enzyme activities. Low PepXP activity is a predictor for *L. lactis* subsp. *lactis*. High PepXP activity and low BcaT activity (if measured in GM17) is a predictor for *L. lactis* subsp. *diacetylactis*

120-fold between strains if grown in GM17 and only approximately 5-fold if grown in CDM. In order to analyse the regulatory control of enzyme activities, correlation coefficients comparing the specific activities after growth in the different media were calculated for the total data set and for subsets defined on the basis of strain origin or phylogenetic clustering (Fig. 1 and 2).

Comparison dairy versus non-dairy isolates

The comparison of dairy and non-dairy isolates revealed that the screening environment is of great importance for the identification of correlations between the investigated groups. For instance, BcaT activities of cells grown in GM17 were not correlated with niche origin, but a highly significant difference was measured when the same analysis was performed in CDM (Fig. 1). Furthermore, the average PepN activity levels measured in non-dairy isolates were significantly lower than in dairy isolates when the strains were grown in GM17 (ca. 35 vs. 63 nmol/min/mg protein, $p=0.015$), while they were significantly higher when the same strains were grown in CDM (ca. 132 vs. 97 nmol/min/mg protein, $p=0.002$). In contrast, HicDH and BcaT activities of non-dairy isolates were significantly lower compared to those of dairy isolates ($p<0.001$), only when the strains were grown in CDM. Furthermore, non-dairy isolates appeared to have a lower PepXP activity level as compared to dairy isolates ($p<0.001$), which was the measurement found to be the most independent of the growth medium. A decision tree analysis showed that based on the PepXP measurements dairy and non-dairy isolates could be predicted with a prediction error of 5.9%. This demonstrates that clustering of the two groups based on specific enzyme activities is possible, but the choice of the investigated enzyme activity and the screening environment are crucial for this successful identification.

Comparison based on taxonomic position

The correlation coefficients between specific activities for all 84 strains grown in the two different media were R=0.17 (BcaT), R=0.35 (HicDH), R=0.42 (PepN), R=0.74 (PepXP) and R=0.63 (Est). Some correlation coefficients increased when only a particular subspecies/biovar was analyzed. For example, HicDH activities in both media displayed a clearly correlated activity level only within *L. lactis* ssp. *cremoris,* while PepXP measurements displayed a distinct higher correlation within *L. lactis* subsp. *lactis*. Moreover, the PepN activities of GM17-grown *L. lactis* ssp. *cremoris* cultures were significantly higher as compared to *L. lactis* ssp. *lactis* (ca. 112 vs 37 nmol/min/mg protein, $p<0.0001$)(Fig 2). This difference was not observed in CDM-grown cultures. A classical decision tree allowed the identification of *L. lactis* subsp. *lactis* based on PepXP activities (prediction error 13.2%) and further showed that biovar *diacetylactis* could be identified by having high PepXP and low BcaT activities in GM17 (prediction error 14.3%) (Fig 3). The identification of biovar *diacetylactis* as shown in Figure 3 is only possible if the BcaT activities are measured in GM17. Error rates of decision trees based on other enzyme activities drastically increased, reflecting the relatively poor correlations between taxonomic position and most enzyme activities measured. Overall, phylogenetically very closely related strains appear to display highly diverse enzyme activities (Fig. 4).

Regulatory diversity

To quantify the level of regulatory diversity the ratio of the specific enzyme activities in the two media employed was calculated and revealed considerable medium-dependent differences. The specific activities of BcaT, HicDH, PepN, PepXP and esterase differed up to 43, 16, 52, 10 and 5 fold between the measurements in the two media, respectively (Fig. 5). To assess to which extent the measurements from one environment can predict the outcome in a different environment we projected the data for the measurements after growth in GM17 onto results obtained after cells were grown in CDM. This comparison showed that the environment-to-environment prediction of strains with specific enzyme activities below the 10^{th} or above the 90^{th} percentile resulted in error rates ranging from 44-100% (Table 1). Six out of 10 predictions have an error rate close to 90%, which is what one would expect if the values were randomly distributed. We therefore consider the predictive value of enzyme measurements obtained from one environment of very limited (or even no) use for different environments.

DISCUSSION

The enzymatic conversion of bio-molecules by means of fermentation is an important process in the food industry. To improve such conversions there is a continuous process of strain improvement with the focus on acquiring and/or selecting microorganisms, which have the desired phenotype. Ongoing developments in the field of high throughput screening open new avenues for the discovery of industrially relevant phenotypes. An important approach is the mining of biodiversity by screening natural bacterial isolates for required enzymatic activities. However, it is often difficult or impossible to perform screening procedures in the

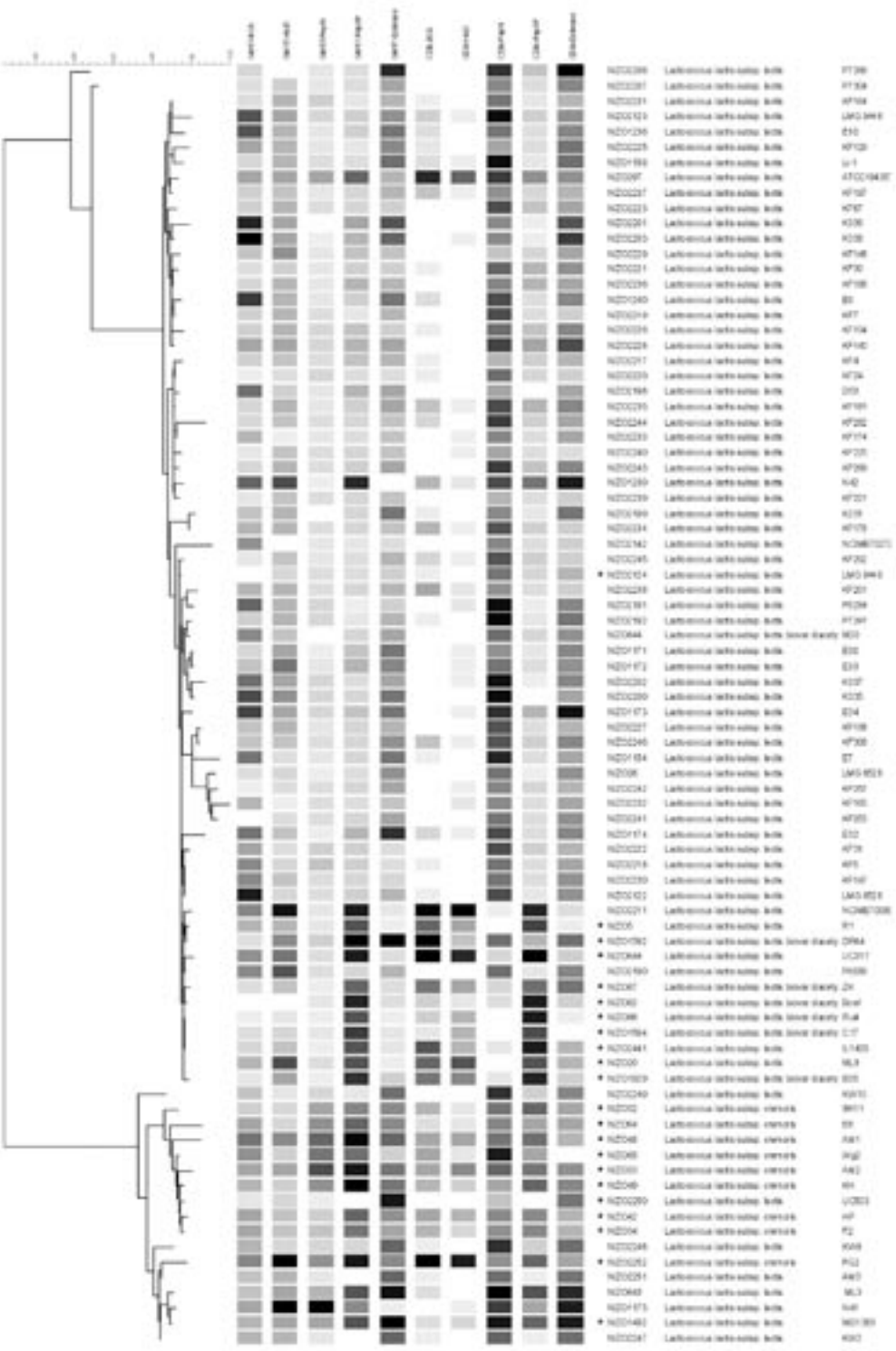

Figure 4: Specific enzyme activities of BcaT, HicD, PepN, PepXP and Est measured GM17 and CDM (respectively from left to right) are shown on a greyscale in the middle. The lowest enzyme activity within each condition is shown in white, the highest enzyme activity is shown in black. NJ cluster analysis based on 5 loci multi locus sequencing from on sequences determined by Rademaker et al (19) is displayed to the left. Specific enzyme activity profiles show relatively little conservation between phylogenetically closely related strains or different screening environments. Dairy isolates are indicated (*).

same environmental conditions envisioned for the application of the strains. It is commonly accepted that the screening conditions are likely to influence the expression level of bacterial enzymes. Consequently, relatively poor predictive value of laboratory-based screening results may be anticipated when extrapolated to the strain's performance in the application environment. To increase our insight in this diversity at a regulatory level, we determined five enzyme activities of 84 closely related *L. lactis* strains in two different growth media. The activities observed with dairy isolates of the *L. lactis* subsp. *lactis* and subsp. *cremoris* are in agreement with a more limited, previously performed analysis by Crow *et al.* (5), which also included some of the strains used in the present study. In this study it was shown that PepN activity levels were almost twice as high in isolates belonging to *L. lactis* subsp. *cremoris* as compared to the subsp. *lactis*. Our measurements in GM17 confirm this finding, but we also show that this difference is not observed if cells are grown in CDM (Fig. 2). It is important to realize that Crow *et al.* grew the strains in reconstituted skimmed milk, which, for certain strains, was supplemented with yeast extract and/or glucose. Various peptidases of dairy strains are known to be important for the bacterial utilization of milk proteins (13), which is in agreement with their high number of amino acid auxotrophies (2, 6, 10). Consequently, the finding that PepXP activity levels in dairy isolates are high compared to non-dairy isolates is probably a reflection of the adaptation to the dairy environment. Nevertheless, the dairy-related regulatory adaptation in terms of control of specific enzyme levels appears to follow different paths for PepN activity. PepN is significantly increased in dairy isolates only if grown in CDM, while an opposite conclusion may be reached when the same activity is measured in dairy isolates grown in M17. PepN and BcaT are both regulated by the pleiotropic regulator CodY, which strongly represses responsive genes in the presence of high peptide concentrations. Consistent with this mechanism, higher PepN activities were found in cells grown in peptide free CDM. In contrast, the BcaT measurements display the opposite response (lower activities in CDM), for which we lack an appropriate explanation, to date. Nevertheless, the observation that dairy isolates display a significantly increased BcaT activity in CDM, supports that some form of regulation underlies this observation. Although the *codY* binding site is well conserved in firmicutes (12) our data indicates that its regulation differs clearly between dairy and non-dairy isolates. Another significant difference found is the increased activity of HicDH in dairy isolates if grown in CDM. HicDH belongs to a family of hydroxy acid dehydrogenases, which play an important role in NAD^+ regeneration in the cell. This finding together with the up-regulation of a *L. lactis* lactate dehydrogenase (*ldh*) during the ripening of cheese (Chapter 6 of this thesis) suggests a dairy-specific redox response either at the level of adaptation or regulation.

The specific activities determined in the two different environments demonstrate a clear correlation for PepXP, suggesting that it is regulated similarly in both media, or alternatively, that it is not regulated at all. When considering the correlations of all other specific enzyme

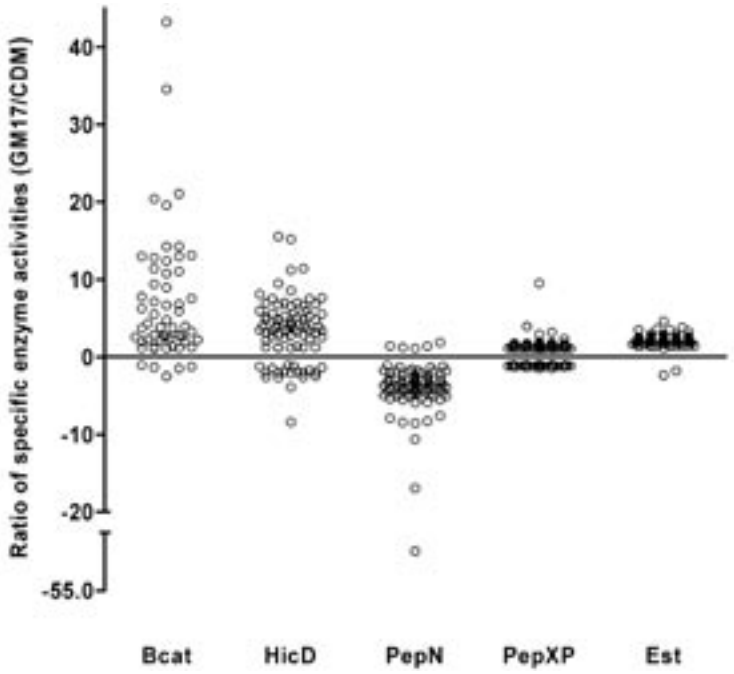

Figure 5: Regulatory diversity as expressed by the ratio of specific enzyme activities if measured either in GM17 or in CDM. Clouds represent the different enzyme measurements indicated on the x-axis. Each circle represents an individual strain. The differential regulation in the two investigated environments show large strain specific variations especially for the activities of BcaT, HicD and PepN.

activities measured in the 84 strains, we conclude these to be rather poor, indicating that these enzymes are strongly and differentially regulated by environmental conditions in a strain-specific manner. The correlation coefficients appeared to increase only for some sub-collections sorted either by strain-origin or by different subspecies/variants. The variation of PepN activities between strains is 25 fold lower if measured after growth on CDM as compared to GM17. This indication of strong regulation, combined with a very low correlation coefficient between the measurements in the two media, suggests very diverse regulatory characteristics in individual strains. Based on the fact that only strains of the species *L. lactis* were used in this study, the observed diversity of regulatory phenotypes was unexpectedly high, in our opinion. Intriguingly the strongest correlation observed in the presented dataset was the co-regulation of the BcaT and HicDH activity levels when cells were grown in CDM (R=0.88). In contrast, only a very weak correlation between these two measurements was observed between strains when they were grown in M17, which is consistent with findings reported previously (3). Since both BcaT and HicDH are involved in branched-chain amino acid metabolism, their co-regulation seems plausible. Taken together the presented data demonstrates that strong correlations can be found in our dataset. However, we only found few of these strong correlations, and we are not aware of a way to predict these, which would be essential for the design and interpretation of e.g. screening procedures in environments other than the intended application. The strong

correlations between strain-origin (Fig 1), the HicDH and BcaT activity level (in CDM p-value $<1^{-10}$), or between the PepXP measurements in the different environments (p-value $<1^{-10}$) are highly significant, which demonstrates the reliability of our data and strengthen the conclusions on correlations that we did not find.

The finding that very closely related strains show a large variation in their regulatory responses is consistent with a number of experimental evolution studies that indicate that the change of regulatory mechanisms belongs to the first events during adaptive evolution (4, 15, 16). In fact drastic changes in gene regulation can be seen after as little as 1000 generations of experimental evolution (14) (Chapter 7 of this thesis).

Despite the extensive characterization of *L. lactis* throughout the last decades the extent of the regulatory diversity was never described in such detail before. To the best of our knowledge, these results represent the most extensive diversity analysis of regulatory phenotypes within a single bacterial species, to date. We report that 4 out of 5 measured activities (PepN, HicDH, BcaT and esterase) show very diverse regulatory responses within the species *L. lactis* and it may be expected that other strain-specific enzyme activity patterns may be expected under alternative growth conditions (7, 11). Although some variation on the regulatory level was to be expected, the obtained results revealed an unexpectedly high level of diversity. Moreover, our data strongly support the notion that enzyme activities measured in a particular medium or specific environmental condition are of limited value (or even no value) for the prediction of activities in a different environment. For this reason it is essential that screening efforts should employ environmental conditions that are as close as possible to those encountered during the process of interest. This conclusion is likely to be valid beyond the field of lactic acid bacteria. Furthermore, our data corroborate findings described for a number of experimental evolution experiments, suggesting that regulatory changes are important drivers in evolutionary adaptation processes.

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Chapter

3

A high throughput cheese manufacturing model for effective cheese starter culture screening

Herwig Bachmann, Zeger Kruijswijk, Douwe Molenaar, Michiel Kleerebezem, Johan E.T. van Hylckama Vlieg

Abstract

Cheese making is a process where enzymatic coagulation of milk is followed by protein separation, carbohydrate removal and an extended bacterial fermentation. The numbers of variables during this complex process that influence cheese quality are so vast that the developments of new manufacturing protocols are cumbersome. To reduce screening costs, several models were developed to miniaturize the cheese manufacturing process. However, these are not able to accommodate the throughputs required for systematic screening programs. Here, we describe a protocol which allows the parallel manufacturing of ~600 cheeses in individual cheese vats that are addressed with individual process specifications. Protocols for the production of miniaturized Gouda- and Cheddar-type cheeses have been developed. Starting with as little as 1.7 ml of milk miniature cheeses of about 170 mg can be produced and they closely resemble conventionally produced cheese in terms of acidification profiles, moisture and salt contents, proteolysis, flavor profiles and micro structure. Flavor profiling of miniature cheeses manufactured with and without mixed strain adjunct starter cultures clearly allowed the distinguishing of the different cheeses. Moreover single strain adjunct starter cultures engineered to over-express important flavor related enzymes revealed effects similar to those described in industrial cheese. Benchmarking against industrial cheese produced from the same raw materials established a good correlation between their proteolytic degradation products and their flavor profiles. These miniature cheeses, referred to as MicroCheeses, open new possibilities to study many aspects of cheese production which will not only accelerate product development but also allow a more systematic approach to investigate the complex biochemistry and microbiology of cheese making.

Introduction

Industrial cheese manufacturing is a process that is characterized by many variables. Firstly, standardized milk is treated with an enzyme to coagulate the milk proteins. This coagulation leads to a gel like structure, the so-called curds, which is cut into small blocks and subsequently stirred, while following a cheese-specific temperature regime. During the stirring of the curds syneresis occurs, a process that leads to the extrusion of the liquid fraction, the so-called whey, from the protein matrix. The whey contains a high amount of lactose and for a typical Gouda-type cheese, a part of this lactose is removed by replacing some of the whey with water. Eventually, the curd is moulded and pressed and, in case of Gouda-type cheese, salt is added by submerging the cheese in brine. Prior to the initial coagulation step a mixed culture of bacteria is added to the milk, the so-called starter culture. These bacteria mostly belong to the lactic acid bacteria (LAB) and they are responsible for a number of essential properties of cheese (31). The acidification resulting from the conversion of lactose into lactic acid by LAB not only determines organoleptic characteristics but also serves as a preservative that prevents the growth of other, non-starter bacteria, which is important in terms of product quality and consistency, shelf live and food safety. The proteolytic activity of the bacterial starter culture catalyzes the degradation of milk proteins, which influences the cheese texture and results in the formation of peptides and free amino acids. Amino acids act as precursors for specific bacterial metabolites, which include potent aroma compounds. In addition to proteolysis, glycolysis and lipolysis are important pathways leading to flavor-active bacterial metabolites. Examples for such molecules resulting from carbohydrate metabolism are acetate, diacetyl, acetaldehyde, propionic acid and acetoin. Examples of relevant flavor derivatives from the amino acid metabolism are 3-methylbutanal, dimethylsulphide or methional (14, 22, 37). For example, the degradation of amino acids leading to the formation of α-keto-acids and their further degradation to important flavor compounds has been relatively well characterized (32, 36, 38). The actual lysis of bacterial cells during the ripening period is also thought to have an influence on cheese properties, mainly through the release of intracellular peptidases (25). In addition to the production of flavor active molecules the bacterial population can also influence the texture of a food product by e.g. the production of exo-polysaccharides (17) or through proteolytic activity (20).

Given this complexity of cheese making, it is obvious that there is a vast number of combinatorial possibilities of variables, which makes the development of novel cheese variants and new production protocols quite cumbersome, laborious, and expensive. For example, starter cultures show a large variation in functional properties and as a result the choice of starter culture can have a major influence on the properties of the final cheese product. Adjunct/secondary starter culture microorganisms are added to certain cheeses with the purpose of improving the sensory quality (11). The positive influence of single or mixed adjunct starter cultures on cheese properties is well recognized (1, 5, 11, 15) and widely applied. Consequently, the screening of strain collections and natural isolates offers an attractive approach for the identification of applicable new starter strains. Screening efforts are commonly performed in laboratory media (8, 29) or in media designed to resemble a product environment (2, 10). Such screens yield valuable insights in strain specific properties. However, they often have little value for predicting strain performance in cheese where environmental conditions alter the

physiological status of the cell. This is illustrated by the relatively poor correlation of volatile compounds measured from different lactococcal cultures grown either in milk or a cheese paste model (2). Moreover in a recent study we have demonstrated that the specific activities of 5 lactococcal enzymes, which are considered to be of key-importance for flavor formation, displayed very limited or even no correlation if measured after bacterial growth in either in a rich laboratory medium or a chemically defined medium (Chapter 2 of this thesis). This study clearly established that the predictive value of in vitro liquid culture screening results is very limited and strongly determined by the culture conditions, indicating that high throughput screening technologies in a product-like environment would be of great value. To the best of our knowledge the smallest model system reported for cheese manufacturing uses 200 ml of milk per cheese and allows an approximate throughput of 10 individual cheeses per person per day (27). Other model systems, which try to mimic the cheese environment, have similar limitations in their throughput capacities. For a review on cheese model systems see reference (26). However, the described models are not effective enough and require considerable investments when applied in more elaborate or large scale screening efforts. Current product development is therefore mainly driven by educated guesses rather than by the systematic screening of processing conditions and/or starter cultures. Here, we present a high-throughput cheese making model which allows the simultaneous manufacturing of individual miniature cheeses from as little as 1.7 ml of milk, enabling an experienced person to handle up to 600 cheeses per day. We demonstrate the modification of flavor profiles as a consequence of adding either a mixed strain adjunct starter culture or single strain adjuncts, which were engineered to over-express key enzymes involved in flavor formation. Furthermore the results establish that the manufacturing process is highly reproducible and that key properties of the MicroCheeses resemble those of conventionally manufactured cheese.

Material and Methods

Milk, enzymes, starter cultures, culturing conditions

The milk used for manufacturing MicroCheese was standardized bovine milk with a fat content of ~ 3.5%, protein content of ~3.4% and a lactose content of ~4.5% (heat treated at 72.5°C for 9 seconds). To allow the manufacturing of cheese on different days with the same batch of milk, 800 ml aliquots were shock frozen by slowly pouring milk directly into liquid nitrogen. Subsequently it was stored at -40°C until usage. Starter cultures for Gouda type cheese, FR18, Bos and APS, were obtained from CSK food enrichment (Ede, The Netherlands). Starter cultures for Cheddar-type cheese, Choozit^{TM} RA21 LYO 250 DCU (named RA21 throughout this paper) and Choozit^{TM} FLAV54 LYO 5D (named Flav54 throughout this paper), were obtained from Danisco (Copenhagen, Denmark). Because of the small quantities of starter culture needed the concentrated cultures were not added directly to the milk, but the cultures were pre-cultured in sterilized reconstituted skimmed milk powder (Promex Spray 1% skimmed milk powder; Friesland foods butter, Lochem, The Netherlands). For pre-culturing the starter cultures were grown at the following conditions: 20 hours at 20°C for FR18 and Bos, 16 hours at 37°C for APS,

18 hours at 30°C for RA21 and 16 hours at 37°C for FLAV54. Determination of bacterial colony forming units in cheese was done by dissolving cheese in 2% sodium-citrate and subsequent plating on M17 agar (Merck, Darmstadt, Germany) supplemented with 0.5% lactose. As a control sample for GC-MS analysis a young Gouda-type cheese (Jonge Beemster) purchased at a local store was taken along.

Gouda type cheese

For cheese manufacturing the frozen milk was thawed and subsequently warmed to 30.5°C. The milk was supplemented with renneting enzyme (Kalase – 150 IMCU, CSK food enrichment, Ede, The Netherlands) at a concentration of 230 µl/liter milk. Furthermore 400 µl of a 33% w/v $CaCl_2$ solution was added per liter milk. As a starter culture one percent of an FR18 pre-culture was added to the milk. For MicroCheeses produced with an adjunct culture 2.5% of an APS pre-culture were added to the milk. Following inoculation the wells of a 2 ml deepwell microplate (Greiner, Alphen a/d Rijn, The Netherlands) were filled with 1.7 ml of milk each and the plates were sealed with a capmat (Greiner, Alphen a/d Rijn, The Netherlands) and incubated at 30.5°C. After 45 minutes the cutting of the curds was started, using a custom made stirring device. This stirring device is made from stainless steel and consists of a plate with a handle at the top and 96 pins attached to the bottom. The pins are 3 mm in diameter and 45 mm long, and they are aligned in a way so they are positioned precisely in the middle of each well if the stirring device is placed in a 96-well microplate (Fig. 1). The stirring device was used for manual cutting and stirring of the curds. Cutting of the curds was carried out by slow horizontal and vertical movements of the stirring device through the curds. This was followed by stirring of the curds and the two steps together took 20 minutes. During these 20 minutes cutting/stirring for 20 seconds and resting for 3 minutes were alternated. After the stirring, the curds where allowed to rest for 5 minutes and then the plates were sealed again and centrifuged at 466 g for 5 minutes to slightly compact the curds. Subsequently 680 µl of whey were removed from each well and replaced with 620 µl of sterile tab water. Throughout cutting, stirring and centrifuging the plates were kept at 30.5°C. The addition of the washing water, which was heated to 45°C, brought the temperature in the wells to approximately 36°C. After the addition of the water the plates were placed in a water-bath tempered at 35.5°C and the curds were re-suspended with the stirring device. This was followed by a 40 minutes incubation period at 35.5°C and regular stirring as described above. Then the plates were incubated at the same temperature for another 20 minutes without stirring. Whey was removed from the curds by centrifuging the microplates at 4800 g for one hour at 30°C. The supernatant was discarded by decanting the plate and keeping the plate upside-down on a tissue for 15 minutes. Finally the plates where covered with a Breathseal (Greiner; Alphen a/d Rijn, The Netherlands) and placed in a climate stove at 30°C and 30% relative humidity to adjust the moisture content of the MicroCheeses. After over night inoculation 17 µl of a sterile 20% sodium chloride solution (w/v) was added to each well which, as calculated from the expected yield (33), should give approximately 3% salt in dry matter in the cheese. The evaluation of moisture content of the MicroCheeses was monitored by calculations based on the total weight of the cheese in each plate and by taking individual samples and determining the moisture content as described below. After cheeses reached the target moisture

content of 42-45% the plates were sealed in 0.8 atmospheres of 100% nitrogen. The cheeses were left for ripening at 17°C for 1 and 6 weeks before further analysis.

Cheddar-type cheese

The ingredients for Cheddar-type cheese, milk, kalase and $CaCl_2$ were used in concentrations identical to Gouda type cheese. As starter culture for Cheddar we used 1% of a RA21 pre-culture. In case of the addition of an adjunct we added, next to RA21, 0.75% of Flav54 pre-culture. After inoculation, the plates were sealed and incubated at 30°C for 45 minutes. The coagulated milk was cut and stirred with the cutting device for 10 minutes as described above. Subsequently stirring was continued and the temperature was raised with 1°C per six minutes to 39°C. The increase in temperature in this protocol is relatively slow which ensures equal temperature increase throughout the plate. Then stirring was continued for another 60 minutes at 39°C. Afterwards the curds were allowed to rest for 10 minutes which was followed by centrifugation of the plates at 466 g for 5 minutes at 30°C. After centrifugation the whey was removed and the sealed plate was incubated for another 90 minutes at 30°C. In the following step 30 µl of a sterile 30% (w/v) sodium chloride solution was added to each well and the plates were kept at 30°C for another 5 minutes. Subsequently the plates were centrifuged at 4800 g and 30°C for 60 minutes. The removal of supernatant, reduction of the moisture content and ripening conditions were performed as described. The target moisture content was 35-38%.

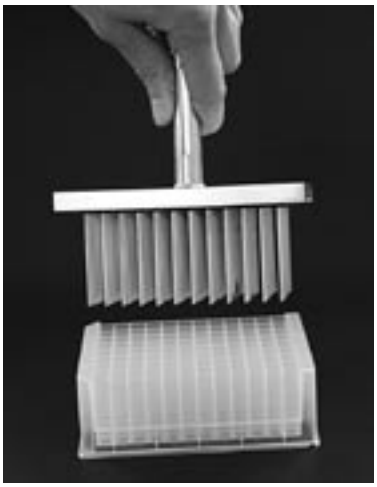

Figure 1: Microplate and stirring device used for the manufacturing of 96 individual MicroCheeses.

BcKAD and MetC mutant strains

L. lactis MG1363 (16), a *bckad* over-expression strain NZ9000 pNZ7500 (30) , a wild type strain with high BcKAD activity *L. lactis* B1157 (2) and it's cognate *bckad* deletion mutant B2083 (30), a *metC* over-expression strain NZ9000 pNZ8136 (13) and a *metC* deletion mutant MG1363Δ*metC* were used as adjunct cultures (Table 1). Strains were pre-cultured over night at 30°C in GM17.

For the over-expression of *bckad* and *metC* the overnight cultures were diluted (1:10) in reconstituted skimmed milk supplemented with 0.5% glucose and incubated for 1 hour at 30°C. If required 5 ng nisin were added per ml culture and cells were incubated for another 2 hours at 30°C. Fifty µl of these cultures were used as adjunct culture to 1.7 ml of milk, which was already inoculated with 1% of a Bos starter culture. The colony forming units of adjunct added to the individual MicroCheeses were determined to be approximately $2*10^7$ per ml milk. Subsequently Gouda-type MicroCheese was manufactured essentially as described above. To MicroCheeses made with strains MG1363 and NZ9000 as adjunct 0.5% glucose was added to the milk to allow growth of these lactose negative strains.

Comparison of industrial cheese and MicroCheese

Cheese was manufactured on a pilot-scale essentially as described earlier (18, 19). In short curds were prepared in a 1500-liter open vat and eventually shaped into 10 kg wheels. After milk inoculation with Bos starter culture an aliquot was taken from the 1500 liter vat and used immediately for further processing according to the Gouda-type MicroCheese protocol. Pilot-scale cheese and MicroCheese were ripened at 13°C and samples were taken after 14, 41 and 124 days.

Moisture, Salt, Fat and pH determination

Moisture, salt and fat analysis were performed according to the Dutch norm NEN 3755, 3762 and 3758 (Nederlands centrum voor normalisatie, Delft, The Netherlands) respectively with the alteration that for each determination only approximately 170 mg of cheese (one MicroCheese) were used. For the butyrometric fat determination 15 MicroCheeses were pooled, to have sufficient material (>2 grams). The pH values were determined with a 3 mm pH electrode (BioTrode, Metrohm; Herisau, Switzerland), which was used according to the manufacturer's instructions. For pH measurements in MicroCheese this electrode was pressed approximately 2 mm into the MicroCheese.

Analysis of proteolysis

Determination of proteolysis in MicroCheese samples was essentially performed as described elsewhere (35). In short, 170 mg cheese were dissolved in 1.5 ml buffer E^+ (0.1 M 1.3-bis[tris(hydroxymethyl)-methylamino]propane, 8 M urea, 1.3 % sodium citrate $\times$ 2 H_2O pH7 and 40 mg/ml di-thiotreitol which is added directly before buffer usage) followed by ultrasonic treatment for 30 minutes. Subsequently 500 µl were mixed with 1500 µl elution buffer A (see below), centrifuged for 5 min at 17200g and finally filtered through a 0.22 µm membrane. Analytical reversed phase chromatography (RP-HPLC) was carried out with equipment as described earlier (35). The equipment was linked to a data acquisition and processing system (Turbochrom, Perkin-Elmer). Solvent A was a mixture of acetonitrile-water-trifluoracetic acid (TFA) (20:980:1, v/v/v) and solvent B contained the same components (900:100:0.8, v/v/v).

Components were eluted with a linear gradient of solvent B in A. The absorbance of the eluent was monitored at 220 nm and 280 nm. The injection volume was 100 µl.

GC-MS analysis

For volatile analysis an Ultra fast GC-MS setup was used. Each MicroCheese was transferred to a 1.5 ml headspace vial. Headspace volatiles were allowed to equilibrate for 20 minutes at 60°C and subsequently directly injected into the GC-MS by a Combi Pal Autosampler (CTC Analytics AG; Zwingen, Switzerland) (GC column - UFM RTX 200 10m x 0.14mm; Thermo Fisher Scientific, Inc.; Waltham, USA). The initial temperature of the GC column was held for 0.4 minutes at 20°C and subsequently it was raised at 200°C/min. to the final temperature of 250°C at which it was held for 0.5 minutes. The total run time, including cooling, was approximately 5 min. Mass spectra were recorded by a Thermo plus Time of Flight mass spectrometer (Thermo Fisher Scientific, Inc.; Waltham, USA). The detection of mass spectra was performed with ionization energy of 70 eV and a scanning rate of 25 scans/s. The detected m/z ratio ranged from 35 – 350. Peak identification was done using the NIST MS search program version 2.0. Quantitation of peak areas was performed using XCalibur 1.4SR1 software (Thermo Fisher Scientific, Inc.; Waltham, USA).
Headspace volatiles of samples with BcKAD and MetC mutant *L. lactis* strains as well as samples for the comparison of factory cheese with MicroCheese were essentially analyzed as described above but equilibration was performed for 16 min at 60°C followed by a focusing and cryofixation at -50°C. Separation of the compounds was performed on a FactorFour VF-1ms column (30m x 0.25mm) (Varian, The Netherlands).

CSLM microscopy

Imaging was performed using a LEICA TCS SP Confocal Laser Scanning Microscope (CSLM) in the fluorescence, single photon mode. The set-up was configured with an inverted microscope (model LEICA DM IRBE) and an Ar/Kr laser. The objective lens used was a 63x/NA1.2/Water immersion/PL APO. Nile Blue A (Sigma, Zwijndrecht, The Netherlands) was used to stain the inclusion of lipids in the cheese matrix.

Statistical analysis

Clustering and analysis of correlation of the compound data of all GC-MS profiles with ripening periods and starter composition was performed using Random Forest (9) as implemented in the Random Forest package for R (24). Pearson correlation coefficients of the logarithm of the peak areas were calculated for the comparison of factory and MicroCheese flavor profiles.

Results

Development of the small-scale cheese model

A protocol for the high-throughput manufacturing of cheese was developed by employing a standard 96 deep-well microplate as an array of cheese vats. In this method each well can be addressed separately with substrate, ingredients or cultures and an individual cheese is produced from as little as 1.7 ml of milk. For the validation of the model we prepared 4 different types of cheese, encompassing Gouda- and Cheddar-type cheeses, both with and without adjunct starter culture. For each of the 4 different types of cheese we manufactured two times 96 MicroCheeses in individual experiments and multiple samples were analyzed for acidification rates, moisture content, salt concentration and volatile flavor compounds. The analysis of volatile flavor compounds was performed on MicroCheese ripened for one and for six weeks. The amount of rennet, $CaCl_2$ and starter culture added to the milk, as well the temperature regime followed throughout cheese manufacturing, were identical to those applied in conventional cheese making protocols. The cutting of the curds with a dedicated cutting/stirring device (Fig. 1) resulted in a curd size of 0.2 – 0.4 mm in diameter for Gouda type cheese and 0.9 – 1.1 mm in diameter for Cheddar-type cheese. Industrially manufactured cheeses typically show curd sizes between 5 and 10 mm. The relatively small curd size in the presented model is inherent to the system, with the size of the cheese vats being 7x7x40 mm. The pressing of the curds was mimicked by centrifugation. The addition of salt to either Gouda or Cheddar-type cheese was not performed by submerging the cheese in brine or kneading the salt into the curds, respectively, but by adding a defined amount of brine to the cheese which was subsequently absorbed in the curd. To avoid positional effects in the individual wells of the microplate, which may be caused by temperature gradients, the milk was kept at the desired temperature during the filling of the wells and subsequently a strict temperature regime was employed.

Physico-chemical parameters

Positional effects are a well-documented cause of artifacts in microplate screening (21). Therefore, we assessed whether there was a positional effect on the acidification profile. The pH in different positions of a microplate were measured 4, 5.5 and 24 hours after the start of the MicroCheese manufacturing, which was defined by the moment of inoculation of milk with enzymes and starter culture. The pH values measured in wells on the outside borders of the plate were compared to the pH values measured in wells that are more centrally located. For none of the assessed time points a significant difference was detected, indicating homogenous conditions across the entire plate during cheese manufacturing (Fig. 2). Overall, the acidification rates for Gouda- and Cheddar-type cheese showed very low standard deviations within a single experiment (< 1.2%) and remained reasonably low (< 2.9%) when comparing experiments carried out on different days (Fig. 3). The measured acidification rates are very similar to the acidification profiles measured during the industrial cheese making process (25).

The moisture content measured after the last centrifugation step of the respective protocol for Gouda and Cheddar MicroCheeses were between 45% and 50%. Based on typical moisture

contents of industrial cheeses we set the target moisture contents to 42-45% for Gouda- and 35-38% for Cheddar-type cheeses. To adjust the moisture content of MicroCheeses after centrifugation, the plates were incubated in a controlled climate stove. After 40 hours of incubation in the climate stove the average moisture content was 42.8% (±1.9) and 44.2% (±0.7) for the Gouda-type MicroCheeses produced on different days and 40.6% (±2.7) and 41.6% (±1.7) for Cheddar-type MicroCheeses produced on different days. The Cheddar-type cheeses were incubated in the climate stove for an additional 24 hours to achieve the target values. Following the moisture content adjustment, MicroCheese plates were sealed in a nitrogen atmosphere to avoid fungal contaminations and ripened at 17°C until further analysis. The addition of salt to the MicroCheese was achieved by adding a defined amount of sterile brine to each cheese. Salt readily diffuses throughout cheese (28), which due to the small size of the manufactured cheeses will rapidly lead to a homogenous distribution. Based on industrial cheese properties the target values for the salt concentrations were 3% in dry matter for both types of cheese. The amounts of sodium chloride added were calculated to achieve the concentration found in the corresponding industrial-cheese protocol. For a Gouda type cheese this is readily predictable, because the total amount of the added brine remains in each well. For Cheddar this process required refinement, because the salt is added before the last centrifugation step, after which excess whey is still being removed, leading to an additional reduction of sodium chloride during this step. This reduction was compensated by increasing the initial amount of sodium chloride added. The analysis of our cheeses showed a NaCl concentration of 3.0% (±0.1) and 2.9% (±0.3) (in dry matter) for Gouda- and Cheddar-type cheese, respectively.

The average fat-content in dry matter was measured in pooled Gouda-type MicroCheeses and was determined to be 40.2% (±0.4), which is considerably lower than the 48% measured in industrial cheese manufactured with the same standardized milk. Based on these results the fat-loss for Gouda-type cheese in the MicroCheese system was estimated to be approximately 19.0% (±0.4), which is significantly higher than the 7% normally observed in industrially manufactured cheese. Similarly, an increased fat loss

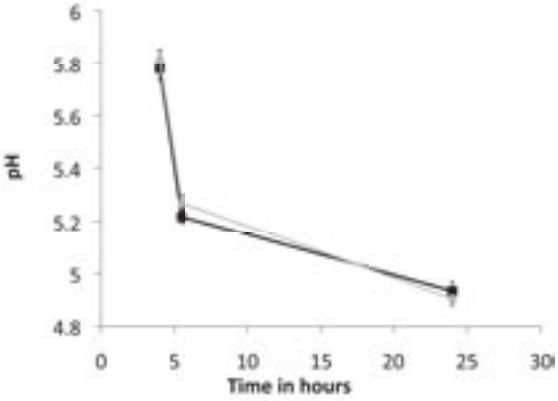

Figure 2: Acidification profiles in border-wells and non-border-wells show no significant positional effects. Grey circles show pH values measured in various non-border-wells (positions B2 to G11) (n=15). Black squares show pH values in border-wells C1, F1, H2, H6, and H12. Error bars show standard deviation.

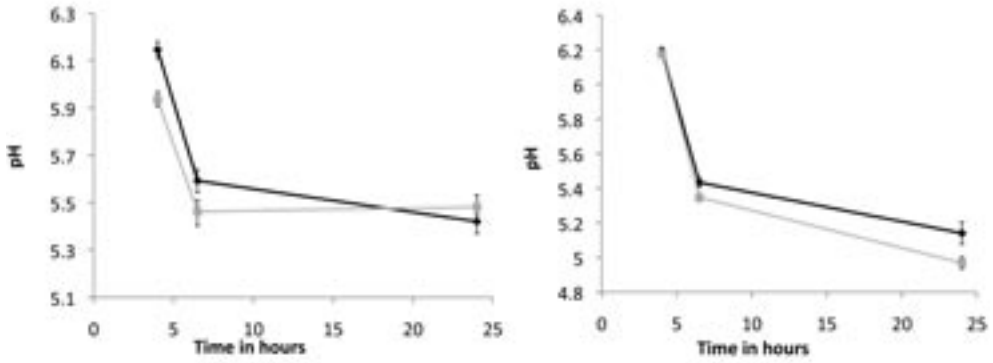

Figure 3: Acidification of Gouda (panel A) and Cheddar (panel B) type MicroCheese during the first 24 hours illustrate the reproducibility between individually manufactured MicroCheeses. Diamonds and squares represent MicroCheeses made on different days. Error bars show standard deviation (n=10)

of 16.2% (±1.3) was observed with the Cheddar-type protocol. The enhanced fat loss in the MicroCheese system is most likely due to the small curd size and the centrifugation steps in our protocol, which favor phase separation. To assess the influence of the lower fat content on the cheese microstructure, CLSM microscopy images were generated for three individually manufactured MicroCheeses, and compared to industrially produced Gouda-type cheese. CSLM images obtained for MicroCheese samples were similar to those obtained with industrially made Gouda-type cheese (Fig 4). Coalescence of the fat globules is clearly present in the MicroCheese model system, indicating a similar microstructure of the two sample-types. These findings illustrate that despite the lower relative fat content in the MicroCheese as compared to the industrial cheese, the structural properties observed in terms of fat-coalescence are still conserved.

Volatile metabolites

To further compare the presented protocol with industrially manufactured cheese, the levels of flavor volatiles were determined in MicroCheeses using a high-throughput GC-MS setup. Typical cheese flavor compounds could be detected, including acetic acid, acetoin, butanoic acid, diacetyl, ethylbutyrate, ethylhexanoate, ethyloctanoate, 3-methyl-butanal, hexanoic acid, limonene, 2-nonanon, pentanone, pentenal, and 2-propanon (Table 2). The areas under all identified peaks were used as a measure to quantify the flavor compounds, and quantified data were subjected to detailed statistical analysis. A multidimensional scaling plot showed that the replicate MicroCheese samples clustered closely together and that the 4 different types of cheese (Gouda- and Cheddar-type with and without adjunct culture) could be clearly distinguished (Fig. 5). Consequently, a full Random Forest classifier (classifying both ripening periods and starter compositions) based on the compound data showed a low out of box (OOB) prediction error estimate of approximately 5%. Similarly, the difference between samples with either 1 or 6 weeks ripening time could be detected with high significance. In general the

6-weeks ripened samples contained higher concentrations of most flavor compounds which is consistent with literature (25). No significant differences were detected for the same type of MicroCheeses produced on different days. Flavor profiles of a control sample (Jonge Beemster) taken along for the GC-MS analysis confirm the global resemblance between MicroCheese and industrial cheese, yet these profiles allowed distinguishing of all analyzed cheeses (Fig. 5). Furthermore Gouda-type MicroCheeses manufactured with the APS adjunct culture show many similarities with it's industrially manufactured counterpart – Proosdij-type cheese (23).

Bacterial growth and survival

The amount of bacterial colony forming units (CFU) per gram MicroCheese manufactured with a gouda-type protocol were determined 2 and 27 days after the cheeses were produced, and approximately $2x10^9$ CFU and $1x10^8$ CFU per gram cheese were recovered after 2 and 27 days of cheese ripening, respectively. These results are comparable to the number of CFUs detected in industrial cheeses (3, 4).

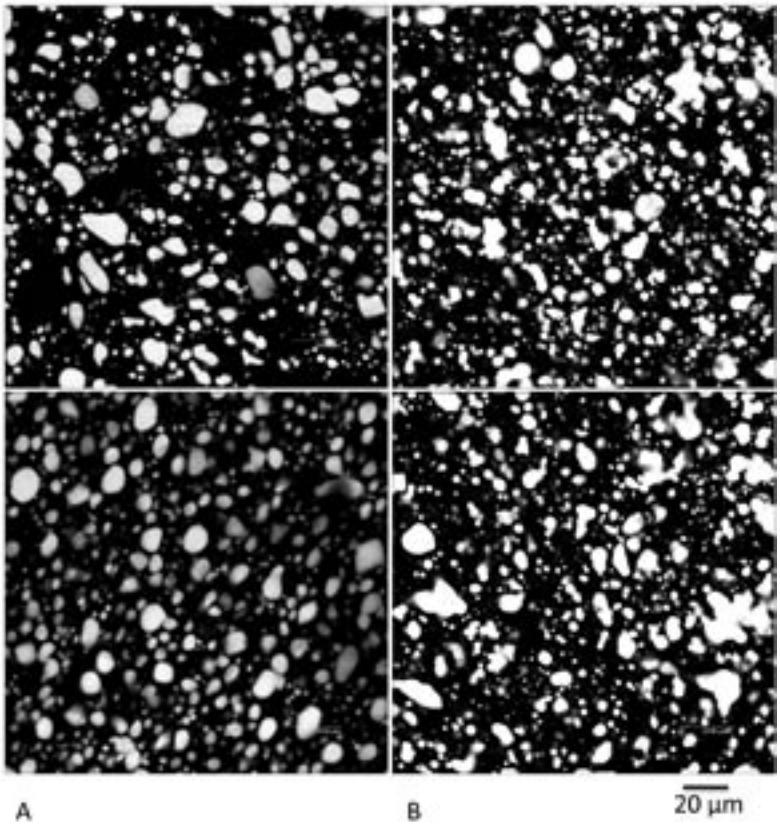

Figure 4: Comparative analysis of cheese microstructure. CLSM image of a 5 weeks old Gouda-type MicroCheese (panel A) and an industrially manufactured 5 weeks old Gouda-type cheese (panel B) show a highly similar coalescence pattern. The cheese was stained with Nile Blue to visualize the lipid inclusions (white) in the cheese matrix. Each panel shows two images from different sections of the preparations.

Comparison of MicroCheese and factory cheese

To obtain a direct comparison between traditionally manufactured cheese and the MicroCheese model, cheese was prepared in a conventional way resulting in 10 kg Gouda-type cheese wheels. After inoculating the cheese vat (1500 liters) in the pilot plant with starter culture, an aliquot of milk was taken and it was immediately used for the preparation of MicroCheese. The MicroCheese was produced with the same specifications as the conventional cheese and both cheese-types were ripened at the same temperature. Samples for GC-MS flavor profiling and RP-HPLC analysis were taken from both cheese-types after 14, 41 and 124 days of ripening.

Based on previously published information (12) RP-HPLC analysis of MicroCheese and factory cheese samples allowed the identification of individual peptide fragments (Fig. 6). In both types of cheese the α_{S1}, α_{S2}, β-cas and para$_{\kappa}$-cas fractions were present at similar levels and their changes in time were in good agreement. Their degradation products α_{S1}-Ia, α_{S1}-Ic and β-cas-I were also in very good agreement between the two types of cheese at all ripening times. The only difference

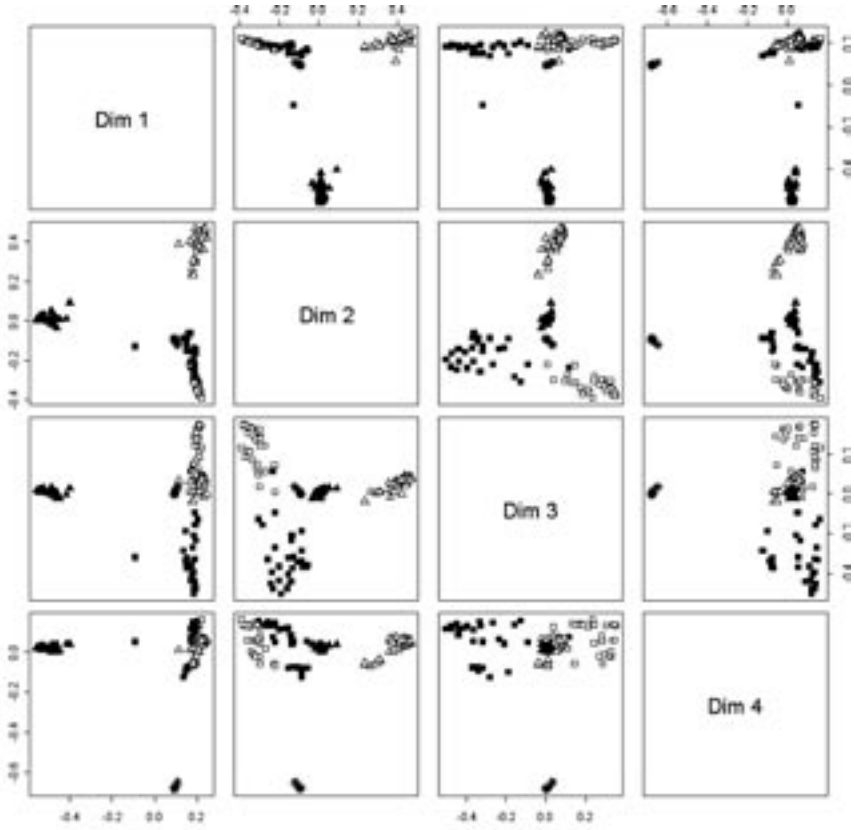

Figure 5: Multidimensional scaling plot of GC-MS peak areas (Table 2) of volatile compounds measured in MicroCheese after 6 weeks of cheese ripening. The analysis is based on supervised random forest and the results clearly allow distinguishing of 4 different types of MicroCheese and industrially manufactured cheese. Gouda without adjunct (FR18 – □), Gouda with adjunct (FR18-APS - ■), Cheddar without adjunct (RA21 - △), Cheddar with adjunct (RA21-FLAV54 - ▲) and industrially manufactured Gouda (Jonge Beemster - ✳).

detected between the RP-HPLC profiles was the appearance of the α_{S1}-Ib fraction, which is increased after 41 days of ripening and decreased after 124 days of ripening in factory cheese as compared to MicroCheese. This suggests a higher turnover rate of this peptide in industrial cheese. Besides the clearly identifiable peaks the overall comparison of the samples of both cheeses yielded very similar elution profiles (data not shown). Taken together the detected time dependent degradation of milk proteins is similar in both types of cheese and consistent with literature (34).

Flavor compounds were quantified by determining peak areas of GC-MS analysis (Fig. 7). The overall correlation coefficients R for the 14, 41 and 124 day old samples were calculated to be 0.95, 0.97 and 0.92, respectively. Small differences were measured for the short chain aldehydes butanal, 3-methylbutanal and 2-methylbutanal, which respectively were 2.6, 2.6 and 2.1 fold higher in MicroCheese as compared to conventional cheese (Fig. 7). In addition, 2-propanone appeared to be present at a higher level in MicroCheese, while 2-butanon levels were higher in conventional cheese. All other compounds measured such as diacetyl, acetoin, butanol, 2-pentanone and dimethyl-disulfide displayed a very good quantitative correlation between the two cheeses.

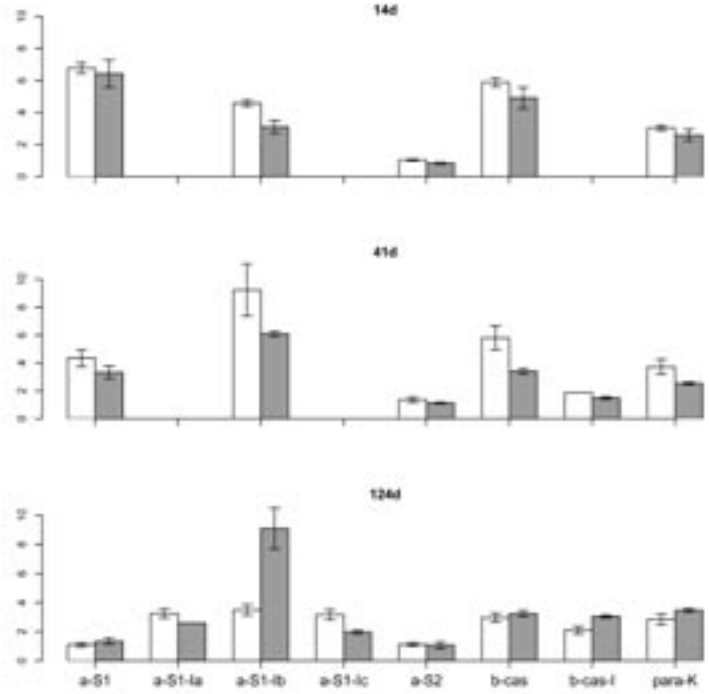

Figure 6: Proteolysis in MicroCheese and industrially manufactured cheese as determined by RP-HPLC. The levels of various casein fractions and their degradation products are shown. Industrially manufactured cheese (white bars) is compared to MicroCheese (grey bars). The three panels represent 14, 41 and 124 days of ripening time from top to bottom respectively. Missing bars indicate that these peptides were not detectable. The identified peptides are indicated on the x-axis and the peak areas are indicated on the y-axis. Error bars show standard deviation (n=4). Fragments α_{s1}-Ia, α_{s1}-Ib and α_{s1}-Ic are degradation products of α_{s1}. Fragment β-cas-I is a degradation product of β-cas. With the exception of peptide α_{S1}-1b the protein degradation in the two types of cheese is very similar

Overall, using the exact same raw materials, the MicroCheese model system generated highly similar results compared to factory cheese over extended ripening times, exemplifying the suitability of the model system for screening purposes with good up-scaling extrapolation characteristics.

MetC and BcKAD overproducers as adjunct

To further investigate the starter culture screening potential offered by the MicroCheese model, the impact of the addition of selected adjunct starter cultures in the Gouda-type MicroCheese was evaluated. The use of an adjunct culture, which overproduced MetC and their cognate deletion mutants, resulted in no significant differences in flavor profiles (data not shown). These results are consistent with data obtained from industrial cheese where sensory analysis revealed no effect by over-expressing *metC* (Bruinenberg *et al.* manuscript in preparation). Moreover, Gouda-type MicroCheeses were manufactured using strain *L. lactis* B1157 as an adjunct culture. This strain is known to express high levels of the α-keto acid decarboxylase (BcKAD) enzyme, which is known to catalyze the formation of aldehydes from α-keto-acids (30). The ability of strain B1157 to form relatively high concentrations of 3-methyl-butanal was established in a laboratory medium (30) as well as in milk a cheese model system (2) and in industrially manufactured cheese (3). In addition, MicroCheeses were manufactured containing adjunct culture B2083, a B1157 derivative in which the *bckad* gene has been inactivated or a genetically engineered strain (NZ9000-pNZ7500) in

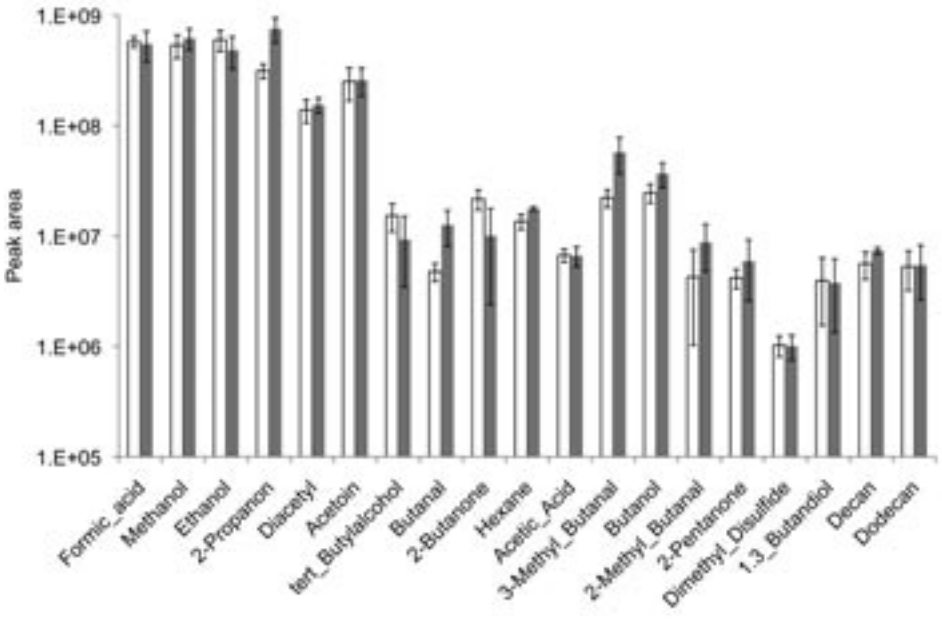

Figure 7: Quantification of volatile flavor compounds as measured in a conventionally produced cheese (white bars) and MicroCheese (grey bars) in after 41 days of ripening. Error bars show standard deviation (n=5). The identified compounds are given on the x-axis and peak areas are given on the y-axis. Overall the data obtained from MicroCheese and industrial cheese shows good correlation.

which BcKAD is expressed at a high level after induction of the nisin controlled expression system (30). After 41 days of cheese ripening MicroCheeses produced with strains B1157 and NZ9000-pNZ7500 cultures respectively contained 7.6- and 5.3-fold increased 3-methylbutanal levels as compared to the control MicroCheeses (Fig. 8). Both differences are highly significant ($p<0.001$) and are consistent with differences observed in cheeses produced with the same cultures at an industrial scale (3). These findings demonstrate that the MicroCheese model allows the accurate prediction of the flavor-profile impact of modulation of a single enzyme in the starter culture, both for enzymes that generate a flavor-effect as well as enzymes that fail to do so. Thereby, these results underline the advanced predictive value of the MicroCheese model in comparison to liquid culture screening results, which suggested that MetC overproduction might impact on cheese-flavor profiles (13).

Discussion

Here we describe a high throughput cheese-manufacturing model, which was benchmarked against industrially manufactured cheese. The results not only demonstrate that physico-chemical parameters resemble industrial cheese very well but also a high reproducibility between individually manufactured MicroCheeses. Undoubtedly, important key parameters measured in relation to the suitability of the MicroCheese model for screening purposes is the determination of proteolytic and

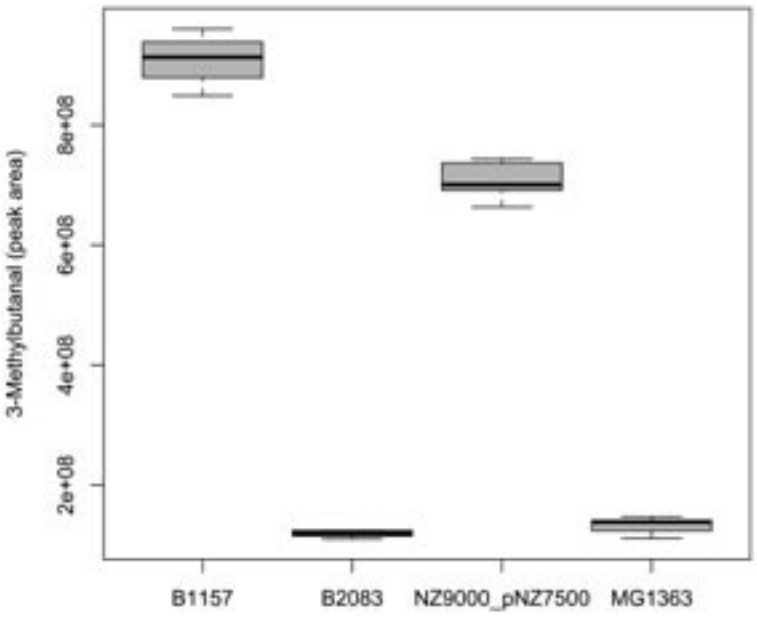

Figure 8: Gouda MicroCheese production with (engineered) adjuncts, high in 3-methylbutanal production and the isogenic control strains. Peak areas of 3-methylbutanal concentrations as measured after 41 days of ripening. The indicated strains are *L. lactis* B1157 (n=4) and a derivative with an inactivated *bckad* gene B2083 (n=5). Furthermore a *bckad* over-expression strain NZ9000 pNZ7500 (n=5) and a corresponding control strain MG1363 (n=5). Whiskers show the total range of measured values. The boxes show the median and the 25th and 75th percentiles.

volatile metabolite profiles during cheese ripening. Many aspects of the degradation pattern of milk proteins in the MicroCheese model were highly similar to industrially produced cheese (Fig. 7). Furthermore, clear parallels were observed in time-dependent volatile flavor compound production profiles during cheese ripening in and industrial cheeses. The Gouda-type MicroCheese produced with the APS adjunct culture resembles the commercially produced Proosdij-type cheese, which was extensively characterized (1, 23). Volatile compounds such as 2-propanon, acetoin, diacetyl, acetic acid, 3-methylbutanal, 2-pentanone, ethyl-butyrate, limonene, hexanoic acid, 2-nonanone, 2-heptanone and ethyl-caproate were found in MicroCheese as well as in the industrial counterpart (23). We sampled MicroCheeses after 1 and 6 weeks of ripening and identified an increase of all of the above compounds during the ripening period, which can be compared to the described flavor development in industrially produced cheese (23). The influence of the APS adjunct culture on Gouda-type cheese flavor profiles are subtle and the difference in taste are partially determined by the ratios of different volatile compounds to each other. The statistical analysis of MicroCheeses manufactured with and without APS adjunct clearly allowed distinguishing the two types of cheese. One of the key flavor compounds which is described to play an important role in the particular flavor of Proosdij-type cheese is 3-methylbutanal. In agreement with industrial cheeses (23) we found increased concentrations of 3-methylbutanal in MicroCheese manufactured with the APS adjunct culture. However this difference is much more pronounced in 1-week-old MicroCheeses (2.9 fold increase with APS) than in 6-week-old MicroCheese (1.2 fold increase with APS). Such a decrease of 3-methylbutanal with ripening time has been observed before in industrially manufactured cheese and it was speculated that this decrease was caused by the conversion of the aldehyde to the corresponding alcohol (1). For the production of Cheddar-type MicroCheese we used commercially available starter cultures. The Cheddar adjunct culture we used is described to enhance the sweet note of cheese. The most distinguished difference caused by the Cheddar adjunct FLAV54 in the MicroCheese system is a significant increase in the concentration of acetic acid (4.1 fold). Increased concentrations of acetic acid were correlated to the sweetness off cheese (7) which would be consistent with our findings. Moreover, the predictive value of flavor volatile patterns obtained from the MicroCheese model was established by a direct comparison of the model with the normal cheese production process, using the same raw-materials, which was further confirmed by the designed manipulation of the 3-methylbutanal levels using engineered adjunct starter cultures (30) (Fig. 7 and 8).

Table 1: Strains and plasmids used to assess the effect of varying *bckad* and *metC* expression levels on flavor profiles.

Strain Name	**description**	**Reference**
MG1363	*L. Lactis*; plasmid free, wild-type strain	Gasson et al. 1983
NZ9000	*L. lactis* MG1363; *pepN::nisRK*	Kuipers et al. 1998
B1157	*L. Lactis*, Branched-chain α-keto acid decarboxylase-positive	Ayad et al. 1999
B2083	Tet^r, Ery^r, Cm^r; pGh9:ISSI integrated in the chromosome of B1157 at position 462 of the decarboxylase gene (*bckad)*	Smit et al. 2005
Plasmid Name		
pNZ8136	Cm^r, 4.5-kb pNZ8048 derivative carrying the MG1363 *metC* gene	Fernández et al. 2000
pNZ7500	Cm^r pNZ8148 derivative containing a 1.8-kb fragment carrying the decarboxylase gene (*bckad*) of B1157	Smit B. et al. 2005

The obvious and unavoidable differences between the two systems used are the smaller curd size and the larger volume/surface ratio, which may be causative for some of the differences observed between the two systems, including the decreased fat-content in the MicroCheese model and the slight deviations in flavor profiles. Nevertheless, despite this lower fat content the microstructure of the MicroCheeses was comparable to industrially produced cheese.

Taken together the presented data support the conclusion that the MicroCheeses manufactured in a high throughput format resemble most key properties of conventionally manufactured cheese. The data convincingly show that the MicroCheese model offers a potential high-throughput system for a variety of screening purposes. Therefore, current activities focus on (further) automation of the described protocol to enhance its throughput and improve e.g the reproducibility of stirring intensities, and the protocols are extended towards additional cheese types like Emmental type and soft cheeses. Besides offering a valuable screening platform for cheese product development the model also enables the effective investigation of fundamental questions related to cheese manufacturing and *in situ* bacterial physiology and genetics. In relation to the latter we have recently developed an

Table 2: GC-MS peak areas of volatile compounds identified in MicroCheese.

Starter culture	**Fr18**				**Fr18APS**			
ripening time	**1 week**		**6 weeks**		**1 week**		**6 weeks**	
n[a]	**4**		**10**		**4**		**10**	
	Peak area[b]	**SDEV in %[b]**	**Peak area**	**SDEV in %**	**Peak area**	**SDEV in %**	**Peak area**	**SDEV in %**
2-Propanon	**138651**	*18,5%*	**6751524**	*20,4%*	**105316**	*14,2%*	**5970845**	*15,2%*
Acetic Acid	**17886**	*25,4%*	**26834**	*48,7%*	**22288**	*57,0%*	**40426**	*47,6%*
Diacetyl	**314425**	*5,1%*	**255557**	*24,2%*	**725639**	*9,7%*	**342150**	*13,2%*
3-Methyl butanal	**5183**	*39,2%*	**41216**	*68,2%*	**15283**	*51,0%*	**49207**	*57,9%*
2 Pentanone	**36095**	*8,4%*	**102737**	*49,4%*	**20573**	*23,7%*	**102119**	*56,7%*
Butanoic acid	**1043**	*42,0%*	**196074**	*91,2%*	**970**	*22,6%*	**128588**	*79,4%*
Acetoin	**1726033**	*18,4%*	**1643147**	*12,5%*	**3504683**	*26,9%*	**2099108**	*10,8%*
Ethylbutyrate	**643**	*31,0%*	**17162**	*128,1%*	**1618**	*33,8%*	**13168**	*113,6%*
Undecane 5,5-dimethyl	**1416**	*30,4%*	**2193**	*59,9%*	**2396**	*90,1%*	**4065**	*49,6%*
Pentamethylheptane	**3514**	*13,2%*	**19684**	*63,2%*	**3668**	*8,2%*	**19861**	*41,6%*
Limonene	**1911**	*15,2%*	**1607**	*43,6%*	**1466**	*16,7%*	**1193**	*48,0%*
2-Heptanone	**15413**	*24,7%*	**61117**	*55,2%*	**18410**	*69,2%*	**57348**	*60,3%*
Hexanoic acid	**1566**	*20,1%*	**77826**	*92,0%*	**1418**	*24,9%*	**46774**	*101,0%*
Ethylcaproate	**350**	*24,2%*	**11742**	*107,0%*	**3262**	*60,2%*	**7007**	*99,8%*
2-Nonanone	**3271**	*17,0%*	**28066**	*75,3%*	**8566**	*123,7%*	**21712**	*81,4%*
Octanoic acid	**8687**	*17,1%*	**14457**	*37,3%*	**8032**	*21,1%*	**11310**	*52,1%*
Ethyloctanoate	**42**	*71,1%*	**4249**	*105,6%*	**nd[c]**		**2352**	*120,4%*

a) n= number of biological replicates analyzed.
b) Averages (bold) and standard deviations in % (italics) are given.
c) nd = not detectable

improved Recombinant *In Vivo* Expression Technology (R-IVET) system in *L. lactis* (6), which has been applied to monitor real-time *in situ L. lactis* gene expression in individually MicroCheeses using luciferase as a reporter (Chapter 5 of this tesis).

Based on the unprecedented throughput offered by the MicroCheese model, a range of microbial screening possibilities can be foreseen, including the screening of microbial culture collections for desired flavor forming capacities, the screening of bacterial mutant libraries, the assessment of microbial viability during the ripening process, or the expression of relevant enzyme activities *in situ*. In addition the model appears suitable for the evaluation of variations in processing conditions, the addition of enzymes, etc. Finally, any of these screening approaches may readily be combined with altered and desired physico-chemical parameters of the cheese matrix, like the reduction of the fat or salt content of cheese, which may offer avenues for the identification of dedicated starter cultures and/or enzymes that are able to compensate for flavor and texture defects in these novel cheese matrices.

	RA21				RA21FLAV54				Beemster	
	1 week		6 weeks		1 week		6 weeks		6 weeks	
	8		10		8		10		4	
	Peak area	SDEV in %	Peak area	SDEV in %	Peak area	SDEV in %	Peak area	SDEV in %	Peak area	SDEV in %
	190791	*85,6%*	**7206944**	*16,8%*	**302558**	*42,7%*	**7268759**	*24,7%*	**230998**	*16,6%*
	59757	*33,5%*	**67595**	*19,4%*	**247777**	*21,4%*	**131222**	*16,1%*	**41126**	*14,5%*
	753509	*28,5%*	**448036**	*40,8%*	**652528**	*10,6%*	**356259**	*11,7%*	**48245**	*23,0%*
	5926	*39,4%*	**51339**	*119,8%*	**10828**	*41,5%*	**22409**	*29,8%*	**14781**	*24,6%*
	59965	*104,9%*	**145937**	*66,3%*	**59182**	*105,8%*	**147077**	*60,4%*	**6975**	*21,2%*
	2405	*17,3%*	**18159**	*40,7%*	**2270**	*12,1%*	**24108**	*61,9%*	**3130**	*6,3%*
	6709909	*13,2%*	**2747033**	*10,6%*	**6112486**	*7,4%*	**2729263**	*8,1%*	**300760**	*17,3%*
	4517	*26,2%*	**18738**	*122,7%*	**3913**	*13,0%*	**22796**	*102,0%*	**54**	*36,2%*
	4226	*87,1%*	**3840**	*51,2%*	**8425**	*14,2%*	**2936**	*26,4%*	**997**	*41,3%*
	37970	*89,1%*	**123570**	*63,2%*	**130139**	*109,7%*	**194199**	*61,7%*	**416**	*18,1%*
	1082	*31,2%*	**999**	*75,6%*	**1285**	*31,6%*	**828**	*52,9%*	**696**	*53,6%*
	16613	*80,7%*	**75339**	*69,2%*	**14157**	*86,0%*	**72808**	*57,0%*	**13425**	*5,3%*
	1850	*22,5%*	**3576**	*29,7%*	**1812**	*15,8%*	**3123**	*53,4%*	**1782**	*15,2%*
	1379	*60,4%*	**6837**	*80,8%*	**1883**	*37,1%*	**7933**	*72,0%*	**2072**	*168,6%*
	5800	*63,4%*	**16981**	*64,8%*	**5124**	*76,3%*	**17891**	*55,5%*	**3403**	*22,3%*
	6523	*14,8%*	**6324**	*39,2%*	**5712**	*32,2%*	**6446**	*53,8%*	**4528**	*31,3%*
	37	*114,4%*	**318**	*65,8%*	**66**	*72,6%*	**411**	*86,7%*	**50**	*9,9%*

Acknowledgements

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Chapter

4

Luciferase detection during stationary phase in *Lactococcus lactis*

Herwig Bachmann, Filipe Santos, Michiel Kleerebezem, Johan E.T. van Hylckama Vlieg

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The luminescence signal of *luxAB*-encoded bacterial luciferase strongly depends on the metabolic state of the host cell, which restricts the use of this reporter system to metabolically active bacteria. Here we show that in stationary phase cells of *Lactococcus lactis* the detection of luciferase is significantly improved by the addition of riboflavin or FMN to whole-cell assay systems.

The *luxAB* encoded luciferase of *Vibrio harveyi* is frequently used as a reporter in a variety of microorganisms (10). Simple detection and high sensitivity underlie the increasing popularity of this system (11). Luciferase catalyses the reaction of molecular oxygen, $FMNH_2$ and a long-chain aldehyde, yielding the corresponding carboxylic acid, FMN, water and light (490 nm). Besides its use as a promoter-probe luciferase is also used for the analysis of the metabolic activity of cells (13). The latter is based on the dependency of the luminescence signal on the intracellular $FMNH_2$ concentration, which is directly correlated to the metabolic activity of a cell (6). This dependency is well documented for Gram-positive (7, 8, 17, 19) and Gram-negative (9, 13) bacteria, and is illustrated by a rapid decline of luminescence upon entry into the stationary growth phase. Here we describe an improved method for the detection of luciferase activity in stationary cells of *Lactococcus lactis*.

In our studies we used the plasmid-encoded luciferase *(luxAB)* of *Vibrio harveyi* in the lactic acid bacterium *L. lactis* MG5267 (16). The reporter construct was generated by digesting plasmid pJIM2374 (5) with HindIII and PstI. The *luxAB* fragment was isolated, made blunt and cloned into pCRblunt (Invitrogen, Breda, The Netherlands) yielding pNZ5512. Subsequently the *luxAB* fragment was isolated from pNZ5512 as an EcoRV, HindIII fragment, made blunt and ligated into PmlI digested pNZ7125 (2), resulting in pNZ5518. The *usp45* promoter (15) was amplified from genomic DNA of *L. lactis* MG1363 with the oligonucleotides usp45REV1 (5'GAACGATCATGCCTGCAGAGTACTTGTTC) and usp45FW2 (5' CTATTACTCGAGACACTTTTGCTC). The amplified fragment was restricted with Sau3AI and ligated into BglII-digested pNZ5518, resulting in pNZ5519, in which the *luxAB* genes are under control of the *usp45* promoter. Furthermore, the oligonucleotides AdaptVI-1 (5'CATGGAATATCCTCCTGAATTGGGGATCCCTCGAGTTAGTTAGTGCCCGGGCTAA) and AdaptVI-2 (5'GATCTTAGCCCGGGCACTAACTAACTCGAGGGATCCCCAATTCAGGAGGATATTC) were annealed and ligated into the BglII, NcoI digested plasmid pNZ5518 which resulted in the introduction of a SmaI restriction site (plasmid pNZ5520). Genomic DNA from *L. lactis* MG1363 was partially digested with AluI and 0.5 – 2 kb fragments were isolated and ligated into *Sma*I digested pNZ5520.

L. lactis was grown in microplates (Greiner, Alphen a/d Rijn, The Netherlands, #780271 or #655180) at 30°C in rich medium M17 (12) supplemented with 0.5 % lactose, 5 µg/ml chloramphenicol and, when indicated, 10 mg/l riboflavin (Sigma, Zwijndrecht, The Netherlands, #R4500). Luminescence and optical density at 595 nm (OD_{595}) measurements were performed by mixing 50 µl of cell suspension with 150 µl of 1.9% w/v glycerol-2-phosphate disodium salt (Sigma, Zwijndrecht, The Netherlands, #G6376) in a white microplate with a transparent bottom (Greiner, Alphen a/d Rijn, The Netherlands, #655095). If indicated 10 mg/l riboflavin or 10 mg/l flavin mononucleotide (FMN) (Sigma, Zwijndrecht, The Netherlands, #F2253) was added to the buffer. Two minutes after mixing the cells with the buffer, 10 µl of 0.1% nonanal (Sigma, Zwijndrecht, The Netherlands, #W278203) in 40% ethanol was added to each well.

Luminescence was determined with 2 minute intervals over a period of 15 minutes after nonanal addition in a Genios microplate reader (Tecan, Zurich, Switzerland). The peak value measured for each sample was used for data analysis.

When cultivated in M17, *L. lactis* MG5267 transformed with the *luxAB* expression plasmid pNZ5519 displayed a rapid decline of the luminescence signal upon entry of the cells into the stationary phase of growth (Fig. 1). We hypothesized FMN could represent the limiting factor in the luminescence reaction and that addition of the FMN precursor riboflavin could complement this limitation. Indeed, addition of riboflavin to either the culture medium M17 or to the assay buffer leads to an up to 100-fold increase of luminescence, enabling detection of luminescence in cells that have entered the stationary phase of growth (Fig. 1 and Fig. 2). Moreover, the introduction of the *luxAB* expression plasmid pNZ5519 in the riboflavin-overproducing *L. lactis* strain CB010 (3) also allowed stable luminescence signal detection in cells entering the stationary phase of growth (Fig. 1). The addition of FMN to the assay buffer had an effect similar to that of the addition of riboflavin (Fig. 2). To exclude that these observations resulted from regulatory effects on the *usp45* promoter, 5 alternative promoter-*luxAB* fusion constructs were analysed under the same conditions. These clones were selected from a pNZ5520-based promoter screening library constructed in MG5267 (data not shown). They contain random fragments of genomic *L. lactis* DNA cloned upstream of the promoterless *luxAB* gene cassette. There was a 100-fold difference in luminescence levels between the clone with the highest and the lowest activity. For all constructs luminescence in stationary

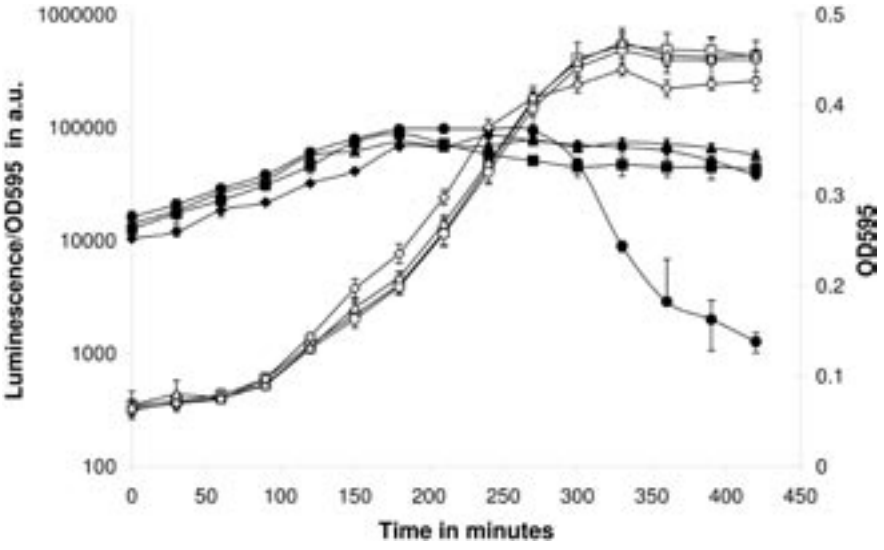

Figure 1: The effect of riboflavin on luminescence of luciferase-expressing *L. lactis* MG5267. Growth was analysed by monitoring optical density at 595 nm (OD_{595}). Filled symbols show luminescence per OD_{595} values and open symbols show the corresponding OD_{595} measurements. Symbols: ■, MG5267(pNZ5519) grown in LM17 measured in buffer plus riboflavin; ●, MG5267(pNZ5519) grown in LM17 measured in buffer only; ▲, MG5267(pNZ5519) grown in LM17 + riboflavin (10 mg/l) measured in buffer only; ♦, CB010(pNZ5519) grown in LM17 measured in buffer only. Each data point represents the average of 12 biological replicates (error bars show standard deviation).

phase could be increased significantly by the addition of either riboflavin or FMN (Fig. 2). The negative control with a promoterless *luxAB* construct pNZ5518 shows a luminescence signal comparable to background measurements irrespective of the addition of riboflavin or FMN (Fig. 2). These results confirm that riboflavin/FMN availability is a limiting factor for the luminescence signal in *L. lactis* cells that are in the stationary phase of growth. Furthermore, they indicate that NADH for the (re-) generation of the luminescence reaction co-factor, $FMNH_2$, is available in these cells.

In a different experimental setup we supplied nonanal in a volatile form to the cultures by placing 2% nonanal diluted in mineral oil in the spaces between the wells of a covered microplate. Luminescence was measured throughout the growth curve in the wells where the cells were cultured. We ensured that neither nonanal nor oxygen was limiting the luminescence reaction in those cultures and found that despite the addition of extra riboflavin to the medium, luminescence signals in stationary phase were variable (data no shown). This finding suggests that a continuous luminescence reaction might have an effect on the metabolism of stationary *L. lactis* cells.

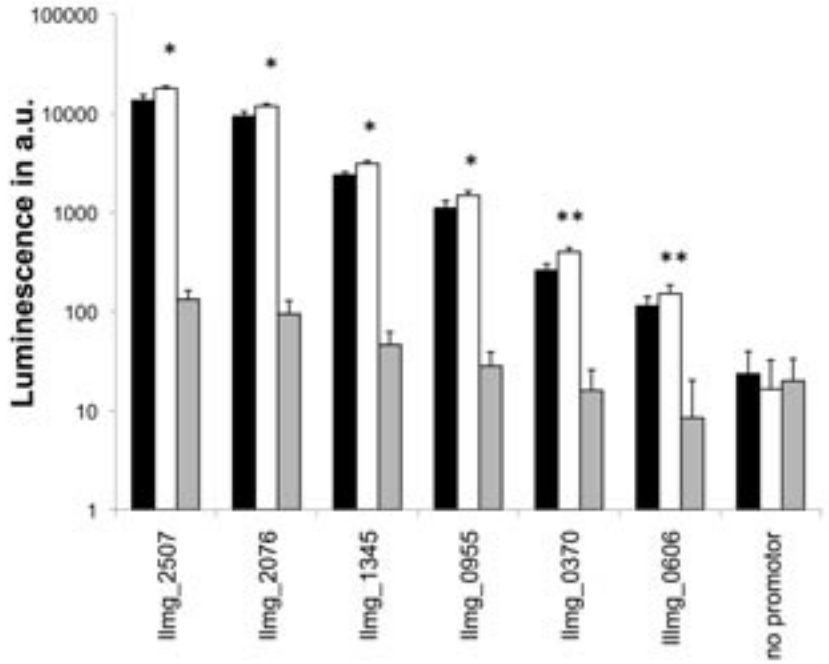

Figure 2: The effect of the addition of riboflavin or FMN on luminescence signals of stationary phase cells of *L. lactis* MG5267 with varying luciferase expression levels. Luciferase activity was measured 3.5 hours after cultures entered the stationary phase. The black bars show measurements performed in buffer supplemented with 10 mg/l riboflavin. White bars show measurements in buffer supplemented with 10 mg/l FMN. Grey bars show measurements in buffer only. Each bar represents the average value of four biological replicates (error bars show standard deviation). * $p << 0.001$ and ** $p<0.002$ (pairwise T-test). The experiment was repeated 4 times with similar results. The names of the samples refer to the promoter sequence upstream of the luciferase genes as annotated in *L. lactis* MG1363 (18).

The presented data show that the detection of bacterial luciferase in stationary phase *L. lactis* can be significantly improved by the addition of riboflavin or FMN. Riboflavin is known to serve as an $FMNH_2$ analogue for the luminescence reaction, but only in its reduced form (14). This excludes that the described effect is caused by transported riboflavin itself and is confirmed by our finding that luminescence in the *luxAB* negative controls was not influenced by the addition of either riboflavin or FMN. Blouin et al. reported that the addition of FMN to *E. coli* cultures shortly before luminescence measurements could increase the signal, but these authors did not relate this observation to luminescence detection in stationary phase cells (1). The phylogeny of the *L. lactis* riboflavin transporter RibU (4) suggests that our finding might also be applicable to a number of other Firmicutes. However, a reliable assessment of the applicability to other species requires additional experimentation. In conclusion, the detection of *luxAB* encoded luminescence for *L. lactis* is significantly improved by the addition of riboflavin or FMN to either the culture medium or the buffer used during the luminescence assay. Furthermore it is important to realize that a continuous luminescence reaction in *L. lactis* might influence the metabolic state of the stationary host cell.

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Chapter 5

High throughput identification and validation of in situ expressed genes in *Lactococcus lactis*

Herwig Bachmann, Michiel Kleerebezem, Johan E.T. van Hylckama Vlieg

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Abstract

Understanding the functional response of bacteria to their natural environment is one of the challenges in current microbiology. Over the past decades several techniques have been developed to study gene expression in complex natural habitats. Most of these methods however are laborious and validation of results under in situ conditions is cumbersome. Here we report the improvement of the Recombinase-based In vivo Expression Technology (R-IVET) by the implementation of two additional reporter genes. The first one is an α-galactosidase (*melA*) which facilitates the rapid identification of in vivo induced genes. Secondly, the bacterial luciferase genes (*luxAB*) are transcriptionally coupled to the resolvase gene, which allows rapid validation and characterization of in vivo induced genes. The system is implemented and validated in the industrially important lactic acid bacterium *Lactococcus lactis*. We demonstrate the applicability of the advanced R-IVET system by the identification and validation of lactococcal promoter elements that are induced in minimal medium as compared to the commonly used rich laboratory medium M17. R-IVET screening led to the identification of 19 promoters that predominantly control expression of genes involved in amino acid and nucleotide metabolism, and transport functions. Furthermore the luciferase allows high resolution transcription analysis and enabled the identification of complex medium constituents and specific molecules involved in promoter control. Rapid target validation exemplifies the high throughput potential of the extended R-IVET system. The system can be applied to other bacterial species, provided the used reporter genes are functional in the organism of interest.

Introduction

Understanding the responses of microorganisms in their natural environments is one of the big challenges of the post-genomic era. Complex niches such as soil, decaying plant material and the (mammalian) gastrointestinal tract show a rich bacterial diversity and complex microbial activity patterns. Sampling complexity and environmental dynamics hamper comprehensive transcriptional analysis of a specific microorganism residing in such a complex environment. To overcome these challenges a number of tools to study in vivo gene expression were developed during the past decades (25). One of the most powerful technologies is termed In vivo Expression Technology (IVET) and is based on transcriptional fusions of genomic DNA fragments to a selectable reporter gene (32). Recombinase-based In vivo Expression Technology (R-IVET) is an IVET variation that employs a resolvase as the primary reporter gene. Upon expression of the resolvase a chromosomally located marker, situated between two recombination sites, is excised from the genome (1). This irreversible step "records" in situ promoter activity and acts as a promoter-trap system, allowing the accumulation of promoter activity detection over extended in situ incubation periods. Moreover, the system is able to detect very low and transient gene expression in complex environmental conditions. An inherent property of IVET systems is that only the up-regulation of genes can be detected. Efforts were made to broaden the spectrum of detectable genes in R-IVET systems by mutating the ribosomal binding site of the resolvase and using these mutants to construct multiple libraries (23). Another limitation of IVET systems is that the validation of induced target sequences in vivo is in many cases laborious and requires advanced transcript detection methodology (27).
In this study we addressed these restrictions and describe the implementation of an advanced R-IVET system in the lactic acid bacterium *Lactococcus lactis*, which is one of the best characterized low-GC gram positive bacteria and is extensively used in complex food fermentations.

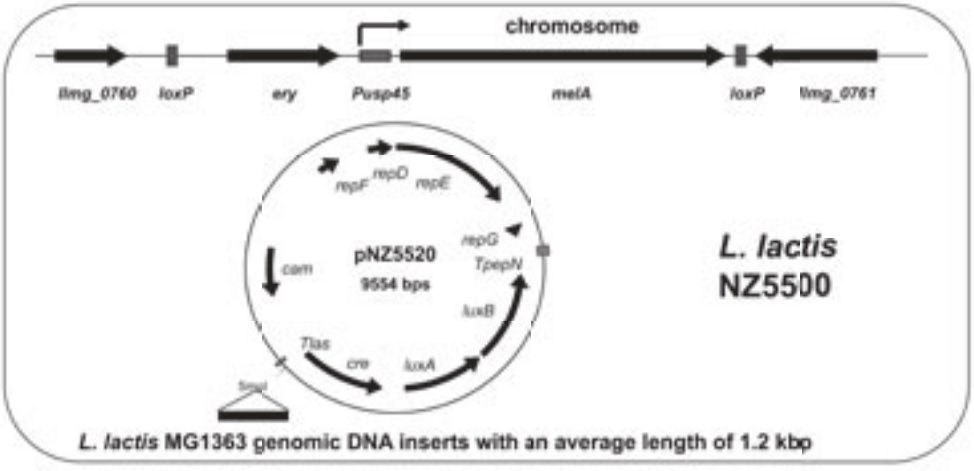

Figure1: Schematic representation of the R-IVET system. On the chromosome the integration locus of the *loxP-ery-Pusp45-melA-loxP* fragment is shown. The plasmid pNZ5520 harbors a *cre-luxA-luxB* construct which is flanked by the terminators *Tlas* and *TpepN*. The SmaI/SrfI restriction site upstream of cre was used for the insertion of chromosomal fragments.

This improved system employs an α-galacotsidase gene (*melA*) for positive primary clone selection without selection pressure and the luciferase genes *luxAB* as a secondary reporter for the validation of selected target genes. Bacterial luciferase allows sensitive detection of promoter activity in vitro and in situ after the primary R-IVET selection process. To exemplify the applicability of the system, 19 promoters specifically activated in a minimal medium as compared to a rich laboratory medium were identified and their regulation by media components was evaluated. The identified sequences predominantly regulate the expression of genes involved in amino acid and nucleotide metabolism and genes coding for transporters. The added value of the presence of *luxAB* in the recombinase vector is also illustrated by the high-resolution analysis of the *purCSQLF* promoter activity pattern in different media and during the different phases of growth.

Material and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* was grown under aerobic conditions in TY broth (33). *Lactococcus lactis* MG1363 was grown in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose. *L. lactis* MG5267 and its derivatives were grown on M17 medium supplemented with 0.5% (wt/vol) lactose. *Escherichia coli* MC1061 (6) and Top10 (Invitrogen, Breda, The Netherlands) were used as an intermediate cloning host for the construction of pNZ5512 and pNZ5517. *L. lactis* MG1363 (12) was used as an intermediate cloning host for pNZ7125, pNZ5515 and pNZ5518. *L. lactis* NZ5500 was used as a cloning host for pNZ5519 and pNZ5520. The minimal medium for *L. lactis* that was used for R-IVET library screening was prepared as described by Poolman and Konings (30), with the modification that only eight amino acids were added (glutamate, histidine, isoleucine, leucine, methionine valine, asparagines and glutamine), which are essential for exponential growth of *L. lactis* (14). When appropriate, antibiotics were added to the media, at a concentration of 10 µg/ml chloramphenicol, 200 µg/ml erythromycin and 50 µg/ml kanamycin for *Escherichia coli* and 5 µg/ml chloramphenicol and 5 µg/ml erythromycin for *L. lactis* unless when indicated differently.

DNA techniques

Plasmid preparation from *E. coli* and *L. lactis* was performed using Jetstar columns according to the manufacturer's manual (Genomed GmbH, Loehne, Germany). For *L. lactis* the manufacturer's protocol was modified by using a solution of 50 mM Tris, 10 mM EDTA, 100 µg/ml RNAse and 4 mg/ml lysozyme, at pH 8.0 for the first step in the cell lysis protocol. Cell suspensions were incubated at 50°C for 1 hour and subsequently plasmid DNA was purified according to the manufacturer's recommendations. DNA techniques were performed as described by Sambrook et al. (33). Restriction endonucleases, DNA polymerases and DNA Ligase were purchased from Invitrogen (Breda, The Netherlands), New England Biolabs (Leusden, The Netherlands) and Stratagene (La Jolla, USA) and used

according to the manufacturer's recommendations. Oligonucleotides were purchased from Proligo (Paris, France) and Invitrogen (Breda, The Netherlands). Transformation of *L. lactis* was performed as described by Wells et al. (41).

Construction of Lactococcus lactis NZ5500

The intergenic region between the convergent genes *llmg_0760* and *llmg_0761* in the genome of *Lactococcus lactis* MG1363 was selected as target site for introduction of the R-IVET resolution cassette. This locus contains a unique BpuI restriction site between the 3' ends of the convergent genes which was used during cloning procedures (see below). The *llmg_0760* and *llmg_0761* 3'-encoding fragments and intergenic region was amplified by PWO superyield DNA polymerase (Roche Diagnostics GmbH; Mannheim, Germany) PCR, using the primers lc0012FW1 and lc0323Rev1 and *L. lactis* MG1363 genomic DNA as template. The 1.6 kbp amplicon was cloned into pCRblunt (Invitrogen, Breda, The Netherlands), yielding pNZ5511 and the identity and intactness of the cloned fragment was verified by DNA sequencing. To construct a *loxP-ery-loxP* resolution cassette the erythromycin resistance gene-cassette was obtained as a PstI fragment from pUC18ery (38), and subsequently treated with T4 DNA polymerase. The fragment was then digested with BamHI and cloned in BamHI-HincII digested pUC19lox2 (4). From the resulting plasmid, the *loxP-ery-loxP* cassette was recovered as a PstI fragment, which was treated with T4 DNA polymerase and cloned in Bpu10I digested and with Klenow fragment treated pNZ5511. The resulting plasmid was designated pNZ5513 and contains a *llmg0760-loxP-ery-loxP-llmg0761* R-IVET resolution cassette. This cassette was recovered from pNZ5513 as a HindIII-PstI fragment, which was treated with T4 polymerase and cloned in ClaI digested and with Klenow fragment treated pNZ5510, yielding pNZ5514. Plasmid pNZ5510 is a pNZ7101 (31) derivative from which the erythromycin gene was removed by HindIII digestion, Klenow treatment, and self-ligation. To obtain the α-galactosidase encoding gene (*melA*) of *Lactobacillus plantarum* under control of a strong lactococcal promoter, the *melA* coding region and its ribosome binding site was PCR amplified using the primers melAF94 and melAF95 and cloned in the SmaI digested derivative of vector pIL253 (35), from which the EcoRI fragment had been removed by EcoRI digestion followed by self-ligation. The resulting vector (pNZ3250) was digested with BamHI and XbaI, treated with S1 nuclease and re-ligated. This was followed by the insertion of a multiple cloning site (composed of the oligonucleotides pRB056_122 and pRB056_123) into the EcoRI site upstream of the *melA* gene, generating the *melA* reporter plasmid pNZ3251. The *melA* gene was subsequently placed under transcriptional control of the strong lactococcal promoter of the *usp45* gene (P*usp45*) (37). P*usp45* was obtained as a 224 bp amplicon using usp45FW3 and usp45REV3 as primers and chromosomal DNA of *L. lactis* MG1363 as a template. The P*usp45* amplicon was digested with NcoI and XhoI and cloned in equally digested pNZ3251, resulting in pNZ5515. The P*usp45-melA* fragment was recovered from pNZ5515 as a DraI-NcoI fragment, treated with Klenow fragment and cloned in pCRblunt (3), generating pNZ5516. The P*usp45-melA* cassette was isolated from pNZ5516 as an EcoRI fragment and protruding ends were removed by Klenow treatment. This fragment was cloned downstream of the erythromycin resistance gene into the R-IVET resolution

Table 1: Bacterial strains, plasmids and oligonucleotides used in this study.

Strain, Plasmid, Oligonucleotide	Relevant markers or characteristics / Sequence	Source or reference
Strains		
E. Coli		
Top10	cloning host	Invitrogen
MC1061	cloning host	6
L. lactis		
MG1363	cloning host	12
MG5267	MG1363 with the lactose operon integrated into the genome	39
NZ5500	MG5267 with a *-loxP-ery-usp45p-melA-loxP-* fragment integrated into the genome	this study
Plasmids		
pCRblunt	positive selection cloning vector	3
pNZ5511	Km^r; pCRblunt containing a genomic fragment *(llmg_0760-llmg_0761)* from MG1363	this study
pUC18ery	Ap^r, Em^r	4
pUC19lox2	Ap^r; pUC18 derivative containing 2 *loxP* sites in tandem repeat	4
pUCloxery	Ap^r; pUC19 containing a *loxP-ery-loxP* fragment	this study
pNZ5513	Km^r; pNZ5511 containing *llmg_0760-loxP-ery-loxP-llmg_0761*	this study
pNZ7101	Cm^r, Em^r, vector for the construction of genomic insertion mutants	31
pNZ5510	Cm^r, pNZ7101 derivative missing Em^r	this study
pNZ5514	Cm^r, pNZ5510 containing *llmg_0760-loxP-ery-loxP-llmg_0761*	this study
pNZ5515	Cm^r, pNZ3251 containing *usp45p upstream of melA*	this study
pNZ5516	Km^r; pCRblunt containing *-usp45p-melA-*	this study
pNZ5517	Cm^r, pNZ5514 containing *llmg_0760-loxP-ery-usp45p-melA-loxP-llmg_0761*	this study
pNZ7125	Cm^r, R-IVET library vector	4
pJIM2374	Em^r; derivative of pORI128 carrying *luxAB* from *V. harveyi* and the Em^r gene from pIL252	8
pNZ5512	Km^r; pCRblunt containing *luxAB* from pJIM2374	this study
pNZ5518	Cm^r, pNZ7125 derivativie containing *luxAB* downstream of cre	this study
pNZ5519	Cm^r; pNZ5518 derivative containing *usp45p* upstream of cre	this study
pNZ5520	Cm^r; pNZ5518 derivative containing a linker with an SrfI restriction site upstream of cre	this study
pIL253	Em^r; high copy vector	35
pNZ3250	pIL253 digested with EcoRI and recircularized; *melA* from L. plantarum intoduced into the SmaI site	this study
pNZ3251	Em^r; multiple cloning site introduced into pNZ3250	this study
Oligonucleotides[1]		
		this study
lc0012FW1	CATTGCTAGTCCAAACGCTCTT	this study
lc0323REV1	GGTCTAAATGAAATTAAGTAAAGTTGC	this study
lc0012 REV1	TCAAGTTCAGGTGCTTACGG	this study
lc0012 FW2	GGATTGCTCGCTCATTTATT	this study
lc0323FW1	GCAAGCTATGAACTGCATCA	this study
lc0323REV2	TGCAAGAAATGAATCTCGAA	this study
pUSPFOR	TAGCGATCACACTTTTGCTC	this study
usp45REV1	GAACGATCATGCCTGCAGAGTACTTGTTC	this study
usp45FW2	CTATTACTCGAGACACTTTTGCTC	this study

usp45FW3	GATATTccatggACACTTTTGCTCAATA	this study
usp45REV3	GATTctcgagCAGAGTACTTGTTCTTTT	this study
Adapt VI-1	CATGGAATATCCTCCTGAATTGGGGATCCCTCGAGTTAGTTAGTGCCCGGGCTAA	this study
Adapt VI-2	GATCTTAGCCCGGGCACTAACTAACTCGAGGGATCCCCAATTCAGGAGGATATTC	this study
xDNA FW2	TGGAATATCCTCCTGAATTGG	this study
xDNA REV2	GAATTTGCTTCTGCAGTTAAAAAC	this study
melAFW	CTCTACACGACTCCCGTTCA	this study
eryREV	TTAGCCAGTTTCGTCGTT	this study
melAF94	AACTGA tgtaca AAAAGCTAATAGCGAAGGG	this study
melAR95	AATATG tgtaca GGCTGAGCTTAGTCCTTAGCC	this study
pRB056_122	AATTGCCTGCAGGCAAACAACCAGGGCGGATCCCGGGCCATGG CTAGCGATCGGGCGGCGCGGCCGCTAGCTCTAGACCCCTCGAGTAACTAAGTAAC	this study
pRB056_123	AATTGTTACTTAGTTACTCGAGGGGTCTAGAGCTAGCGGCCGCG CCGCCCGATCGCTAGCCATGGCCCGGGATCCGCCCTGGTTGTTTGCCTGCAGGC	this study

Km^r, kanamycine resistant; Cm^r, chloramphenicol resistant; Em^r erythromycin resistant; Ap^r, ampicillin resistant;

[1]lower case letters indicate a restriction site

cassette in SmaI digested pNZ5514, yielding pNZ5517. This final R-IVET resolution vector contains a chromosomal integration cassette composed of the *loxP-ery-Pusp45-melA-loxP* resolution cassette flanked by *llmg_0760* and *llmg_0761* regions. Strain MG5267 was transformed with pNZ5517 and integrants were selected on LM17 supplemented with 2.5 µg/ml erythromycin. Replica plating revealed that out of 63 primary integrants, three colonies displayed chloramphenicol sensitivity, suggesting direct double cross-over integrants. The anticipated genetic organization in these candidate double-cross over integrants was verified by PCR using the primer combinations melAFW-lc0012FW2 and lc0323REV2-eryREV, leading to the identification of the desired R-IVET double cross-over integrant (designated NZ5500) that harbors the *loxP-ery-Pusp45-melA-loxP* cassette between the convergent genes *llmg_0760* and *llmg_0761*.

Library construction

A random chromosomal R-IVET library for *L. lactis* was constructed based on the previously described R-IVET vector pNZ7125 (4) that contains the *cre* gene as recombinase reporter, which was supplemented by the secondary reporter *luxAB*. The *luxAB* genes were isolated as a 2,5 kbp HindIII-PsiI fragment from pJIM2374 (8), treated with Klenow fragment and cloned in pCRblunt (Invitrogen, Breda, The Netherlands), yielding pNZ5512. The *luxAB* fragment was re-isolated from pNZ5512 as an EcoRV-HindIII fragment, protruding ends were removed by Klenow treatment and subsequently it was cloned downstream of the *cre* gene in PmlI digested pNZ7125, resulting in pNZ5518. To verify the functionality of the *cre-luxAB* fragment in pNZ5518 the *L. lactis usp45* promoter was amplified from genomic DNA of MG1363 with the primers usp45REV1 and usp45FW2. The amplified fragment was restricted with Sau3AI and ligated into BglII digested pNZ5518 resulting in pNZ5519. A blunt-end cloning site was introduced upstream of the *cre* gene by oligonucleotide-linker (linker was composed of AdaptVI-1 and AdaptVI-2) ligation in NcoI-BglII digested pNZ5518, yielding pNZ5520.

This R-IVET vector is based on a low-copy replicon (pIL252 (35)) and contains a blunt end cloning site (SrfI/SmaI) upstream of the *cre-luxAB* expression-detection cassette.
To construct the R-IVET library, genomic DNA of MG1363 was partially digested with AluI and fragments ranging from 0.5 – 1.5 kilo base pairs were isolated from an agarose gel. The isolated DNA fragments were ligated into the SmaI digested pNZ5520. The ligation mixture was digested with SrfI (the genome of MG1363 contains only 2 SrfI restriction sites), to eliminate self-ligated plasmids prior to transformation to NZ5500. Transformants were plated on LM17, containing 5 µg erythromycin/ml and 5 µg chloramphenicol/ml and incubated for 3 days. Colonies of approximately 23000 transformants were collectively re-suspended in 100 ml LM17 broth supplemented with chloramphenicol. Subsequently, multiple aliquoted glycerol stocks containing approximately 10^{10} colony forming units each were prepared and stored at -80°C. The excision rate during the counter-selection procedure was determined by plating an aliquot of the primary transformation of the R-IVET library on LM17 supplemented with chloramphenicol and 5-Bromo-4-Chloro-3-indolyl α-D-galactopyranoside (X-α-Gal) (MP Biomedicals, Amsterdam, The Netherlands). The excision rate was determined as the percentage of colonies showing a white phenotype on this *melA*-indicator medium.

Screening procedure

Ten ml of minimal medium were inoculated with approximately 10^6 cells of the R-IVET library and incubated at 30°C for 24 hours. Cells were plated on LM17 supplemented with chloramphenicol and 30 µg/ml X-α-Gal and incubated for 3 days. Colonies that had undergone excision of the *loxP-ery-melA-loxP* fragment during the screening procedure in minimal medium showed a white phenotype and they were transferred to 96 well microplates and glycerolstocks were prepared. Luminescence screening in LM17 was performed throughout growth as described below. A subset of identified clones, covering the whole range of observed luminescence activity levels, was selected for detailed analysis. Individual clones were pre-cultured in minimal medium and luminescence screens were performed in subcultures in minimal medium or minimal medium supplemented with 0.25 % yeast extract (Sparks, MD, USA), 0.25% Bacto tryptone (Sparks, MD, USA), 0.5% meat extract (Merck, Darmstadt, Germany) or 0.5% soy broth (Sparks, MD, USA). To ensure growth in the microplate reader under non anaerobic conditions the amino acid arginine (125 mg/l) was added to the minimal medium in those cases where no rich-nutrient supplements were added.
For the supplementation of M17 medium with purines, adenine, guanine and xanthine (Fluka, Zwijndrecht, The Netherlands) the components were dissolved in a 0.1 molar solution of sodium hydroxide at a concentration of 10 g/l for each compound. Using these purine stock solutions growth media were supplemented to final concentrations as indicated. Single amino acid supplementation of minimal medium was done with the following concentrations, serine 1 g/l, glycine 0.5 g/l threonine 0.68 g/l and tryptophan 0.1g/l. Experiments with the *arcA* clone were performed under anaerobic conditions in minimal medium with either 10, 2, 0.4, 0.08, 0.016 or 0 mM of arginine added and samples were taken during logarithmic growth.

DNA Sequencing

Total DNA from candidate clones was isolated using the InstaGene™ Matrix (Bio-Rad; Hercules, USA) according to the manufacturer's instructions. Amplification of the DNA inserts of the library plasmid pNZ5520 was performed by Herculase-PCR (Stratagene, La Jolla, USA) or Taq-PCR (Promega, Madison, USA) using the primers creREV2 and BglIICre (Table 1). Amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and used for end-sequencing of the insert, using primer creREV2 as a sequencing primer and the ABI PRISM® Big Dye Terminators v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions.

Detection of luminescence

The detection of luminescence was performed in white microplates with a transparent bottom (Greiner, Alphen a/d Rijn, The Netherlands). Cells were grown at 30°C in a Safire II microplate reader (Tecan; Zürich, Switzerland) and nonanal (Sigma, Zwijndrecht, The Netherlands) was supplied as a volatile by placing 50 µl of 1% nonanal diluted in silicon oil (Fluka, Zwijndrecht, The Netherlands) in all the empty spaces between the wells of the mircroplate. The plates were covered with a lid to entrap the volatile in the plate and luminescence and optical density at 600 nm (OD_{600}) were measured in 10 minute intervals throughout growth. The measured values are expressed in lux/OD_{600} (in arbitrary units – a.u.). The maximum values of the lux/OD_{600} measurements were taken for primary data analysis. For all luminescence measurements riboflavin was added to the medium to improve the luminescence assay (2). Luminescence measurements for the anaerobically grown *arcA* clone were performed as described elsewhere (2).

Results

Adapted R-IVET screen for L. lactis

The *cre-loxP* system is functional in *L. lactis* (5), which suggests that the *cre*-resolvase is suitable for the construction of a R-IVET screening system in this organism. For R-IVET screening purposes, a *cre*-resolution target locus, *loxP-ery-Pusp45-melA-loxP*, was constructed and integrated into the chromosome of *L. lactis* MG5267, resulting in strain NZ5500 (Fig. 1). This strain is a derivative of *L. lactis* MG1363 that harbors the lactose operon integrated into its chromosome (39), and was chosen based on the fact that a full genome sequence is available (40) and its ability to utilize lactose as a carbon source. The *cre*-resolution cassette confers erythromycin resistance to *L. lactis* NZ5500. The *Lactobacillus plantarum* WCFS1 gene *melA*, encoding an α-galactosidase (18) under control of the lactococcal *usp45* promoter, leads to a blue phenotype of NZ5500 on media containing X-α-Gal (15). The introduction of the *cre*-resolution cassette in the chromosome of MG5267 did not effect the growth rate (data not shown). To verify the chromosomal stability of the *cre* resolution cassette in NZ5500 cells were

propagated in LM17 for 200 generations and, subsequently, approximately 1000 colonies were plated on LM17 supplemented with X-α-Gal. Only a single white colony that still displayed erythromycin resistance was observed, suggesting a mutation in the *melA* gene had occurred and corroborating that the *loxP-ery-Pusp45-melA-loxP* locus is stable.

Library construction

An advanced R-IVET promoter-trap vector was constructed based on the previously described R-IVET vector pNZ7125 (4). This novel R-IVET vector (pNZ5520) harbors a promoter-less *cre-luxAB* cassette downstream of a SfrI/SmaI cloning site. Introduction of pNZ5520 into NZ5500 resulted in a stable erythromycin resistant and MelA positive phenotype that remained unaffected by 200 generations of growth The pNZ5520 derivative harboring the strong *usp45* promoter upstream of *cre-luxAB* cassette (pNZ5519) was also introduced in this strain. As anticipated, introduction of this plasmid in strain NZ5500 instantly led to an erythromycin sensitive and MelA negative phenotype in all transformant colonies. Moreover, the NZ5500 derivative harboring pNZ5519 displayed a more than 1000-fold increased level of luminescence in comparison to its pNZ5520 containing counterpart (data not shown). These experiments confirm the functionality of the *cre/loxP* resolution system as well as the *luxAB* reporter and validate the suitability of the constructed system for R-IVET screening. During further experiments, the pNZ5519 and pNZ5520 harboring derivatives of strain NZ5500 were used as luminescence positive and negative controls, respectively.

A plasmid library was constructed by cloning 0.5 – 1.5 kbp random-fragments of MG1363 chromosomal DNA into the SfrI/SmaI site of pNZ5520. The resulting R-IVET library was transformed into NZ5500 cells and transformants were plated on LM17 supplemented with chloramphenicol and erythromycin for counter-selection. During this counter-selection, active promoter elements e.g. promoters of household genes upstream of *cre* will lead to the expression of the resolvase leading to clones with an erythromycin sensitive and MelA negative phenotype. Thereby, counter-selection on medium containing erythromycin will eliminate these clones from the library. During the counter-selection process approximately 10% of all clones showed excision of the *loxP-ery-Pusp45-melA-loxP* fragment as determined by blue/white screening on plates lacking erythromycin and containing X-α-Gal. The growth and culture manipulation steps during counter-selection conditions were kept to a minimum, aiming to maintain the broadest possible diversity of library clones. PCR analysis, amplifying the insert present in the upstream region of individual NZ5500 transformants containing a specific pNZ5520 derivative, showed that more than 95 % of all R-IVET library clones contained an insert with an estimated average size of 1.2 kbp. Based on these numbers, the genomic coverage of the library was calculated according to the formula of Clarke and Carbon (7) and estimated to be higher than 99%. To analyze the diversity of the library inserts, the inserts of 90 randomly selected library clones were amplified by PCR and subjected to Sau3AI and AluI digestion. No common restriction patterns could be identified among these 90 clones, confirming the library's diversity. In addition, the inserts of 30 randomly picked library clones were subjected to single-end sequencing, establishing that the inserts were randomly distributed over the chromosome of *L. lactis* MG1363 (data not shown).

Table 2: Effect of the addition of rich medium constituents to minimal medium on a subset of R-IVET clones activated during growth in CDM. Luminescence was measured in 10 minute intervals throughout growth and the maximum values were used for data analysis. The numbers in bold show the fold change of the luminescence signal in comparison to minimal medium.

			Effect of[b]							
Open reading frame[a]	**Gene**	**product**	**Tryptone** (0.25%)		**Yeast Extract** (0.25%)		**Soy Broth** (0.5%)		**Meat Extract** (0.5%)	
			fold change	P value	fold change	P value	fold change	P value	fold change	P value
llmg_0091	*cysD*	O-acetylhomoserine sulfhydrylase	**-1,21**	0,317	**-1,03**	0,865	**-1,42**	0,172	**-4,57**	0,027
llmg_0113	*relA*	Guanosine polyphosphate pyrophosphohydrolases/synthetases	**-1,19**	0,200	**-1,16**	0,046	**1,40**	0,007	**-1,81**	0,005
llmg_0118	*bcaP*	Branched chain amino acid permease	**-8,67**	0,025	**-13,96**	0,026	**-1,76**	0,106	**-22,17**	0,022
llmg_0138	*argG*	Argininosuccinate synthase	**-1,07**	0,730	**-5,07**	0,040	**-7,14**	0,035	**-4,28**	0,043
llmg_0198		Abortive infection protein	**-1,04**	0,269	**-2,20**	0,019	**-1,39**	0,021	**-2,04**	0,002
llmg_0226		Predicted outer membrane protein	**1,03**	0,720	**-7,88**	0,024	**-1,19**	0,177	**-1,95**	0,024
llmg_0332		Putative biotin synthesis protein	**-1,69**	0,115	**-3,35**	0,043	**-1,63**	0,155	**-3,59**	0,041
llmg_0335	*plpA*	ABC-type metal ion transport system	**-1,48**	0,067	**-3,23**	0,235	**-1,62**	0,099	**-1,34**	0,100
llmg_0390	*rlrG*	Transcriptional regulator, LysR family	**-10,04**	0,009	**-8,96**	0,021	**-1,47**	0,167	**-23,10**	0,008
llmg_0447	*nifJ*	Pyruvate:ferredoxin oxidoreductase, alpha subunit	**-3,07**	0,012	**-2,83**	0,014	**-1,23**	0,191	**-3,68**	0,010
llmg_0497		Histone acetyltransferase HPA2 and related acetyltransferases	**-1,30**	0,680	**-3,21**	0,327	**-1,26**	0,691	**-1,71**	0,499
llmg_0565	*serC*	Phosphoserine aminotransferase	**-3,13**	0,041	**-2,11**	0,066	**-1,81**	0,100	**-5,43**	0,029
llmg_0645	*pacL*	Cation transport ATPase	**-1,33**	0,408	**-2,96**	0,466	**-1,74**	0,220	**-3,21**	0,106
llmg_0740	*dexC*	Neopullulanase	**2,22**	0,006	**-6,87**	0,010	**1,51**	0,026	**1,60**	0,027
llmg_0858	*uxuB*	Fructuronate reductase	**-3,13**	0,059	**-2,29**	0,085	**-2,22**	0,089	**-5,07**	0,044
llmg_0945		Putative glycerol dehydrogenase	**-1,82**	0,067	**-8,37**	0,019	**-3,77**	0,027	**-3,58**	0,028
llmg_0973	*purC*	Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase	**1,57**	0,003	**-3,90**	0,004	**-1,43**	0,011	**-2,07**	0,004
llmg_1030	*trpE*	Anthranilate/para-aminobenzoate synthases component I	**-5,00**	0,018	**-10,26**	0,014	**-4,59**	0,019	**-10,86**	0,014

Open reading frame[a]	Gene	product	Effect of[b] Tryptone (0.25%)		Yeast Extract (0.25%)		Soy Broth (0.5%)		Meat Extract (0.5%)	
			fold change	P value	fold change	P value	fold change	P value	fold change	P value
llmg_1054		Predicted transcriptional regulators	**-1,32**	0,238	**-38,96**	0,022	**-2,14**	0,069	**-2,23**	0,065
llmg_1106	*pyrDB*	Dihydroorotate dehydrogenase	**4,12**	0,009	**1,25**	0,420	**2,85**	0,051	**4,96**	0,002
llmg_1121	*nupC*	Purine/cytidine ABC transporter permease protein	**-1,35**	0,173	**-5,54**	0,022	**-2,07**	0,056	**-3,10**	0,033
llmg_1168		Predicted xylanase/chitin deacetylase; on antisenses strand	**-6,58**	0,012	**-15,10**	0,010	**-8,19**	0,011	**-22,16**	0,010
llmg_1210		Permeases of the major facilitator superfamily	**-1,67**	0,017	**-10,96**	0,003	**-1,97**	0,010	**-2,89**	0,005
llmg_1509	*pyrE*	Orotate phosphoribosyltransferase	**2,07**	0,004	**1,32**	0,317	**4,97**	0,000	**2,98**	0,008
llmg_1741	*leuS*	Leucyl-tRNA synthetase	**-3,81**	0,065	**-4,04**	0,066	**-2,75**	0,090	**-6,07**	0,052
llmg_2313	*arcA*	Arginine deiminase	**-6,89**	0,000	**-16,51**	0,000	**-4,13**	0,000	**-10,97**	0,000
llmg_2325		FMN-binding split barrel	**1,03**	0,239	**-1,79**	0,003	**-1,17**	0,046	**-1,24**	0,007
nd[d]			**-1,67**	0,108	**-30,06**	0,000	**-14,66**	0,001	**-27,53**	0,001

a) ORF (open reading frame) indicates the locus tag of the identified clone as described in the genome of L. lactis MG1363 (GenBank AM406671).
b) Each column refers to samples grown in minimal medium supplemented with the indicated component. The percentage next to each component gives the final concentration in the medium.
c) For each sample 2 biological replicates were measured. The numbers in italics give the p-value of a pairwise t-test.
d) not determined

Plating the library that was retained after primary counter-selection on LM17 supplemented with chloramphenicol and X-α-Gal resulted in ~1% white colonies. These colonies can be considered false-positives and were most likely the consequence of the limited counter-selection procedure applied during library construction. The sensitivity of the luminescence measurements was demonstrated by randomly picking 25 of those false positives and determining their luminescence signal when grown on LM17 medium, showing that the vast majority (24 out of 25) of the white colonies generated significantly higher luminescence signals than those observed with the negative control, NZ5500 harboring pNZ5520. These measurements establish the sensitivity of the luminescence measurements and illustrate that false positive clones can readily be identified in R-IVET screens using this secondary reporter.

Screen on minimal medium

To demonstrate the functionality of the advanced R-IVET system it was applied to identify promoters of *L. lactis* that are specifically induced when cells grow on minimal medium as compared to the rich laboratory medium used during library construction. In short, during library construction a counter-selection was performed to eliminate all genes induced on the rich medium M17 which is achieved by the addition of erythromycin to the medium. The obtained R-IVET library was then cultured in minimal medium and subsequently plated on LM17 medium supplemented with chloramphenicol and X-α-Gal. After 3 days of incubation about 3000 colonies were screened for the excision of the *loxP-ery-Pusp45-melA-loxP* cassette and 163 (~5.4%) white colonies were identified and transferred to a 96 well microplate. All 163 clones were subjected to luminescence screening in M17. Luminescence and optical density

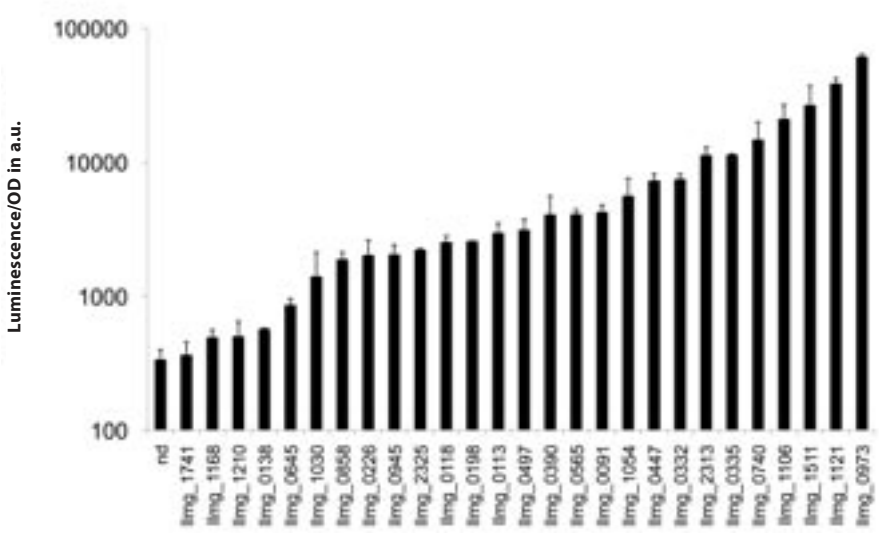

Figure 2: Luminescence signal in M17. The clones are of a subset of the initial screen and they were chosen to represent the entire range of luminescence activity levels observed. Luminescence was measured in 10 minute intervals throughout growth and the maximum values were used for data analysis. Each bar shows the average of 2 biological replicates. Error bars show standard deviation.

were measured at 10 minute intervals throughout the growth curve. Based on the results a subset of 28 clones covering the entire range of luminescence signal strengths was selected for further characterization and sequencing (Table 2). The luminescence measurements of these individual clones in M17 displayed an almost 200-fold difference between the strongest and the weakest signal observed, indicating the presence of a broad range of promoters in the library (Fig. 2) We were able to sequence 27 out of the 28 clones, identifying clones containing genes involved in amino acid metabolism (7 clones), nucleotide metabolism (5 clones), transport functions (5 clones), and several other functions (10 clones). One out of the 27 sequences did not contain a genuine promoter sequence. This clone (llmg_1168) contained a chromosomal fragment that was apparently wrongly oriented since it corresponded to the antisense strand of locus llmg_1168. The finding of target sequences located on the antisense strand of an open reading frame is not an uncommon result of IVET screens (32, 34).

The subset of 28 clones was subjected to a detailed characterization in which the main components of M17 were added separately to minimal medium in a concentration identical to that present in M17. The M17 components added to minimal medium were either, yeast extract (0.25%), meat-extract (0.5%), soy broth (0.5%) or trypton (0.25%). The results showed that 19 out of the 28 selected clones displayed significantly decreased luminescence levels upon the addition of at least one of these rich medium constituents as compared to the level observed in minimal medium (Table 2). In general, yeast and meat extract repressed the *luxAB* expression of the selected clones more effectively as compared to soy broth and trypton. Several genes are differentially regulated, depending on the rich medium component added. This is exemplified by the results observed with *bcaP* a branched chain amino acid permease (9), which is down-regulated 14- and 22-fold upon the addition of yeast extract and meat extract, respectively. At the same time this clone showed no significant change of promoter activity upon the addition of soy broth. Another example is the clone containing a fragment encoding the O-acetylhomoserine sulfhydrylase (*cysD*), which was only significantly down-regulated by the addition of meat extract but not by the other components. Notably, two of the selected clones, corresponding to inserts from the *pyrE*, and *pyrDB* loci displayed up-regulation of the luminescence signal upon addition of any of the M17 constituents. These 2 clones rank among the clones with the highest luminescence signal in M17, indicating that they are expressed at a relatively high level also in this rich laboratory medium that was also employed during counter-selection (Fig. 2). These clones may either be false positives or they have escaped elimination from the library during counter-selection procedure due to variation in the conditions employed (i.e. solid versus liquid media). Importantly, most clones that express high levels of *luxAB*-luminescence in M17, also failed to show significant differential regulation in minimal medium upon the addition of single constituents from the rich medium, supporting their classification as false-positive.

Supplementation with individual compounds

To further analyze the transcriptional response of identified clones we performed additional experiments where we aimed at identifying the molecules regulating the differential expression of target sequences. One of the identified clones harbored the promoter region of *trpE*, a gene involved in tryptophan biosynthesis. With this clone a

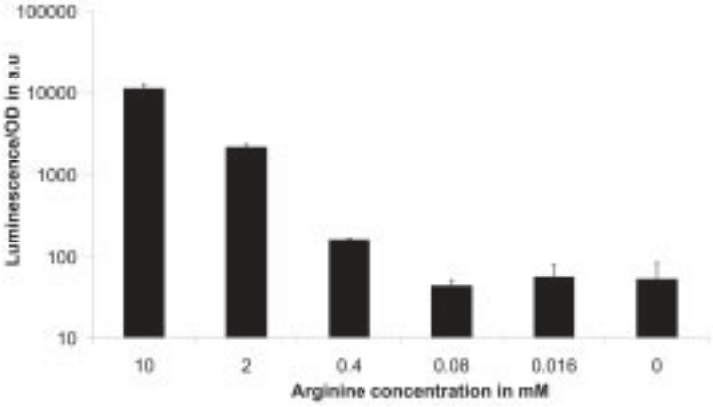

Figure 3: Luciferase signal of the *arcA* R-IVET clone (llmg_2313) grown anaerobically in minimal medium. Arginine was added to the medium in the indicated concentrations. Samples were taken from exponentially growing cells and the result of the luciferase measurement is expressed in arbitrary units (a.u.) per unit of OD_{600}. Error bars show standard deviation (n=4).

significant decrease of the luminescence signal was observed upon addition of any of the M17 components tested, with yeast and meat extract having the largest effect leading to a 10-fold decrease of the luminescence signal (Table 2). The supplementation of minimal medium with the amino acid tryptophan (100 mg/L) resulted also in a 10 fold decrease of luminescence, indicating that tryptophan may be the regulatory molecule in these complex media components.

We also investigated the effect of arginine on the expression of the arginine deiminase *arcA* a clone which was down-regulated 16- and 11-fold by the addition of yeast and meat extract respectively (Table 2). The addition of arginine to the medium clearly showed an up-regulation of *arcA* (Fig.3) which is consistent with previously published results (22). Furthermore, we investigated the effect of serine, glycine and threonine on the phosphoserine aminotransferase *serC*. Bacto trypton and yeast extract led to a 3- and 5-fold down-regulation of *serC*, respectively (Table 2), while no effect was observed upon the addition of serine, glycine and threonine to the minimal medium (data not shown).

A clone containing the promoter sequence of the *purCSQLF* operon upstream of *luxAB* showed the highest expression level when grown on M17 (Table 2) and displayed a very distinct expression pattern throughout the growth-curve. Its luminescence signal increased approximately 1000-fold within less than 2 hours during the mid-logarithmic growth phase. To exemplify this rapid switch of gene expression the activity pattern of this clone was compared to that of a control construct where *luxAB* expression is controlled by the lactococcal *usp45* promoter (Fig. 4). Since the *purCSQLF* operon is involved in purine biosynthesis (16), the influence of the addition of purines to M17 was also investigated. The addition of either adenine, guanine or xanthine to M17 clearly led to a decrease of the luminescence signal where the quantitative effect for equal concentrations decreases from adenine to guanine to xanthine (Fig. 5). To investigate how the *purC* target sequence, which displays such a high expression level of luciferase, can escape elimination during the counter-selection process we isolated plasmid DNA of the clone and re-introduced it into

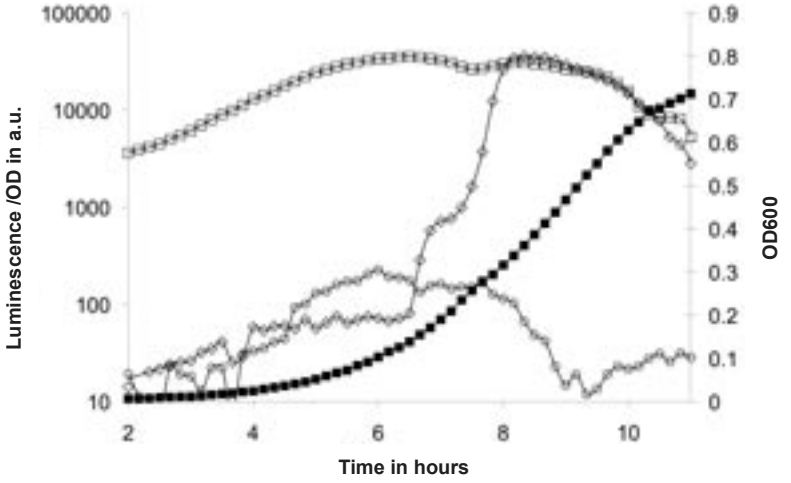

Figure 4: Effect of purine addition on luciferase activity during growth in M17 of the R-IVET clone llmg_0973 containing a plasmid with the *purCSQLF* promoter sequence. Luminescence and optical density at 600 nm (OD_{600}) were measured with 10 minute intervals. The luminescence signals per unit of OD_{600} are depicted for the clone harbouring the promoter sequence of *purCSQLF* upstream of the *cre-luxAB* genes with no additional adenine (◇) in the medium or with the supplementation of 36.7 mg/l adenine (○) to the growth medium. As a control strain NZ5500(pNZ5519) harbouring the usp45 promoter sequence upstream of the cre-luxAB genes was included (□). The corresponding OD_{600} measurement is shown for NZ5500(pNZ5519) (■), which is representative for all three clones. Each data point represents the average of multiple biological replicates – n=11 (◇), n=3 (○) and n=8 (□).

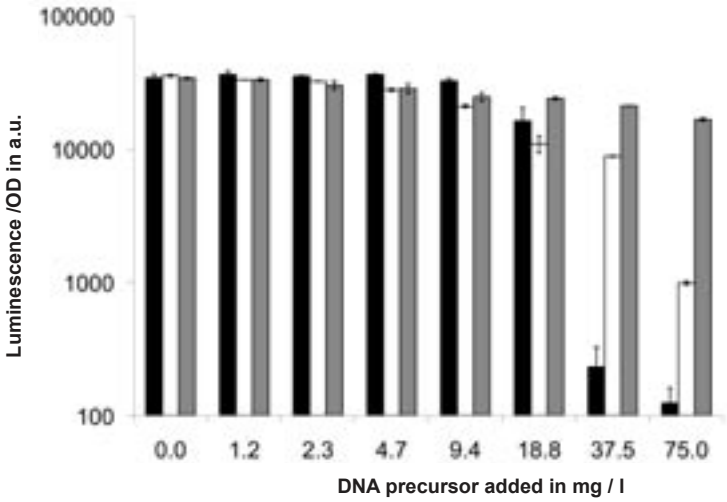

Figure 5: Luciferase expression controlled by the *purCSQLF* promoter in clone llmg_0973 after the addition of purines to M17 medium. Luminescence was measured with 10 minute intervals throughout growth and the maximum values were used for data analysis. Black bars indicate the addition of adenine (n=3), white bars the addition of guanine (n=2) and grey bars the addition of xanthine (n=2) to the medium. As a control experiment equal amounts of the solution in which no purines were dissolved (0.1 molar NaOH) were added and the results showed no effect on growth and *purCSQLF* expression (data not shown). Error bars show standard deviation.

the original R-IVET strain NZ5500. The transformants were plated on medium containing X-α-Gal and supplemented with either chloroamphenicol or with chloramphenicol and erythromycin. The results showed more than 99% blue colonies and that the addition of erythromycin had neither an effect on the amount of colonies nor on the colony size, indicating that the resolvase was not expressed at high levels in these clones. Subsequently, a single colony was isolated and propagated overnight in LM17 broth. When a sample of this culture was plated we found that all colonies showed a MelA-negative phenotype. These findings show that the purine shortage which triggers expression of *purCSQLF* operon is only encountered during mid-logarithmic growth-phase in M17 liquid medium and not on the same medium used in agar plates.

Discussion

In vivo Expression Technology (IVET) has been used widely and a number of variant versions of the system have been developed (32). Especially R-IVET systems have been successfully applied to study regulatory responses of various bacterial species in their natural habitats, where the analysis of gene expression with alternative methods is difficult (32). However, currently used R-IVET systems also have some drawbacks of which several were addressed in the present study. Firstly, the introduction of an α-galactosidase encoding gene (*melA*) between the *loxP* sites of the excision cassette on the chromosome allows simple blue-white screening under non-selective conditions on media containing X-α-Gal. This enabled simple quality control, without the need of replica plating of the library during counter-selection, as well as rapid and reliable detection of clones from which the *loxP-ery-Pusp45-melA-loxP* cassette was excised. Secondly, a major challenge in many R-IVET-based studies is the validation of promoter activation and cognate gene expression patterns in the natural environment that follows the primary R-IVET clone selection. The R-IVET system presented here overcomes this drawback by the inclusion of the bacterial luciferase encoding *luxAB* genes of *Vibrio harvey* as a secondary promoter probe in combination with the primary promoter-trap resolvase (*cre*). Bacterial luciferase (LuxAB) catalyzes the reaction of a long chain aldehyde, reduced Flavin Mononucleotide ($FMNH_2$) and molecular oxygen yielding the corresponding carboxylic acid, Flavin Mononucleotide (FMN), water and light (490 nm). It is functional in many microbes (28), and detection methods are very sensitive and allow in situ measurement in complex environments, including several tissues of living animals (13) and food products (26). Including luciferase as a secondary reporter allowed us to analyze promoter activities in a quantitative and high-throughput manner. Furthermore, the developed assay allows the systematic screening of R-IVET clones in vitro to identify the biochemical trigger for the activation of gene expression.

The R-IVET system developed was implemented in *L. lactis* and used to identify promoters activated in minimal medium as compared to rich laboratory medium. The 163 R-IVET clones identified in the primary R-IVET screen were further analyzed by measuring luminescence continuously throughout the growth curve in rich medium. A subset consisting of 28 clones representing luminescence signal levels covering the entire range observed for all clones was selected for further evaluation. The detailed analysis of this subset showed that

the luciferase reporter gene allows the rapid identification of potentially false-positives through their relatively high luciferase signals in the rich laboratory medium used during counter-selection. Moreover, we also show that some promoter sequences are only partially repressed in rich medium e.g. *arcA*, *serC* and *bcaP*. These moderately expressed genes would normally be considered false positives and would therefore be expected to be eliminated from a R-IVET library that has been subjected to extensive counter-selection. This demonstrates that the luciferase-based, secondary attribute broadens the detection range of the developed R-IVET system. Measurement of luciferase activity in minimal medium supplemented with specific components present in the rich laboratory medium used during counter selection, allowed the identification of rich medium constituents, and even specific molecules that regulate the activity of specific R-IVET promoter clones. This is exemplified by the down-regulation of the *trpE*-R-IVET clone by all rich medium components and by the subsequent demonstration that this effect is achieved specifically by the tryptophan present in these components. These findings are consistent with previous reports that describe the tryptophan mediated transcriptional control of the *trpEFG* locus in *L. lactis* (11). Analogously, we evaluated the effect of arginine on the promoter element of the *arcA*. The results clearly showed an up-regulation of *arcA* with an increasing arginine concentration in the medium, which is consistent with literature (22). However, *arcA* was down-regulated by the addition of all rich M17 components. We currently can not explain which compound of the rich components is causing this effect. Furthermore, we investigated the effect of serine, glycine and threonine on the phosphoserine aminotransferase *serC*. In this case we did not observe an effect of the amino acid addition on their regulation even though the rich nutritional sources clearly reduced their activity. This may be explained by the energetically less efficient uptake of free amino acids as compared to uptake of oligopeptides containing these amino acids, which are the predominant nitrogen source in the rich-medium components employed (19). Another possible explanation could be that the interconversion of amino acids influences the expression level of the investigated promoter sequences.

Additional analyses focused on the strongest promoter sequence identified in the initial screening. This *purC*-R-IVET clone displayed a very distinct expression pattern compared to most other clones. This is evident in an almost 1000 fold increase of the luminescence signal during mid-logarithmic growth phase, reaching activity levels similar to that observed for the luciferase-positive control. The strict temporal expression pattern observed for this clone demonstrates that the luciferase reporter is available in its active form extremely rapidly upon promoter activation. This is an important attribute of the luciferase reporter making it very valuable for the validation of in situ expression of genes. Using the *purC*-R-IVET clone we also showed a clear dose response relationship following the addition of the purines adenine, guanine and xanthine. The demonstrated effect of purines on *purC* is consistent with literature findings (16) and it was recently found that purine addition has a profound effect on high density cultures even in a rich culture medium (20). Notably, our control experiments have unambiguously shown that the positive-control R-IVET vector that contains the *cre-luxAB* cassette under control of the strong *usp45* promoter resulted in 100% resolution of the chromosomal target locus

(*loxP-ery-Pusp45-melA-loxP*), suggesting that it is highly unlikely that promoter of similar strength would escape elimination from the R-IVET library during counter-selection. This consideration led us to reintroduce the *purC*-R-IVET clone into the original R-IVET strain and investigate its expression pattern on agar plates in more detail. The results clearly showed that on a solid medium this promoter sequence is not activated to a level that would lead to the resolution of the two *lox* sites. However, liquid medium cultures with the same medium led to a 100% resolution of the target locus of the clones recovered from that culture. Unraveling the differential activity patterns of the *purCSQLF* promoter in solid and liquid media, was experimentally straightforward and demonstrates the strength of the current system in validating identified R-IVET clones.

The organism chosen for this study, *L. lactis*, is extensively used in (multi-species) starter cultures in the dairy industry for the production of cheese. The presented R-IVET method will enable elucidation of gene activation in *L. lactis* in such complex dairy fermentation environments. The system can be directly applied in product matrices as these are compatible with luminescence measurements, because the substrate of the luminescence reaction can be supplied either as a volatile or by submerging the product in e.g. silicon oil containing the substrate. Moreover, for in vivo luminescence measurements in the gastro intestinal tract the *luxCDE* genes, coding for substrate generation of the luminescence reaction, could readily be incorporated in the system and, thereby, provide the luciferase substrate from endogenous metabolism (10). Such autotrophic luciferase constructions have been shown to offer tremendous possibilities for in situ measurements in living model animals (13).

The R-IVET system described here overcomes some of the R-IVET shortcomings by broadening the detection range of promoter activity and allowing high throughput validation of target sequences in vitro and in situ. Based on literature many of the system's components are functional in other bacterial species, which should allow the transfer of the developed tool to other micro-organisms. For example, the *cre-loxP* resolvase (17, 24, 36, 21) and *luxAB* reporter system (28) have been successfully used in a variety of bacteria. It can be anticipated that the *usp45* promoter will be active in various backgrounds (29) and the pIL252 replicon has been reported as a broad host-range replicon (35). However, depending on the target organism some changes might be needed. Taken together, the system presented here offers significant advantages over the existing R-IVET systems based on its dual-selection possibilities and it could potentially be applied to other bacterial species.

Acknowledegments

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Chapter

6

Gene expression analysis of *Lactococcus lactis* during the manufacturing and ripening of cheese

Herwig Bachmann, Leonie de Wilt, Michiel Kleerebezem, Johan E.T. van Hylckama Vlieg

Abstract

Lactococcus lactis is one of main bacterial species used for the production of semi-hard cheese. Because of its industrial importance numerous studies have been reported on physiological and genetic traits relevant for its application as a dairy starter culture. The vast majority of studies were carried out in laboratory media with a pure culture, which may not always reflect the response as seen under in situ conditions. In this study we applied an advanced Recombinant In Vivo Expression Technology (R-IVET) assay in combination with a high throughput cheese manufacturing protocol for the identification and subsequent validation of promoter sequences specifically induced during the manufacturing and ripening of cheese. The system allowed gene expression measurements in an undisturbed product environment without the use of antibiotics and in combination with a mixed strain starter culture. The utilization of bacterial luciferase as reporter enabled the real-time monitoring of gene expression in cheese for up to 200 hours after the cheese manufacturing process was initiated The results revealed a number of genes that were clearly induced in cheese such as *cysD*, *bcaP*, *dppA*, *hisC*, *gltA*, *rpsE*, *purL*, *amtB* as well as a number of hypothetical genes, pseudogenes and notably genetic elements located on the non-coding strand of annotated open reading frames. The results clearly establish that genes belonging to the CodY-regulon are induced in cheese. Expression analysis using the luminescence reporter under a variety of environmental conditions allowed the identification of genes that are likely involved in interactions with bacteria used in the mixed strain starter culture. The presented approach uncovered some of the key-responses of lactococci to the important application environment of cheese and enabled the identification of novel molecular targets for future functional analysis in situ.

Introduction

Lactococcus lactis is one of the main bacteria used for the manufacture of semi-hard cheeses and its metabolic activities determine various characteristics of cheese, such as pH, texture and flavor profiles (29). A variety of studies have elucidated the importance and specific role of lactococcal activities in the process of cheese manufacturing. Enzymatic activities related to amino acid metabolism are key contributors to numerous cheese properties. The degradation of casein by proteases and peptidases influence cheese texture and supplies amino acids for further reactions that lead to the formation of flavor metabolites (28). For example, branched chain and aromatic amino acid transaminases convert amino acids to α-keto-acids which are precursors for several important flavor molecules, such as 3-methylbutanal (21, 37). Next to the impact of amino acid metabolism, the conversion of lactose to lactate has an important influence on organoleptic properties of cheese, while it also affects shelf-life and food safety. The expression of technologically important enzymes depends on the environmental condition the bacterial cell resides in and in case of cheese these conditions are complex and they change drastically throughout the manufacturing and ripening process. The types of changes include protocol dependent temperature regimes, a drastic decrease in pH by the conversion of lactose to lactate, a sudden increase in osmolarity during brining, carbon starvation after the depletion of lactose, a severe decrease in water activity after the separation of the curds from the whey and a temperature downshift during ripening. The influence of the different types of environmental changes on gene expression and the metabolism on *L. lactis* has been subject of many studies (6, 16, 20, 23, 24, 33), which resulted in the description of many functionalities that are possibly important during dairy fermentation, such as various stress responses. As an example, it has been shown that genes coding for a glutamate decarboxylase and a glutamate-γ-aminobutyrate antiporterter (*gadCB)* are chloride inducible and contribute to acid resistance. Given the low pH and the high NaCl concentrations in cheese these genes are suggested to be important for the survival of lactococci in this environment (23). However, most of these studies address response to a single environmental stress that is typically evaluated in a laboratory media.

To further increase our understanding of bacterial responses in situ, there is an increasing focus on the molecular characteristics of an organism in its natural habitat (4), including specific industrial application environments. As an example, proteome, transcriptome and metabolome studies were performed with *L. lactis* when cultured in milk (7, 18). Despite the resolving power of methods like transcriptome analysis, there are some limitations when these technologies are applied to complex samples originating from natural ecosystems like soil, plant material or the gastro intestinal tract. With respect to transcriptome analyses of *L. lactis* in the dairy environment, the use of mixed strain starter cultures and the complexity of the cheese matrix have seriously hampered functional genomics approaches in situ.

An alternative method allowing the investigation of the transcriptional response under environmental conditions has been designated Recombinant In Vivo Expression Technology (R-IVET). R-IVET employs a recombinase as a primary reporter gene, which upon expression leads to the irreversible excision of a chromosomal genetic marker flanked by two recombinase target-sites. This allows the identification of excision events on the basis of an altered phenotype. We recently developed an improved R-IVET system for *L. lactis* (Chapter

5 of this thesis). This system not only allows the identification of in situ activated promoters and their cognate genes, but also facilitates promoter validation under environmental conditions using an effective luciferase reporter system (2). We used this improved R-IVET methodology to identify genetic elements specifically expressed by *L. lactis* during cheese manufacturing and ripening. The in situ induction of the identified promoter sequences was validated by the manufacturing of individual cheeses using each identified clone as a secondary/adjunct culture. This set-up allowed real-time monitoring of promoter activity and facilitated detailed analysis of the identified promoter clones.

Material and Methods

Bacterial strains and DNA protocols

The R-IVET system used in this study (2) was implemented in strain *L. lactis* MG5267, which is a lactose-positive derivative of the fully sequenced strain MG1363 (34). The R-IVET derivative of MG5267, designated *L. lactis* NZ5500 harbors the erythromycin resistance marker as well as an α-galactosidase gene (*melA*) flanked by two recombination sites (*loxP)* integrated into its genome (3). The R-IVET library consisted of NZ5500 harboring plasmid DNA in which random genomic DNA fragments are cloned upstream of the recombinase encoding *cre* gene, that is transcriptionally fused to the bacterial luciferase genes *luxAB* (2). Cells were grown either in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) lactose, in reconstituted skimmed milk (RSM) (Promex Spray 1% skimmed milk powder; Friesland foods butter, Lochem, The Netherlands), or in Milk Protein Concentrate (MPC) (Milei, Stuttgart, Germany). If indicated RSM or MPC were supplemented with 0.2% casein hydrolysate (Bacto tryptone) (Becton Dickinson, Sparks, MD, USA). Pre-cultivation of the mixed strain starter culture Bos (CSK food enrichment, Ede, The Netherlands) was performed in RSM at 20°C. If appropriate chloramphenicol and erythromycin were used at 5 µg/ml. DNA sequencing of identified clones and transformation of NZ5500 was performed as described earlier (2).

Cheese manufacturing

Recombinant in vivo expression technology (R-IVET) was used to assess gene expression in cheese. For this purpose a lactococcal R-IVET library (2) was used as an adjunct/secondary culture during cheese manufacturing. In short, a laboratory-scale protocol was used to make a Gouda-type cheese from 2 liters of pasteurized bovine milk (3.5% fat, 3.2% protein). The milk was supplemented with renneting enzyme (Kalase - 150 IMCU/ml , CSK food enrichment, Ede, The Netherlands) at a concentration of 230 µl/liter milk, 400 µl of a 33% w/v $CaCl_2$ solution per liter milk and $5*10^6$ colony forming units (CFUs) per milliliter of Bos starter culture (1) as well as $5*10^6$ CFUs per milliliter of the R-IVET library (2). The milk was then incubated at 30.5°C until coagulation (45 minutes) and it was subsequently cut into curds of a diameter of approximately 0.3-0.5 cm. After 10 minutes of cutting, the curds were washed by removing 40% of the whey, which was replaced with sterile tab water of 45°C. Then the curds were stirred in 10 minute

intervals for 1 minute, at 36°C for 1.5 hours. Subsequently, the whey was removed by pressing in a cheese mould for 1.5 hours. The temperature throughout pressing and the following 18 hours was kept at 30°C. The following day the cheese was submerged in an industrial brine solution (~23% NaCl) (NIZO food research, Pilot plant, Ede, The Netherlands) for one hour. After brining the cheese was sealed in a plastic foil filled with 0.8 atmospheres of nitrogen and ripened at 17°C.

The described protocol was used to manufacture cheese with the original R-IVET library (2) of which clones were isolated after 2 days of cheese ripening. The manufactured cheese had a moisture content of 45%, salt content of 2.1% in dry matter, pH of 5.27 and $6.7*10^8$ CFUs per gram cheese, which is representative for a typical Gouda-type cheese. Two days after cheese manufacturing $8.3*10^7$ chloramphenicol resistant CFUs were recovered per gram cheese, representing the R-IVET clones. The mixed starter culture Bos (1) showed no bacterial growth on medium containing chloramphenicol and based on the CFUs given above it was calculated that the average R-IVET clone divided approximately 4 times during cheese manufacturing.

The described cheese production protocol was also used for the screening of a R-IVET sub-library of which induced clones were isolated after 25 days of cheese ripening. The sub-library was generated by counter-selection of the original R-IVET library after it had been used in the process of cheese making. To achieve this, R-IVET clones from cheese made with the original library were recovered after two days of cheese ripening and plated on the counter-selection medium, i.e., LM17 supplemented with chloramphenicol and erythromycin. During this step clones harboring sequences that (transiently) displayed sufficient promoter activity during the first two days of cheese ripening were eliminated from the library, since such promoter activity would induce the irreversible excision of the erythromycin resistance encoding gene from the genome leading to an erythromycin sensitive (Ery^-) clone. The recovered counter-selected clones were washed-off the Petri-dishes, re-suspended in LM17 medium and stored as glycerol stocks at -80 °C. Prior to cheese production a sub-library aliquot was thawed, plated one more time on medium containing erythromycin and washed of the Petri-dishes directly before usage. This sub-library was used as an adjunct/secondary culture in cheese production as described above followed by a recovery of R-IVET clones with an α-galactosidase negative phenotype ($MelA^-$) after 25 days of cheese ripening (Fig. 1).

Isolation of R-IVET clones

Cheese samples were taken and dissolved in a 2% solution of sodium citrate by vigorous mixing for 5 minutes. Subsequently, dilutions of this solution were plated on LM17 medium supplemented with chloramphenicol (5 μg/ml) and 5-Bromo-4-Chloro-3-indolyl α-D-galactopyranoside (X-α-Gal) (MP Biomedicals, Amsterdam, The Netherlands). Colonies with a $MelA^-$, white phenotype on this medium were picked, transferred to LM17 medium in 96 well microplates and glycerol stocks were prepared.

Promoter characterization

In vitro characterization of identified promoter sequences was performed in LM17, reconstituted skimmed milk or Milk Protein Concentrate (MPC) (Milei, Stuttgart, Germany). MPC contains

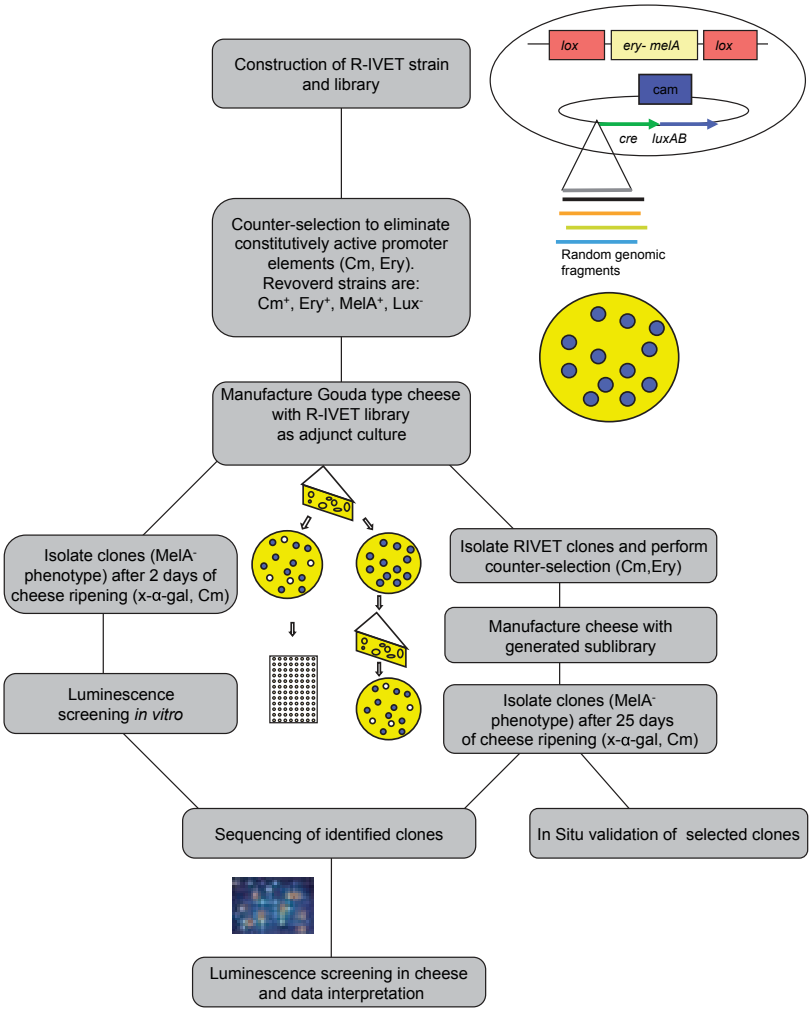

Figure 1: Schematic representation of the R-IVET screening procedures. The construction of the R-IVET library was described elsewhere (2). After counter selection in M17 to remove constitutively expressed promoter sequences, the library was used as an adjunct/secondary culture to manufacture Gouda-type cheese. Clones with inserts carrying a promoter that was induced were isolated after two days of cheese ripening by screening for colonies with MelA- phenotypes. The isolated clones were transferred to 96 well microplates and further characterized by luminescence screening in different environments (left branch). In addition, after 2 days of cheese ripening R-IVET clones were isolated and subjected to a second round of counter-selection on medium supplemented with erythromycin. This resulted in sub-library that was used for another R-IVET screen in cheese that aimed at identifying clones that were induced during the later stages (25 days) of cheese ripening (right branch). Clones isolated after 25 days of cheese ripening were also re-introduced into the original R-IVET host L. lactis strain NZ5500. These clones were used individually as adjunct/secondary cultures to manufacture miniature cheeses and excision rates after different cheese ripening periods were determined with the aim to confirm in situ activation of promoter elements. Identified clones were eventually sequenced and further characterized.

lower concentrations of lactose compared to RSM. A fully-grown R-IVET culture in MPC resulted in a final pH of ~ 5.5 as compared to a pH of ~4.6 in RSM. The pH value of a fully-grown culture in MPC is therefore much closer to the final pH values reached in cheese (typically between 5.4 and 5.2). Tested conditions also included RSM and MPC supplemented with 0.2% of a casein hydrolysate or pre-inoculated with 1/100 of a fully-grown Bos starter culture. For all conditions 10 μl of fully grown over-night cultures of the R-IVET target clones were inoculated into 200 μl of the indicated medium.

The high throughput manufacturing of miniature cheeses (~ 170 mg per cheese) was performed in 2 ml deep-well microplate model using a protocol that was described previously (Chapter 3 of this thesis). In short individual R-IVET clones with identified sequences of interest were pre-cultured at 30°C in RSM supplemented with 0.2% casiton. After over night growth the cultures were fully grown and 35 μl of this culture were used as an adjunct/secondary culture to manufacture Gouda-type miniature cheeses (manufactured from 1700 μl of milk) prepared with the mixed strain starter culture Bos. At the start of the cheese manufacturing process the inoculation-density ratio Bos : R-IVET library was approximately 1:2 (as measured in CFU).

Luminescence measurements

Luminescence in MicroCheeses was measured either directly in the deep-well microplate (where the cheeses were manufactured) with a CCD camera (Princeton Instruments, Trenton, NJ, USA) or in a Safirell microplate reader (Tecan; Zürich, Switzerland) after transfer of the MicroCheeses to a 96 well microplate (μClear-white - Greiner, Alphen a/d Rijn, The Netherlands). Luminescence measurements in all other media were also performed in 96 well μClear-white plates. For all luminescence measurements the wells were covered with 50 μl of silicon oil (Fluka, Zwijndrecht, The Netherlands) containing 0.3% nonanal (Sigma, Zwijndrecht, The Netherlands), which serves as a substrate for the luminescence reaction. For measurements in M17 riboflavin was added to enhance luciferase signals, as has been described previously (3) (Chapter 4 of this thesis).

Data analysis

Gene expression time series results were averaged over three consecutive measurements to eliminate outliers (outliers appeared occasionally during the late stationary phase when expression levels were low). The signal of luminescence traces over extended periods of time are not only determined by the promoter strength that drives the expression of the *luxAB* genes, but also by the amount of CFUs present, and by the metabolic state of the bacterial cells. Throughout a batch culture the luminescence signal typically increases proportionally with cell density, then drops rapidly when cells enter stationary phase and during prolonged incubation in the stationary phase the signal drops under the detection limit (3). For the time resolved analysis of luminescence in a growing culture, it is therefore necessary to normalize the measured luminescence activity by the bacterial density and metabolic activity. Unfortunately, the opacity of milk-like media and cheese does not allow the real-time monitoring of cell densities, thereby requiring alternative cell-density correction methods. Simultaneous

inoculation of individual cultures in a microplate can be assumed to generate similar cell-density increases and similar metabolic activities over time, provided that the cultures do not have severe individual growth differences. This assumption is supported by the observation that growth curves recorded for individual R-IVET library clones in M17 closely overlapped with each other and that the sudden decrease of luminescence activity, normally associated with stationary phase entry, in all media analyzed occurred within a very short time-window for the vast majority of the investigated clones (Fig. 2). Based on this assumption, luminescence measurements were corrected for cell density and metabolic activity by dividing each real-time activity measurement of individual cultures by the median luminescence activity of all 96 cultures in the same microplate at that same time-point. For the comparison of regulatory similarities the obtained values for each clone were normalized to the maximum luminescence value measured throughout the time series. Hierarchical cluster analysis (average linkage) of corrected and normalized luminescence time series data was based on Euclidean distance matrices as implemented in the Genesis software package (31). Based on the result of the hierarchical cluster analysis K-means clustering was performed to identify 12 cluster of each time series experiment also using the Genesis software. The maximum luminescence activity observed for individual clones was used for comparative analysis of medium-dependent promoter activation.

Results

Identification of in situ induced genes

An advanced R-IVET procedure for *L. lactis* (2) was used to identify promoter elements induced in cheese and to assess the corresponding promoter activity profiles during cheese manufacturing and ripening. The R-IVET methodology employed contains next to the recombinase as a primary promoter activity reporter, a secondary, semi-quantitative promoter reporter system by transcriptional fusion to the bacterial luciferase genes (*luxAB*). This allows real-time monitoring of gene expression in the in situ environment of choice, e.g. cheese. The R-IVET library consisted of approximately 23000 individual clones and was used as an adjunct/secondary culture to manufacture Gouda-type cheese. After two days of cheese ripening 7200 individual R-IVET colonies were recovered from cheese, of which 445 (6.1%) displayed a MelA$^-$ phenotype indicating that these clones contained an in situ activated promoter and these clones were isolated for further characterization.

In a separate R-IVET screening approach, promoter sequences were selected that were induced during later stages of cheese ripening. For this purpose a sub-library was constructed by re-isolating erythromycin resistant clones from cheese after 2 days of cheese ripening. The resulting sub-library consisted of more than 10000 individual clones and promoter elements that displayed activity during growth in M17 or during the first two days of cheese manufacturing were eliminated. In contrast to the screen aiming at the identification of early induced genes (first 2 days of cheese ripening) in this secondary screen in situ activated clones were identified after 25 days of cheese ripening. Approximately

9000 colonies were recovered from cheese after 25 days and 38 of these displayed a MelA$^-$ phenotype (0.42%). The more than 14-fold decreased frequency of induced promoter elements indicates that lower amounts of transcriptional changes appear to be specific for the later phases of cheese ripening as compared to the differential changes occurring during the first two days of cheese fermentation.
To verify the specificity of their in situ induction in cheese, plasmid DNA was isolated from 24 of the 38 in situ activated R-IVET clones identified after 25 days of cheese ripening. Each of these plasmids was re-introduced into the original Ery$^+$, MelA$^+$ R-IVET host strain NZ5500 (2). The re-transformed clones were plated on LM17 supplemented with X-α-Gal, and the LM17 induced excision rates for each of these clones were less than 1%. Subsequently, the excision rates in these 24 individual clones were determined after manufacturing miniature cheeses and the subsequent recovery of colonies after 2 and 27 days of cheese ripening. The proportion of MelA$^-$ colonies revealed that with one exception, all clones displayed an increased frequency of excision during cheese ripening. With these 23 clones, the fraction of MelA$^-$ colonies already clearly increased after 2 days in cheese, and for the vast majority of clones increased further after extended ripening times (Table 3). Taken together these results confirm that the sequences identified after 25-days of cheese ripening are containing promoters that are significantly and specifically induced in cheese as compared to M17.

Promoter identification

The original R-IVET library used was subjected to relatively limited counter-selection procedures, with the purpose of also allowing the identification of weakly regulated sequences. This strategy was chosen because the luciferase reporter allowed the subsequent elimination of false-positive clones by high throughput promoter activity screening (2). In order to further study the environmental stimuli regulating promoter activity in the 445 clones identified during the primary, short-term cheese production experiment the luciferase activity was determined when these clones were grown in LM17, RSM, RSM supplemented with 0.2% casiton, or RSM co-inoculated with a mixed strain starter culture. This analysis resulted in the identification of 95 R-IVET clones that were differentially regulated (more than 3-fold difference, $p<0.05$) in at least one of the 4 conditions (data not shown), and these clones were selected for further characterization and sequencing of the sequence inserted upstream of the *cre-luxAB* genes. The 38 clones induced during the later stages of cheese ripening were isolated from a strongly counter-selected library and 23 out of 24 of these clones were individually tested to be very specifically induced in cheese. Therefore all 38 late induced clones were taken for further characterization and sequencing (Fig. 1). A total of 121 R-IVET clones were successfully sequenced and detailed analysis revealed that 19 R-IVET clones were isolated multiple times during the short-term, 2-day cheese production experiment, while only a single R-IVET clone was isolated in duplicate during the extended, 25-day cheese production experiment (Table 1). Notably, the clones selected from the short-term experiment are enriched for clones with at least a 3-fold differential expression difference (see above), which may partly explain the relatively high frequency of multiple isolations of identical clones. The observation that during the

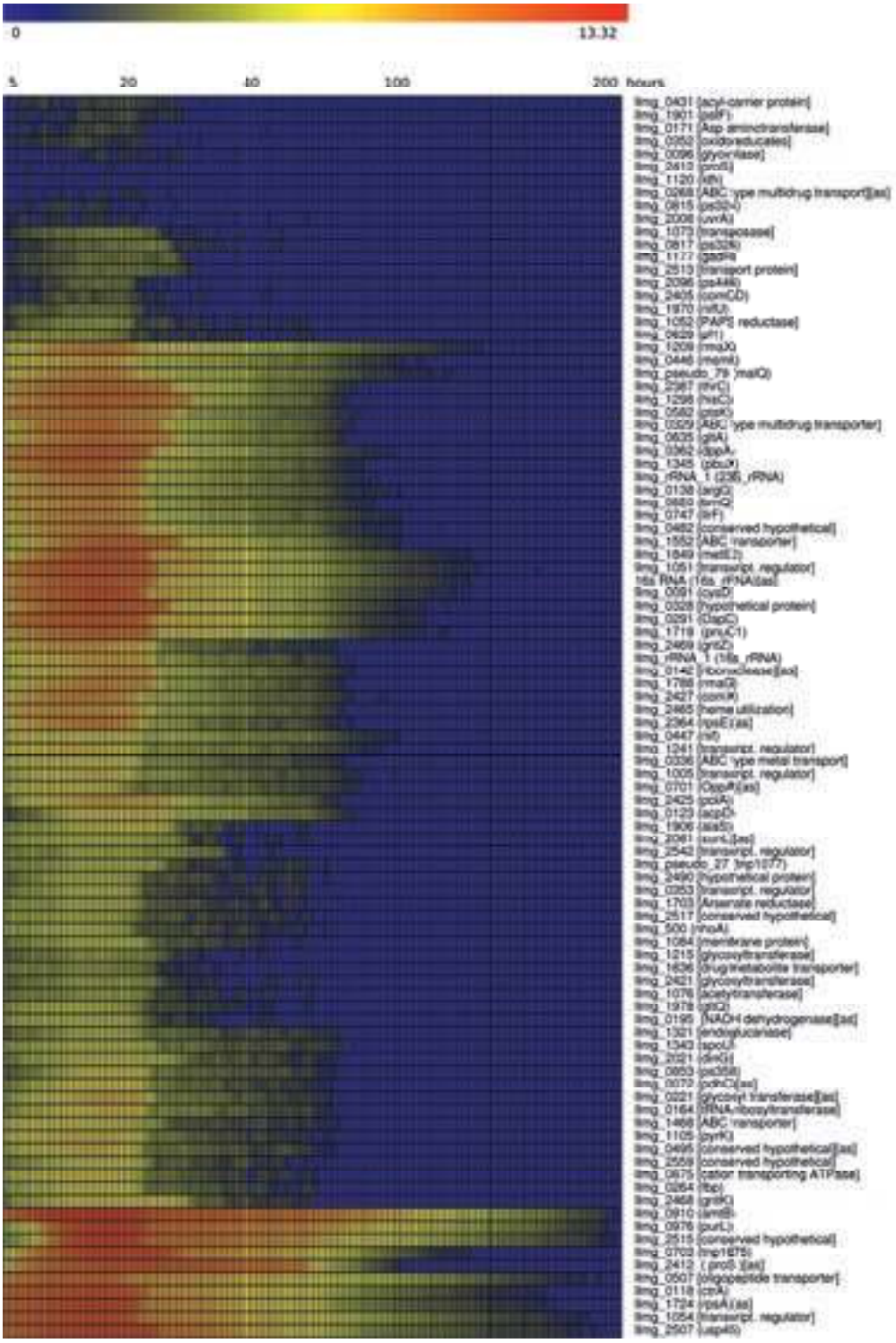
0
13.32
5
20
40
100
200
hours
lmg_0431 [acyl-carrier protein]
lmg_1901 (pdF)
lmg_0171 [Asp aminotransferase]
lmg_0252 [oxidoreducates]
lmg_0096 [glycosidase]
lmg_2413 (proS)
lmg_1120 (dh)
lmg_0268 [ABC type multidrug transport][as]
lmg_0815 (pc02c)
lmg_2008 (uvrA)
lmg_1073 [transposase]
lmg_0817 (pc02b)
lmg_1177 (gatPII
lmg_2513 [transport protein]
lmg_2096 (pc44b)
lmg_2405 (comCD)
lmg_1970 (relU)
lmg_1052 [PAPS reductase]
lmg_0629 (pri)
lmg_1209 (mqJG
lmg_0446 (mqmI)
lmg_pseudo_79 (malQ)
lmg_2387 (thrC)
lmg_1298 (hisC)
lmg_0582 (ptsK)
lmg_0359 [ABC type multidrug transporter]
lmg_0635 (glnA)
lmg_0362 (dppA-
lmg_1345 (pbuX)
lmg_rRNA_1 (23S_rRNA)
lmg_0138 (argG)
lmg_0689 (ermQ)
lmg_0747 (trF)
lmg_0482 [conserved hypothetical]
lmg_1552 [ABC transporter]
lmg_1849 (metE1)
lmg_1051 [transcript. regulator]
16s RNA (16s_rRNA)[as]
lmg_0091 (cysD)
lmg_0328 [hypothetical protein]
lmg_0291 (DapC)
lmg_1719 (pruC1)
lmg_2469 (grsZ)
lmg_rRNA_1 (16s_rRNA)
lmg_0142 [ribonuclease][as]
lmg_1788 (rmaD)
lmg_2427 (comM)
lmg_2465 [heme utilization]
lmg_2364 (rpsE)[as]
lmg_0447 (rir)
lmg_1241 [transcript. regulator]
lmg_0336 [ABC type metal transport]
lmg_1005 [transcript. regulator]
lmg_0701 (OppA)[as]
lmg_2425 (pcIA)
lmg_0123 (acpD)
lmg_1906 (asiS)
lmg_2081 (sunL)[as]
lmg_2542 [transcript. regulator]
lmg_pseudo_27 (mp1077)
lmg_2490 [hypothetical protein]
lmg_0353 [transcript. regulator]
lmg_1703 [Arsenate reductase]
lmg_2517 [conserved hypothetical]
lmg_500 (mnuA)
lmg_1084 [membrane protein]
lmg_1215 [glycosyltransferase]
lmg_1636 [drug/metabolite transporter]
lmg_2421 [glycosyltransferase]
lmg_1076 [acetyltransferase]
lmg_1978 (gtQ)
lmg_0195 [NADH dehydrogenase][as]
lmg_1321 [endoglucanase]
lmg_1343 (spoU)
lmg_2021 (dmG)
lmg_0653 (pc05B)
lmg_0070 (pdhC)[as]
lmg_0221 [glycosyl transferase][as]
lmg_0164 [tRNA-ribosyltransferase]
lmg_1468 [ABC transporter]
lmg_1105 (pyrK)
lmg_0496 [conserved hypothetical][as]
lmg_2559 [conserved hypothetical]
lmg_0675 [cation transporting ATPase]
lmg_0264 (fbp)
lmg_2468 (gntK)
lmg_0910 (amtB-
lmg_0976 (purL)
lmg_2515 [conserved hypothetical]
lmg_0703 (mp1875)
lmg_2412 (proS)[as]
lmg_0507 [oligopeptide transporter]
lmg_0118 (cpA)
lmg_1724 (rpsA)[as]
lmg_1054 [transcript. regulator]
lmg_2507 (asp45)

Figure 2: Time-resolved promoter activity patterns in cheese. Luminescence activity levels were determined in cheese prepared with the 95 R-IVET identified clones. These clones were used as adjunct/secondary cultures for the manufacturing of individual miniature cheeses. Log_2-transformed absolute luminescence levels are shown color-coded as indicated by the top bar. The time series comprises the first 200 hours of cheese ripening as shown on the x-axis (note that the scale is not linear). Measurements were performed every 30 minutes throughout the first 40 hours and every 85 minutes from 40 to 200 hours of cheese ripening. To the right the locus tag and the gene names (between round brackets) are shown. If no gene name was available the putative annotation is given [between square brackets]. If the identified sequence was located on the non-coding strand it is given indicted with [as] at the end of the description. The results show that maximum expression levels between clones differ more than 1000 fold. Most clones reach the peak value about 25 hours after the cheese manufacturing process started. The subsequent decrease of luminescence after this is attributed a decreased metabolic activity in the stationary phase. Clone llmg_2507 (pNZ5519 (2)) harbors one of the strongest known lactococcal promoter sequences (*Pusp45*) and it was used as a positive control in all experiments.

extended experiment only a single clone was isolated in duplicate indicates that further screening would likely result in the identification of additional clones that are in situ induced under these conditions.

The total number of uniquely identified in situ induced promoters was 75 and 24 for the short-term and extended experiments, respectively. Chromosomal mapping of the recovered sequences and analysis of the genetic functions controlled by the identified promoters revealed a broad range of functional categories that were induced during the ripening of cheese. The functional categories of amino acid transport and metabolism, transcription and translation were the most prominent ones identified (Table 2). Notably, 11 out of the 99 promoter sequences were located in the opposite direction of the genes encoded within the identified chromosomal locus, and they might represent promoters that drive the expression of non-coding or antisense RNA molecules. The identification of such antisense transcripts has been described previously in numerous R-IVET screens (19). Moreover, 43 of the identified promoters were located within genetic loci encoding genes organized in operon structures (15) (Table 1).

Genes involved in amino acid metabolism (*cysD*, *argG*, *aspC*, *dapD*, *hisC*, *metE2* and *thrC*) and amino acid transport (*ctrA* (*bcaP*), *dppA*, *brnQ*, *oppA*, and *gltQ*) form the largest functional category that was induced during cheese manufacturing. Furthermore, 10 in situ induced (putative) transcriptional regulators were identified, which probably reflects the drastic change in environment between the investigated conditions. Another important identified functional category is carbohydrate transport and metabolism, which seems plausible in view of the fact that lactose is depleted in Gouda-type cheese approximately 12 hours after the initiation of the cheese production process (22). Interestingly, 4 out of 10 promoters identified within the loci that encode genes belonging to the functional category "translation, ribosomal structure and biogenesis" are oriented to drive expression of the non-coding strand of these genomic regions, suggesting enriched in situ antisense transcription of genes belonging to this functional category as compared to the other functional categories.

Overall the identified promoters belong to a broad class of functional categories with a relatively high proportion belonging to amino acid transport and metabolism and intriguingly about 10% of all identified sequences are located on non-coding DNA regions.

Table 1: Description of identified R-IVET sequences and the genes that are under control of these promoter elements.

Clone name	Direction on DNA	Identified target gene	Loci (of complete operon)	Annotated gene(s) (of complete operon)	Product (of first gene in operon)	Annotated COGs for all genes in operon	Early/late induction[1]
cs175, cs166		llmg_0091	llmg_0091	*cysD*	O-acetylhomoserine sulfhydrylase	E	early
cs038, cs171, cs198		llmg_0096	llmg_0094, llmg_0095, llmg_0096, llmg_0097		putatve glyoxylase protein	ERER	early
cs144		llmg_0118	llmg_0118	*ctrA (bcaP)*	putative amino-acid transporter (branched chain amino acid permease)	E	early
cs129		llmg_0138	llmg_0138, llmg_0139	*argG, argH*	ArgG protein	EE	early
cs206, cs023, cs351[a,c]	antisense	llmg_0142	llmg_0142, llmg_0143	*rnpA,*	ribonuclease P	JU	early
cs206, cs023, cs351[a,c]	antisense	llmg_0144	llmg_0144		hypothetical protein llmg_0144	R	early
cs206, cs023, cs351[a,c]	antisense	llmg_0145	llmg_0145	*rpmH*	50S ribosomal protein L34	J	early
cs148		llmg_0171	llmg_0171	*aspC*	aminotransferase AlaT	E	early
cs216		llmg_0291	llmg_0291	*dapD*	2,3,4,5-tetrahydro- pyridine- 2-carboxylate N-succinyltransferase	E	early
cs103, cs242[b]		llmg_0328	llmg_0328, llmg_0329		hypothetical protein llmg_0328	V	early
cs130		llmg_0329	llmg_0328, llmg_0329		hypothetical protein llmg_0328	VV	early
cs222		llmg_0336	llmg_0336	*plpB*	D-methionine-binding lipoprotein plpB precursor	P	early
cs443		llmg_0352	llmg_0351, llmg_0352	*araJ*	oxidoreductase, aldo/keto reductase family	GR	early
cs457		llmg_0354	llmg_0353, llmg_0354		putative glyoxylase	KE	early
cs231		llmg_0362	llmg_0362	*dppA*	dipeptide-binding protein precursor	E	early
cs108		llmg_0431	llmg_0431		putative acyl carrier protein phosphodiesterase 2	I	early
cs066		llmg_0446	llmg_0446	*msmK*	multiple sugar-binding transport ATP-binding protein	G	early
cs145		llmg_0447	llmg_0447	*nifJ*	NifJ protein	C	early
cs422, cs295[b]		llmg_0482	llmg_0481, llmg_0482		hypothetical protein llmg_0482	S	early
cs393, cs392[a]	antisense	llmg_0495	llmg_0492, llmg_0493, llmg_0494, llmg_0495	*nagZ*	hypothetical protein llmg_0495	SEGS	early
cs122		llmg_0507	llmg_0507		peptide binding protein	E	early
cs153		llmg_0550	llmg_0549, llmg_0550	*tnp981*	hypothetical protein llmg_0550	L	early
cs016, cs298[b,c]	antisense	llmg_0581	llmg_0581		hypothetical protein llmg_0581		early

cs016, cs298[b,c]	antisense	llmg_0582	llmg_0582, llmg_0583	*ptsK, lgt*	HPr kinase/phosphorylase	TM	early
cs254		llmg_0635	llmg_0635, llmg_0636, llmg_0637	*gltA, citB, icd*	methylcitrate synthase	CCC	early
cs176		llmg_0650	llmg_0650	*brnQ*	Branched-chain amino acid transport system II carrier protein	E	early
cs158, cs345, cs278[a,b]		llmg_0703	llmg_0703	*tnp1675*	putative transposase	L	early
cs073		llmg_0747	llmg_0747	*llrF*	two-component system regulator llrF	TK	early
cs105		llmg_0817	llmg_0813, llmg_0814, llmg_0815, llmg_0816, llmg_0817, llmg_0818,	*ps322, ps323, ps324, ps325, ps326, ps327*	hypothetical protein llmg_0817	KFV	early
cs311		llmg_0875	llmg_0875		cation-transporting ATPase	S	early
cs003, cs005[b]		llmg_0910	llmg_0910	*amtB*	ammonium transporter AmtB	P	early
cs086		llmg_0976	llmg_0976, llmg_0977	*purL, purF*	phosphoribosylform-ylglycinamidine synthase II	FF	early
cs407		llmg_1005	llmg_1004, llmg_1005		sugar kinase and transcriptional regulator	GKG	early
cs088, cs387[b]		llmg_1051	llmg_1051		transcriptional regulator	K	early
cs169		llmg_1054	llmg_1052, llmg_1053, llmg_1054, llmg_1055,		hypothetical protein llmg_1054	RKK	early
cs248		llmg_1073	llmg_1073		transposase for insertion sequence element IS904E (fragment)	L	early
cs302, cs360[b]		llmg_1106	llmg_1106	*pyrDB*	PyrDB protein	F	early
cs053		llmg_1177	llmg_1177	*gadR*	positive regulator GadR	K	early
cs263		llmg_1210	llmg_1209, llmg_1210, llmg_1211	*rmaX*	multidrug resistance protein	KGEPR	early
cs162		llmg_1215	llmg_1214, llmg_1215		hypothetical protein llmg_1215 (glycosyltransferase)	M	early
cs375		llmg_1225	llmg_1225, llmg_1226	*metE, metF*	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	EE	early
cs273		llmg_1241	llmg_1240, llmg_1241, llmg_1242, llmg_1243, llmg_1244, llmg_1245		hypothetical protein llmg_1241	MKGKGGR	early
cs055		llmg_1298	llmg_1297, llmg_1298	*hisZ, hisC*	histidinol-phosphate aminotransferase	EE	early
cs060		llmg_1321	llmg_1321		hypothetical protein llmg_1321	G	early
cs081, cs264[b]		llmg_1343	llmg_1343	*spoU*	putative rRNA methyltransferase	J	early
cs399		llmg_1345	llmg_1345, llmg_1346	*pbuX, xpt*	Xanthine/uracil permease	FF	early
cs370		llmg_1468	llmg_1467, llmg_1468		Putative ABC transporter ATP binding protein	VV	early

Clone name	Direction on DNA	Identified target gene	Loci (of complete operon)	Annotated gene(s) (of complete operon)	Product (of first gene in operon)	Annotated COGs for all genes in operon	Early/late induction[1]
cs056		llmg_1552	llmg_1552, llmg_1553		putative ABC type transport system permease protein	MV	early
cs326		llmg_1703	llmg_1703		hypothetical protein llmg_1703	P	early
cs445		llmg_1719	llmg_1719, llmg_1720	*pnuC1, udp*	PnuC1 protein	HF	early
cs280	antisense	llmg_1724	llmg_1724	*rpsA*	30S ribosomal protein S1	J	early
cs331		llmg_1788	llmg_1787, llmg_1788	*fabH, rmaG*	transcriptional regulator, MarR family	IK	early
cs173		llmg_1849	llmg_1849	*metE2*	hypothetical protein llmg_1849	E	early
cs112		llmg_1901	llmg_1901	*pstF*	phosphate transport substrate binding protein pstF	P	early
cs010		llmg_1906	llmg_1906	*alaS*	alanyl-tRNA synthetase	J	early
cs479		llmg_1970	llmg_1970	*nifU*	hypothetical protein llmg_1970	C	early
cs127		llmg_1978	llmg_1978, llmg_1979	*gltQ, gltP*	glutamate ABC transporter ATP-binding protein	EE	early
cs236		llmg_2021	llmg_2021	*dinG*	Probable ATP-dependent helicase dinG homolog	KL	early
cs019[c]	antisense	llmg_2080	llmg_2079, llmg_2080	*pknB, pppL*	putative phosphoprotein phosphatase	RTKLST	early
cs019[c]	antisense	llmg_2081	llmg_2081	*sunL*	Ribosomal RNA small subunit methyltransferase B	J	early
cs100		llmg_2096	llmg_2096, llmg_2097, llmg_2098, llmg_2099, llmg_2100,	*ps446, ps445, ps444, ps443, ps442,*	putative major head protein precursor	OUSR	early
cs460	antisense	llmg_2364	llmg_2363, llmg_2364, llmg_2365	*rpmD, rpsE, rplR*	30S ribosomal protein S5	JJJ	early
cs036		llmg_2387	llmg_2386, llmg_2387	*thrC, thrC*	threonine synthase	VE	early
cs177		llmg_2405	llmg_2402, llmg_2403, llmg_2404, llmg_2405, llmg_2406, llmg_2407	*comGD, comGD, comGC, comGB*	putative competence protein ComGD	UNUN-UUNU	early
cs102		llmg_2412	llmg_2412, llmg_2413, llmg_2414, llmg_2415	*proS, cdsA, uppS*	prolyl-tRNA synthetase	JMII	early
cs007		llmg_2421	llmg_2421		hypothetical protein llmg_2421	M	early
cs299		llmg_2427	llmg_2427	*comX*	competence regulator ComX		early
cs456		llmg_2465	llmg_2465		hypothetical protein llmg_2465, (large exo proteins involved in heme utilization or adhesion)		early
cs317		llmg_2469	llmg_2469	*gntZ*	6-phosphogluconate dehydrogenase-like protein	G	early
cs123, cs113		llmg_2490	llmg_2490		hypothetical protein llmg_2490		early

cs313		llmg_2514	llmg_2513, llmg_2514, llmg_2515		hypothetical protein llmg_2515	GEPRT	early
cs398		llmg_2517	llmg_2517		hypothetical protein llmg_2517	K	early
cs048		llmg_2542	llmg_2542		Putative HTH-type transcriptional regulator	K	early
cs420		llmg_2559	llmg_2559		hypothetical protein llmg_2559		early
cs270, cs455, cs475[b]		llmg_ pseudo_79	llmg_pseudo_79	*malQ*	pseudogene; malQ disrupted by tnp905		early and late
cs354, cs259[a]	antisense	llmg_ pseudo74	llmg_pseudo74	*noxD*	NADH oxidase , frameshift around position 860		early
cs126		llmg_ rRNA_2	llmg_rRNA_1, llmg_ tRNA_18, llmg_rRNA_2 ...llmg_tRNA_32	*23sr-RNA*	23S ribosomal RNA		early
cs012		llmg_ tRNA_18	llmg_rRNA_1, llmg_ tRNA_18, llmg_rRNA_2 ...llmg_tRNA_32	*tRNA-Ala*			early
cs137	antisense	llmg_ rRNA_3	llmg_rRNA_1, llmg_ tRNA_18, llmg_rRNA_2 ...llmg_tRNA_32	*16sRNA, t_RNA-Ala, 23sRNA*	16s RNA, t_RNA-Ala, 23s RNA (see also CS012 - same locus in sense direction)		early
cs476		llmg_0033	llmg_0030, llmg_0031, llmg_0032, llmg_0033, llmg_0034, llmg_0035, llmg_0036	*ps126, ps125, ps124, ps123, ps122, ps121*	hypothetical protein llmg_0033		late
cs472		llmg_0070	llmg_0070		putative permease	GEPR	late
cs485		llmg_0123	llmg_0123	*acpD*	AcpD protein	I	late
cs478		llmg_0164	llmg_0164	*tgt*	queuine tRNA-ribosyltransferase	J	late
cs484	antisense	llmg_0195	llmg_0195		putative NADH dehydrogenase	C	late
cs480		llmg_0264	llmg_0264	*fbp*	fructose-bisphosphatase	G	late
cs465	antisense	llmg_0268	llmg_0268, llmg_0269		ABC transporter ATP binding protein	VV	late
cs462		llmg_0500	llmg_0500	*nhoA*	Arylamine N-acetyltransferase 2	Q	late
cs481		llmg_0629	llmg_0629	*pfl*	formate acetyltransferase	C	late
cs477	antisense	llmg_0701	llmg_0701	*oppA*	Oligopeptide-binding protein oppA precursor	E	late
cs459		llmg_0815	llmg_0813, llmg_0814, llmg_0815, llmg_0816, llmg_0817, llmg_0818	*ps322, ps323, ps324, ps325, ps326, ps327*	hypothetical protein llmg_0815	KFV	late
cs464		llmg_0853	llmg_0853	*ps358*	hypothetical protein llmg_0853		late

Clone name	Direction on DNA	Identified target gene	Loci (of complete operon)	Annotated gene(s) (of complete operon)	Product (of first gene in operon)	Annotated COGs for all genes in operon	Early/late induction[1]
cs458, cs169[b]		llmg_1052	llmg_1052, llmg_1053, llmg_1054, llmg_1055		hypothetical protein llmg_1052, (PAPS reductase)	RRKK	early and late
cs483		llmg_1076	llmg_1076		putative acetyltransferase	J	late
cs482		llmg_1084	llmg_1084		hypothetical protein llmg_1084		late
cs454		llmg_1120	llmg_1120	*ldh*	L-lactate dehydrogenase (in las operon according to Llanos et al. 1993.)	C	late
cs452,cs486[b]		llmg_1636	llmg_1636		putative permease protein	GER	late
cs467		llmg_2008	llmg_2008	*uvrA*	UvrABC system protein A	L	late
cs488		llmg_2131	llmg_2131	*ps412*	hypothetical protein llmg_2131		late
cs466		llmg_2302	llmg_2302,	*dpsA*	non-heme iron-binding ferritin		late
cs451		llmg_2425	llmg_2425	*polA*	DNA polymerase I	L	late
cs471		llmg_2513	llmg_2513, llmg_2514, llmg_2515		putative transport protein	GEPRT	late
cs475		llmg_2532	llmg_2528, llmg_2529, llmg_2530, llmg_2531, llmg_2532	*ps604*, *ps605*, *ps606*, -, *ps608*,	hypothetical protein llmg_2532	G	late
cs453		llmg_ pseudo_27	llmg_pseudo_27	*IS1077I*	transposase and inactivated derivatives		late

a identical clone idetified multiple times
b independent clones with same promoter sequence identified multiple times
c identified target sequence aligns with antisense strand of multiple genes

1 Clones were identified either after 2 days of cheese fermentation (early induced) or after 25 days of cheese ferementation (late induced)

Characterization of identified promoters

To further specify the precise physiochemical conditions that activate the promoter elements (and their cognate downstream genes) identified by R-IVET screening in cheese, 95 R-IVET clones were subjected to a variety of in vitro conditions and luminescence levels were measured for individual clones. The in vitro culture-media evaluated were L-M17, MPC, MPC pre-inoculated with the mixed starter culture Bos or in MPC supplemented with 0.2% casiton. In addition, all clones were used as an adjunct/secondary culture to manufacture individual miniature cheeses and luminescence activity could be followed for up to 200 hours after cheese manufacturing (Fig. 2). The vast majority of promoters were activated in cheese as compared to the rich laboratory medium M17 (Table 2), which is consistent with their isolation during the R-IVET screening procedure. However, some promoters were displaying reduced activity in cheese, which may be a consequence of the limited counter-selection of the R-IVET library in combination with other selection specific parameters e.g. liquid versus semi-solid environment (2). This category of promoters includes those that drive the expression of genes encoding a putative rRNA methyltransferase, a putative permease, pyruvate formate-lyase and a putative glycosyltransferase. Based on the maximum luminescence levels measured in the different environments, specific environmental factors that influence the regulation of activity of some clones could be clearly identified. For example, the luminescence signal of the oligopeptide transporter *dppA* was more than 200 fold up-regulated in cheese relative to M17. However, the expression level of *dppA* appeared 3.7 fold lower in MPC as compared to cheese, but more than 20- and 100-fold lower in MPC supplemented with casiton or a mixed strain starter culture, respectively. These observations clearly indicated down-regulation of *dppA* by either the addition of hydrolyzed casein or the co-culturing with a mixed starter culture. It is known that the mixed starter culture harbors strains with significant levels of proteolytic activity, and which are thereby able to liberate peptides and amino acids from the degradation of milk casein. The regulatory characteristics of genes like *hisC*, *bcaP* (*ctrA*), *cysD, gltA* and *amtB*, appeared to be similar to those observed for *dppA,* which is especially relevant since all of these genes are regulated by the pleiotropic regulator CodY (5, 11). This data establishes the induction of CodY-regulated genes in situ and that they clearly respond to other bacteria in co-culture. Most likely this response is mediated through the ability of co-cultured strains to modify the peptide and amino acid concentrations in the environment. Another identified gene directly controlled by amino acid availability is an alanyl tRNA synthetase (*alaS*) that is preceded by a T-box element, which was identified in silico, as described earlier (35). The in silico analysis also revealed that the CodY regulated gene *hisC* is preceded by a T-box element indicating a different level of regulation of this gene/operon. Most validated genes show little difference in their expression levels if the results obtained in cheese were compared to those obtained in MPC, indicating that growth in a milk-like medium is sufficient to induce the expression of these genes. Genes that clearly deviate from this typical activation pattern are *metE2*, a transcriptional regulator (llmg_1209), a transposase (llmg_0703), a putative ABC-type transport system (llmg_1552), *purL*, *amtB* and some hypothetical genes, indicating that the expression of these genes appears highly specific for cheese conditions.

The highest levels of luciferase expression in cheese were found to associate with the promoter regions of an ammonium transporter (*amtB*), purine biosyntheses gene (*purL*), the hypothetical

gene llmg_2515, a transposase (*tnp1675*), the pseudogene *noxD*, the putative oligopeptide transporter llmg_0507, the transcription regulator llmg_1054, the branched chain amino acid permease (*bcaP/ctrA*) and a sequence located on the opposite strand of the 30s ribosomal protein *rpsA*. The putative oligopeptide transporter llmg_0507 is also highly expressed in environments other than cheese, but all the other promoter sequences displayed a 13- to 220-fold activation in situ in cheese as compared to M17. One of the promoter clones driving the highest luciferase expression measured in cheese is positioned upstream of the hypothetical gene llmg_2515, which is positioned in an operon with the universal stress protein A (*uspA*), a putative transport protein, and the transcriptional regulator *rcfB*. RcfB has been reported to be induced under acidic conditions and it regulates the expression of two acid inducible promoters (P1 and P170 (14)). A *rcfB* mutant was shown to have a 130 fold reduced survival rate if exposed to a lethal acid challenge (14). Since the promoter we identified is located upstream of the *rcfB* containing operon, we investigated the survival of the *rcfB* mutant in cheese. No significant difference in survival rate was observed between the *rcfB* mutant strain and the parental strain MG1363 during the first 23 days of cheese ripening (data not shown). However, this result does not exclude the possibility that one of the other three genes located in the same operon might be of importance during cheese production and/or ripening. The universal shock protein A is described to be induced during stationary phase of *E. coli* and *uspA* mutants are impaired in the survival during stationary phase (17), suggesting that the high expression levels of *uspA* in cheese might be important for the survival in this environment.
Relative expression levels were used to perform K-means clustering (12 clusters) of the real-time luciferase measurements. The results revealed clear differences in the activation patterns of the promoters present in individual clones, which can be extrapolated to differences in gene expression patterns of the corresponding genes. The identified clusters grouped genes with relatively high expression level at the beginning of the measurements followed by a subsequent decrease of activity to levels below the detection limit, but also genes with low initial expression levels followed by a peaking of expression levels after 80 – 100 hours of cheese ripening (Fig. 3). The cluster with relative peak expression levels after 80 - 100 hours of cheese ripening (cluster VIII) consists of 6 genes and it includes two transcriptional regulators (llmg_1054 and *rmaX*), of which *rmaX* is the first gene in an operon that also encodes genes annotated as drug resistance transporters. Furthermore, this cluster contains an ammonium transporter (*amtB*), a putative oligopeptide transporter (llmg_0507), a gene involved in purine biosynthesis (*purL*) and the hypothetical llmg_2515, which is in an operon with *uspA* and *rfcB*. The expression profiles of these genes indicate that they might be of importance during stationary phase of *L. lactis* in cheese.

Discussion

99 promoter elements that were induced during the manufacturing and ripening of cheese were identified. 22 genes downstream of the identified promoters are involved in amino acid transport and metabolism, indicating amino acid starvation during cheese fermentation. Several of those genes are regulated by the pleiotropic regulator CodY (5, 11), which functions as a transcriptional repressor. Many of the genes belonging to the CodY regulon are involved in nitrogen metabolism, but CodY also regulates expression of genes that belong to other

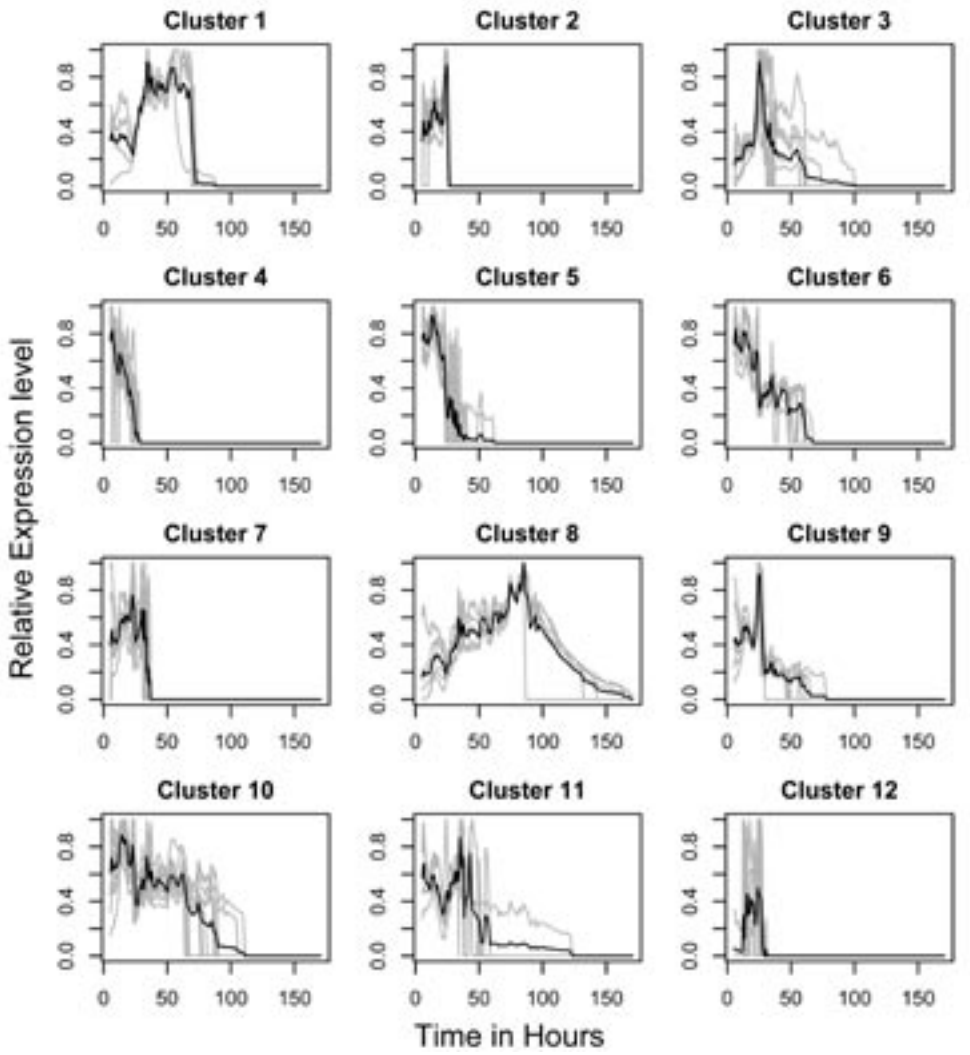

Figure 3: K-means clusters of luciferase activity profiles as shown in Fig. 2. Prior to cluster analysis the activities were corrected for cell growth and metabolic activity and normalized to the peak measurement of each gene. The resulting relative activities are given on the y-axis. Relative activities are displayed for the first 170 hours of cheese ripening (x-axis). Grey curves show profiles of individual clones[a)] and the black line shows the average.

a) **The indicated clusters show isolated sequences that are associated with the following genes: Cluster1:** llmg_0447 (*nif*), llmg_0446 (*msmk*), llmg_pseudo_79 (*malQ*), llmg_2412 (*proS*)[as]. **Cluster2:** llmg_pseudo_27 (*tnp1077*), llmg_1084 [membrane protein], llmg_2421 [glycosyltransferase], llmg_1215 [glycosyltransferase], llmg_1073 [transposase]. **Cluster3:** llmg_1552 [ABC transporter], llmg_2542 [transcript. regulator], llmg_1298 (*hisC*), llmg_0703 (*tnp1675*), llmg_0142 [ribonuclease] [as], llmg_2021 (*dinG*), llmg_2425 (*polA*), llmg_0123 (*acpD*), llmg_2468 (*gntK*). **Cluster4:** llmg_0500 (*nhoA*), llmg_1978 (*gltQ*), llmg_0875 [cation transporting ATPase], llmg_1703 [Arsenate reductase], llmg_1468 [ABC transporter], llmg_2517 [conserved hypothetical], llmg_1636 [drug/metabolite transporter], llmg_1052 [PAPS reductase], llmg_1970 (*nifU*), llmg_0629 (*pf1*), llmg_1076 [acetyltransferase], llmg_0195 [NADH dehydrogenase][as]. **Cluster5:** llmg_0264 (*fbp*), llmg_2469 (*gntZ*), llmg_0495 [conserved hypothetical][as], llmg_1345 (*pbuX*), llmg_2559 [conserved hypothetical], llmg_2364 (*rpsE*)[as]. llmg_2490 [hypothetical protein], llmg_1105 (*pyrK*), llmg_0353 [transcript. regulator]. **Cluster6:** llmg_rRNA_1 (*16s_rRNA*), llmg_0650 (*brnQ*), llmg_2465 [heme utilization], llmg_rRNA_1 (23S_rRNA), llmg_2427 (*comX*), llmg_0362 (*dppA*). **Cluster7:** llmg_0336 [ABC type metal transport], llmg_0164 [tRNA ribosyltransferase], llmg_0072 (*pdhC*)[as], llmg_1343 (*spoU*), llmg_1321 [endoglucanase], llmg_0853 (*ps358*), llmg_0221 [glycosyl transferase][as]. **Cluster8:** llmg_1054 [transcript. regulator], llmg_0910 (*amtB*), llmg_0976 (*purL*), llmg_0507 [oligopeptide transporter], llmg_1209 (*rmaX*), llmg_2515 [conserved hypothetical]. **Cluster 9:** llmg_2387 (*thrC*), llmg_0328 [hypothetical protein], llmg_0582 (*ptsK*), llmg_2081 (*sunL*)[as], llmg_0329 [ABC type multidrug transporter], llmg_0635 (*gltA*), llmg_1788 (*rmaG*). **Cluster10:** llmg_1051 [transcript. regulator], llmg_0118 (*ctrA*), llmg_0291 (DapD), llmg_1724 (*rpsA*)[as], 16s RNA (*16s_rRNA*)[as], llmg_1849 (*metE2*), llmg_0091 (*cysD*), llmg_0138 (*argG*), llmg_1719 (*pnuC1*), llmg_0747 (*llrF*), llmg_0482 [conserved hypothetical]. **Cluster11:** llmg_2507 (*usp45*), llmg_0701 (*oppA*)[as], llmg_1005 [transcript. regulator], llmg_1241 [transcript. regulator]. **Cluster12:** llmg_1906 (*alaS*), llmg_2096 (*ps446*), llmg_1177 (*gadR*), llmg_0817 (*ps326*), llmg_2405 (*comGD*), llmg_2513 [transport protein].

Table 2: Comparison of luciferase activity levels of cells residing in M17, MPC, MPC supplemented with 0.2% casiton, MPC inoculated with the mixed starter culture Bos and cheese. Luminescence activity of the individual clones was measured in 10-minute intervals for up to 200 hours after medium inoculation/cheese manufacturing. The maximum luminescence signal measured for each clone was used for comparison of the expression levels in the different environments.

Functional category/Locus name[1]	Gene name[2]	Comparison conditions[3]								Product description
		Cheese / M17		Cheese / MPC		Cheese / MPC_ Bos		Cheese / MPC_ casiton		
		fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	
Amino acid transport and metabolism										
llmg_0091	*cysD*	**18,7**	0,005	**6,2**	0,007	**3,5**	0,013	**1,6**	0,033	Cystathionine beta-lyases/cystathionine gamma- synthases
llmg_0096[c]		**nd**		**nd**		**nd**		**nd**		Lactoylglutathione lyase and related lyases [EC_number=1.13.11.-]
llmg_0118	*bcaP (ctrA)*	**16,7**	0,006	**1,0**	0,746	**2,6**	0,018	**-1,8**	0,008	putative amino-acid transporter, Amino acid permease-associated region
llmg_0138	*argG*	**-1,7**	0,071	**1,2**	0,229	**-1,2**	0,374	**-3,4**	0,001	Argininosuccinate synthase
llmg_0171	*aspC*	**nd**		**nd**		**nd**		**nd**		Aspartate aminotransferase, Transaminase A (ASPAT)
llmg_0291	*DapD*	**4,9**	0,003	**1,7**	0,019	**1,6**	0,006	**-1,6**	0,005	Tetrahydrodipicolinate N-succinyltransferase
llmg_0362	*dppA*	**213,0**	0,020	**3,7**	0,036	**106,5**	0,020	**20,1**	0,022	Conserved hypothetical; ABC-type oligopeptide transport system, periplasmic component; dipeptide-binding protein precursor
llmg_0507		**-2,5**	0,023	**1,0**	0,887	**-1,4**	0,320	**-5,1**	0,003	Similar to oligopeptide ABC transporter substrate binding proteins Conserved hypothetical protein; peptide binding protein
llmg_0650	*brnQ*	**-1,2**	0,114	**1,4**	0,003	**-1,5**	0,060	**-4,3**	0,001	branched-chain amino acid transport system II carrier protein; branched-chain amino acid:cation transporter
llmg_0701[a,b]	*OppA*	**-2,0**	0,038	**-2,0**	0,293	**-3,4**	0,257	**-3,9**	0,003	ABC type dipeptide transport system periplasmic component
llmg_1298	*hisC*	**37,5**	0,002	**2,3**	0,009	**16,5**	0,002	**1,6**	0,204	histidinol-phosphate aminotransferase [EC:2.6.1.9]
llmg_1849	*metE2*	**6,7**	0,029	**7,7**	0,026	**7,1**	0,028	**1,2**	0,389	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [EC:2.1.1.14]
llmg_1978	*gltQ*	**1,1**	0,576	**-1,2**	0,023	**-1,1**	0,693	**-5,9**	0,006	ABC-type polar amino acid transport system, ATPase component
llmg_2387	*thrC*	**2,7**	0,030	**1,1**	0,500	**1,3**	0,739	**-2,6**	0,133	threonine synthase
Transcription										
llmg_0353		**1,3**	0,217	**1,5**	0,082	**1,5**	0,116	**-1,4**	0,064	putative transcription regulator
llmg_0815b	*ps324*	**nd**		**nd**		**nd**		**nd**		hypothetical protein predicted by glimmer /critica
llmg_1005		**1,7**	0,074	**2,5**	0,024	**1,2**	0,511	**-2,0**	0,012	Transcriptional regulator/sugar kinase; EC_number=2.7.1.-

llmg_1051[c]		**8,2**	0,004	**1,9**	0,014	**2,0**	0,016	**-1,2**	0,076	transcriptional regulator, TetR_N HTH_11
llmg_1054		**22,8**	0,025	**1,4**	0,196	**1,8**	0,148	**-1,2**	0,492	conserved hypothetical protein, ParBc; Similar to ParB-like nucleases; Predicted transcriptional regulators
llmg_1177	*gadR*	**-4,2**	0,015	**1,1**	0,853	**-1,1**	0,902	**-4,8**	0,070	"positive regulator for GadR, activates chloride dependent transcription of gadCB"
llmg_1241		**-1,1**	0,477	**1,8**	0,010	**2,5**	0,039	**-1,4**	0,016	conserved hypothetical protein, Pfam: SIS or Pfam: ROK; Transcriptional regulator/sugar kinase
llmg_1788	*rmaG*	**-3,1**	0,016	**1,0**	0,992	**-3,2**	0,089	**-4,2**	0,018	transcriptional regulator, MarR family
llmg_2517		**-4,2**	0,004	**1,4**	0,044	**-2,1**	0,492	**-11,0**	0,000	conserved hypothetical protein, Transcriptional regulator
llmg_2542		**1,0**	0,804	**1,3**	0,088	**2,3**	0,030	**-1,9**	0,085	transcriptional regulator, putative HTH-type
llmg_1209		**29,6**	0,001	**6,3**	0,002	**7,5**	0,001	**2,5**	0,002	transcriptional regulator, MarR family
Translation, ribosomal structure and biogenesis										
llmg_0142[a,c]		**-1,5**	0,182	**1,3**	0,289	**1,4**	0,345	**-7,8**	0,004	ribonuclease P; EC_number=3.1.26.5
llmg_0164[b]	*tgt*	**1,1**	0,330	**1,1**	0,716	**-1,6**	0,384	**-1,5**	0,056	Queuine/archaeosine tRNA-ribosyltransferase
llmg_1076[b]		**-1,3**	0,179	**-1,3**	0,195	**-2,5**	0,289	**-3,4**	0,022	acetyltransferases, including N-acetylases of ribosomal proteins
llmg_1343[c]	*spoU*	**-7,1**	0,012	**1,8**	0,060	**-1,6**	0,584	**-17,0**	0,000	putative rRNA methyltransferase
llmg_1724[a]	*rpsA*	**16,6**	0,006	**1,4**	0,066	**3,6**	0,011	**-1,1**	0,346	30S ribosomal protein S2
llmg_1906	*alaS*	**-2,0**	0,063	**-1,1**	0,656	**1,2**	0,668	**-3,5**	0,008	AlaS protein [EC:6.1.1.7]; alanyl-tRNA synthetase
llmg_2081[a]	*sunL*	**2,4**	0,005	**-1,3**	0,039	**1,5**	0,287	**-3,7**	0,187	Ribosomal RNA small subunit methyltransferase B, NusB/NOL1/ NOP2/sun family
llmg_2364[a,b]	*rpsE*	**44,3**	0,003	**1,1**	0,648	**1,8**	0,061	**-1,9**	0,103	ribosomal protein 5S
llmg_2412	*proS*	**nd**		**nd**		**nd**		**nd**		Prolyl-tRNA synthetase
Carbohydrate transport and metabolism										
llmg_0264[b]	*fbp*	**-1,5**	0,152	**-2,0**	0,142	**-3,6**	0,172	**-5,0**	0,018	fructose-bisphosphatase [EC:3.1.3.11]
llmg_0446	*msmk*	**1,2**	0,262	**2,0**	0,014	**-1,5**	0,355	**-3,5**	0,025	multiple sugar-binding transport ATP-binding protein
llmg_1321		**1,3**	0,042	**1,5**	0,052	**1,6**	0,251	**-2,1**	0,008	putative Endoglucanase
llmg_1636[b,c]		**-6,7**	0,003	**-1,9**	0,143	**-7,6**	0,045	**-7,5**	0,000	permeases of the drug/ metabolite transporter (DMT) superfamily; Carboxylate/Amino Acid/Amine Transporter Conserved hypothetical protein
llmg_2468		**1,7**	0,043	**1,2**	0,472	**-1,4**	0,511	**-2,4**	0,003	FGGY family of carbohydrate kinases

Functional category/Locus name[1]	Gene name[2]	Comparison conditions[3]								Product description
		Cheese / M17		Cheese / MPC		Cheese / MPC_ Bos		Cheese / MPC_ casiton		
		fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	
llmg_2469	*gntZ*	**-2,3**	0,004	**1,4**	0,061	**-1,1**	0,539	**-2,9**	0,003	6-phosphogluconate dehydrogenase, NAD binding domain of 6-phosphogluconate dehydrogenase
llmg_2513[b]		**nd**		**nd**		**nd**		**nd**		permeases of the major facilitator superfamily; putative transport protein
Energy production and conversion										
llmg_0072		**1,9**	0,053	**1,3**	0,497	**-1,0**	0,976	**-1,3**	0,123	pyruvate dehydrogenase complex E2 component
llmg_0447	*nif*	**-4,2**	0,013	**3,5**	0,194	**-1,4**	0,616	**-9,5**	0,005	oxidoreductase containing iron-sulfur protein
llmg_0629[b]	*pf1*	**-6,5**	0,000	**-2,7**	0,101	**-7,5**	0,257	**-7,6**	0,005	pyruvate-formate lyase
llmg_0635	*gltA*	**91,5**	0,012	**1,9**	0,068	**37,3**	0,012	**5,0**	0,018	Citrate synthase
llmg_1120[b]	*ldh*	**nd**		**nd**		**nd**		**nd**		malate/lactate dehydrogenases
llmg_1970[b]	*nifU*	**-1,2**	0,270	**-1,2**	0,785	**-3,8**	0,255	**-2,9**	0,009	NifU homolog involved in Fe-S cluster formation
llmg_0195[a]		**1,3**	0,202	**-1,1**	0,613	**-1,4**	0,484	**-2,4**	0,038	putative NADH dehydrogenase [EC:1.-.-.-]
Replication, recombination and repair										
llmg_0703[c]		**13,4**	0,013	**34,2**	0,012	**2,8**	0,061	**-1,2**	0,197	Transposase and inactivated derivatives; putative transposae
llmg_1073	*tnp904*	**-1,2**	0,652	**1,0**	0,879	**1,1**	0,737	**-2,2**	0,072	Transposase and inactivated derivatives, possibly inactive, high confidence in function and specificity
llmg_2008[b]	*uvrA*	**nd**		**nd**		**nd**		**nd**		Exinuclease ATPase subunit
llmg_2021	*ding*	**-3,1**	0,010	**-1,3**	0,284	**-1,8**	0,276	**-5,0**	0,003	probable ATP-dependent helicase DinG homolog
llmg_2425[b]	*polA*	**-1,2**	0,126	**1,4**	0,005	**1,4**	0,564	**-2,0**	0,005	DNA polymerase I 3'-5'exonuclease and polymerase domains
Cell wall/membrane/envelope biogenesis										
llmg_0221[a]		**6,9**	0,002	**2,8**	0,006	**6,1**	0,003	**2,0**	0,005	Glycosyl transferase, family 2 Conserved hypothetical protein
llmg_1215		**-7,1**	0,008	**-1,3**	0,042	**-3,4**	0,020	**-7,3**	0,001	conserved hypothetical protein; (glycosyltransferase)
llmg_1552		**7,7**	0,006	**7,7**	0,007	**6,6**	0,006	**1,0**	0,828	putative ABC type transport system permease protein
llmg_2421		**-1,2**	0,259	**-1,4**	0,182	**-1,3**	0,491	**-3,6**	0,114	Glycosyltransferase; conserved hypothetical protein
Function unknown										
llmg_0482[c]		**-1,0**	0,891	**-1,1**	0,762	**-1,3**	0,745	**-3,0**	0,012	Uncharacterized protein conserved in bacteria

llmg_0495a		**1,0**	0,909	**1,8**	0,003	**1,3**	0,247	**-2,3**	0,010	Uncharacterized protein conserved in bacteria
llmg_0875		**-3,9**	0,033	**-1,1**	0,551	**-2,2**	0,106	**-5,2**	0,007	Uncharacterized membrane protein (cation-transporting ATPase)
llmg_2507		**1,5**	0,307	**1,8**	0,278	**-1,0**	0,972	**-1,4**	0,286	Secreted 45 kDa protein precursor
Nucleotide transport and metabolism										
llmg_0976	*purL*	**132,9**	0,000	**37,4**	0,000	**2,7**	0,035	**-2,0**	0,001	phosphoribosylformylglycinamidine synthase II
llmg_1105b		**-6,8**	0,003	**-2,0**	0,072	**-2,0**	0,358	**-12,1**	0,007	dihydroorotate dehydrogenase, electron transfer subunit
llmg_1345	*pbuX*	**3,5**	0,001	**-1,3**	0,082	**-1,1**	0,755	**-2,0**	0,000	Xanthine/uracil permeases
Inorganic ion transport and metabolism										
llmg_0336	*plpB*	**-4,6**	0,013	**-1,2**	0,456	**-1,8**	0,015	**-6,2**	0,001	ABC type metal ion transport system
llmg_1703		**-4,8**	0,002	**-1,1**	0,567	**-2,4**	0,162	**-4,8**	0,003	conserved hypothetical protein, Arsenate reductase and related proteins, glutaredoxin family
llmg_1901	*pstF*	**nd**		**nd**		**nd**		**nd**		ABC-type phosphate transport system, periplasmic component; phosphate transport substrate binding protein
Signal transduction mechanisms										
llmg_0582a,c		**10,8**	0,004	**-1,6**	0,012	**5,4**	0,006	**-3,5**	0,046	Serine kinase of the HPr protein, regulates carbohydrate metabolism
llmg_0747	*llrF*	**-1,2**	0,295	**-1,0**	0,956	**1,0**	0,973	**-2,8**	0,001	two-component system regulator llrF
llmg_0909	*amtB*	**105,3**	0,053	**5,1**	0,077	**13,8**	0,060	**2,0**	0,172	ammonium transporter AmtB
Defense mechanisms										
llmg_0268a,b		**nd**		**nd**		**nd**		**nd**		ABC-type multidrug transport system, ATPase and permease components
llmg_0329		**7,9**	0,033	**2,4**	0,069	**3,1**	0,059	**-2,0**	0,025	ABC type multidrug transport system, ATPase and permease components
llmg_1468		**-1,9**	0,030	**1,0**	0,930	**3,2**	0,060	**-3,4**	0,004	putative ABC transporter ATP binding protein
Lipid transport and metabolism										
llmg_0123b	*AcpD*	**5,2**	0,073	**3,2**	0,100	**1,1**	0,785	**-1,6**	0,145	acyl carrier protein phosphodiesterase
llmg_0431		**nd**		**nd**		**nd**		**nd**		Acyl carrier protein phospodiesterase; EC_number=3.1.4.14; putative
General function prediction only										
llmg_0352		**nd**		**nd**		**nd**		**nd**		oxidoreductase, aldo/keto reductase family
llmg_1052b		**3,7**	0,041	**1,9**	0,093	**2,4**	0,061	**-1,2**	0,376	hypothetical protein (PAPS reductase)

Functional category/Locus name[1]	Gene name[2]	Comparison conditions[3]								Product description
		Cheese / M17		Cheese / MPC		Cheese / MPC_ Bos		Cheese / MPC_ casiton		
		fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	
Coenzyme transport and metabolism										
llmg_1719	*pnuC1*	**6,2**	0,039	**-1,1**	0,658	**1,5**	0,530	**-1,8**	0,057	Nicotinamide mononucleotide transporter
Cell motility										
llmg_2405	*comGD*	**nd**		**nd**		**nd**		**nd**		putative competence protein ComGD
Secondary metabolites biosynthesis, transport and catabolism										
llmg_0500	*nhoA*	**-1,1**	0,349	**-1,2**	0,044	**-1,2**	0,681	**-2,3**	0,001	Arylamine N-acetyltransferase 2
Not classified										
llmg_pseudo_27		**1,2**	0,133	**1,6**	0,021	**-1,4**	0,083	**-3,0**	0,002	Transposase and inactivated derivatives
llmg_pseudo_74[a,c]	*noxD*	**16,0**	0,000	**1,0**	0,709	**1,6**	0,145	**-1,7**	0,005	NADH oxidase , frameshift around position 860
llmg_pseudo_79	*malQ*	**7,7**	0,019	**2,7**	0,034	**10,5**	0,017	**2,1**	0,049	pseudogene; malQ disrupted by tnp905
llmg_rRNA_1		**-2,2**	0,042	**1,1**	0,505	**1,3**	0,257	**-5,3**	0,097	16S ribosomal RNA
llmg_tRNA18[a]		**122,9**	0,000	**4,9**	0,001	**27,2**	0,000	**4,9**	0,000	tRNA_Ala, aligns also on antisesense strand of 16s and 23s RNA
llmg_0328[c]		**98,0**	0,001	**4,6**	0,002	**20,9**	0,002	**3,5**	0,002	hypothetical protein predicted by Glimmer/Critica
llmg_0817	*ps326*	**nd**		**nd**		**nd**		**nd**		hypothetical protein predicted by Glimmer/Critica
llmg_0853[b]	*ps358*	**1,8**	0,134	**3,8**	0,048	**3,0**	0,071	**1,2**	0,353	conserved hypothetical protein
llmg_1084[b]		**-1,2**	0,256	**-1,3**	0,064	**-2,7**	0,217	**-3,5**	0,003	putative membrane protein
llmg_2096	*ps446*	**nd**		**nd**		**nd**		**nd**		Predicted phage phi-C31 gp36 major capsid-like protein
llmg_2427	*comX*	**11,0**	0,002	**1,4**	0,029	**7,9**	0,005	**-1,1**	0,540	competence regulator ComX
llmg_2465[B]		**-1,3**	0,286	**-1,5**	0,022	**-1,2**	0,329	**-2,2**	0,002	conserved hypothetical; (large exo proteins involved in heme utilization or adhesion)
llmg_2490		**1,2**	0,423	**1,4**	0,101	**1,3**	0,380	**-2,4**	0,020	hypothetical protein predicted by Glimmer/Critica
llmg_2515		**219,3**	0,054	**31,3**	0,057	**3,7**	0,110	**-1,2**	0,516	hypothetical protein predicted by Glimmer
llmg_2559		**1,1**	0,627	**-1,0**	0,886	**-1,1**	0,843	**-2,3**	0,024	conserved hypothetical protein;

n.d. - below detection limit, [a]) located on antisense strand, [b]) identified in second screen, [c]) identified multiple times, [1]) locus number, gene annotation and functional categories of the target sequence are shown as given in the genome of *L. lactis* MG1363 , [2]) Indicates genes which are controlled by/in the vincinity of the the identified R-IVET sequence, [3]) Shows the fold differences of the maximum signals in the indicated environment

functional categories (5). The dipeptide transport system *dppA* is one of the identified members of the CodY regulon that is induced in cheese. Our results clearly establish that *dppA* promoter activity is down-regulated either by the addition of hydrolyzed casein to milk or by co-culturing with a mixed strain starter culture. This is likely to reflect a CodY mediated response to the availability of branched chain amino acids (10), either supplied by casiton or by casein proteolysis by the protease producing strains of the mixed starter culture used. A clear dose/response curve of *dppA* expression can be obtained by the addition of varying amounts of casiton to milk, confirming that peptides supplied in trans can explain the observed down-regulation (Chapter 8 of this thesis). Moreover, additional experiments demonstrated that the co-culturing of a protease-negative host strain with a protease positive strain leads to *dppA* promoter repression in the protease negative host, and that the level of this repression correlates with the relative fraction of protease-positive strains in the mixed culture. This result indicated that the luciferase reporter fused to the *dppA* promoter enables the detection of cooperative metabolic traits of functionally different bacteria in a mixed culture (Chapter 8 of this thesis). Remarkably, the expression of *dppA* in cheese is very high compared to all other conditions, even though the cheese was manufactured with the same mixed starter culture as the one used for the in vitro experiment with MPC. A similar trend is seen with all other CodY regulated genes, which may indicate that either the proteolytic activity of the proteolytic positive strains or the peptide availability is lower in cheese as compared to MPC. This seems plausible seen the lower water activity and consequently lower diffusion rates in cheese (26) as compared to the liquid medium MPC. Other genes identified during the R-IVET screen that belong to the CodY regulon are *hisC*, *bcaP* (*ctrA*), *cysD, gltA* and *amtB* (5, 11), all of which were clearly induced in cheese. Furthermore, with the exception of *cysD* all of these genes were down-regulated in the presence of a proteolytic mixed starter culture in MPC, indicating a similar global response of the CodY-responsive genes.

However cluster analysis also revealed that the CodY-regulated genes, despite their shared regulation do not all cluster together if the expression profiles over extended periods are compared (Fig. 3). A different level of regulation might cause such regulatory differences for some of the identified genes. Such different mechanisms are for instance the regulation of *cysD* by sulphur-containing amino acid metabolism (30) or the presence of a regulatory T-box element upstream of *hisC*. Genes of the CodY-regulon, which were not identified as being induced during cheese production and/or ripening are likely to be transcribed during the counter-selection process of the R-IVET library and were therefore eliminated prior to the screening. The proposed amino acid limitation in cheese as identified by the up-regulation of CodY regulated genes is further corroborated by the induction of *alaS*, which is regulated by a T-box element that is directly controlled by amino acid availability (35). Nevertheless, the overall results establish an amino acid limitation during growth in the cheese matrix. This is consistent with studies that established that culturing in milk induced prominent expression responses of genes belonging to the category amino acid transport and metabolism (7, 27). However, a macroarray study using *L. lactis* IL1403 concluded from various stress exposure experiments performed in vitro, using M17 medium, that the *dtp*-operon (equivalent to the *dpp*-operon in MG1363) is likely to be down-regulated under cheese conditions (36). In contrast, our data indicate a very high expression of the *dpp*-operon in cheese and we consider it highly unlikely

that such an up-regulation would occur in a peptide rich medium like M17, underlining the importance of the screening environment

Two other major functional categories that were induced in cheese are related to transcription and translation. In total 10 (putative) transcriptional regulators were isolated during the screening procedure. GadR is an activator of *gadCB*, two chloride inducible genes, which are involved in acid resistance, whereas *gadR* expression itself is independent of the chloride concentration (23). Furthermore, the identified sequences include a number of t-RNA synthetases as well as ribosomal proteins. As the overall translational activities are expected to drop upon the decline of growth rates it seems a rather unexpected result that we identified a relatively high proportion of genes from this functional group. However, the over-expression of ribosomal proteins in stationary phase cells was described earlier for *L. lactis* cultured in milk (18).

Four out of 10 promoter sequences related to translational activities are located on the antisense strand, of which one was independently identified in triplicate. The identification of the antisense sequences has been reported by various (R-) IVET studies, including the identification of an antisense transcript of 16s RNA (19), which was also identified in the study presented here. A R-IVET study in *X. campestris* revealed the location of as many as 80 out of 219 identified promoter sequences to be oriented in the non-coding direction (32). Recently, it was shown that an IVET sequence identified on the opposite strand was actually coding for a functional protein and the authors argue that this could mean that coding density in bacterial genomes is higher than generally assumed (25). In the study presented here most of the identified antisense oriented promoters drive luciferase expression over extended periods in cheese, and some displayed clearly different activity levels under the different in vitro growth conditions analyzed. However, searching for previously unidentified open reading frames longer than 30 amino acids that are located down-stream of the promoters identified on the non-coding DNA strand failed to identify any gene that may encode a known functional proteins or a protein of unknown function that is conserved in different bacterial genomes.

Amongst the identified sequences are fructose-1.3-bisphosphatase (*fbp*) and 6-phosphogluconate dehydrogenase (*gntZ*) that are both involved in the pentose-phospate pathway. Together with the identification of genes involved in nucleotide transport and metabolism (llmg_0976, llmg_1106 and llmg_1345) this finding indicates a limitation of nucleotides when cells are grown in milk. Such a nucleotide limitation, especially during the later phases of a batch fermentation would be consistent with previous studies reporting a purine limitation during mid logarithmic growth in a nutritionally rich medium (2) or the up-regulation of nucleotide biosynthesis in *L. helveticus* if grown in milk (27). The identified sequence coding for the lactate dehydrogenase (*ldh*) during the later ripening stages was unexpected, as it is the last of three genes in the *las* operon, an operon encoding *pfk*, *pyk* and *ldh*. Because of the indispensible role of these three genes in central metabolism one would expect the operon to be induced also during the counter-selection and therefore it should not be retained in the R-IVET library. However, the R-IVET clone identified does not contain the promoter of the *las* operon, but only the upstream region of the *ldh* gene itself, indicating that an alternative promoter is present within this operon. The presence of such a promoter upstream of the *ldh* gene has been discussed previously (12, 13) on basis of the detection

Table 3: Excision rates after re-introduction of plasmids isolated from 24 clones identified during the 25-day fermentation experiment. Several of these clones showed low absolute activation levels in luciferase screening and therefore their specific activation in cheese was validated by re-introduction into the original R-IVET host strain NZ5500. The resulting clones were used as adjunct/secondary cultures to manufacture individual miniature cheeses that were ripened for up to 27 days. Excision rates were determined after plating the individual strains on L-M17 and after recovering cells from cheese ripened for 2 and 27 days. The data establishes that none of the sequences identified during the later stages of cheese ripening showed sufficient promoter activity on the laboratory medium L-M17 to result in excision of the chromosomally located marker cassette harboring the erythromycin resistance and α-galactosidase genes (some showed the sporadic appearance of one MelA$^-$ colony out of approximately 200-300 colonies plated). With the exception of one clone (cs487) all of them revealed increased excision rates if the cells were residing in cheese and as expected the excision rates increase with increased ripening times.

Clone Number	Locus[a]	Gene[b]	MelA$^-$ recovery rate[c]		
			LM17	Cheese - 2 days	Cheese - 27 days
cs451	llmg_2425	*polA*	<1	93	82
cs452	llmg_1636		<1	99	100
cs462	llmg_0500	*nhoA*	<1	14	38
cs463	n.d.		<1	27	57
cs464	llmg_0853	*ps358*	<1	17	48
cs466	llmg_2302	*dpsA*	<1	4	10
cs467	llmg_2008	*uvrA*	<1	7	27
cs468	n.d.		<1	3	29
cs469	n.d.		<1	23	41
cs471	llmg_2513		<1	2	17
cs472	llmg_0070		<1	7	39
cs473	n.d.		<1	49	58
cs474	n.d.		<1	36	69
cs476	llmg_0033	*ps126*	<1	20	56
cs477[d]	llmg_0701	*oppA*	<1	49	77
cs478	llmg_0164	*tgt*	<1	3	15
cs479	llmg_1970	*nifU*	<1	5	32
cs480	llmg_0264	*fbp*	<1	49	60
cs481	llmg_0629	*pf1*	<1	20	59
cs482	llmg_1084		<1	49	87
cs483	llmg_1076		<1	89	96
cs486	llmg_1636		<1	74	94
cs487	n.d.		<1	0	0
cs488	llmg_2131	*ps412*	<1	31	66

[a,b]) Locus number and gene annotation of the identified clone are shown as given in the genome of *L. lactis* MG1363

[c]) Percentage of identified MelA$^-$ R-IVET clones after residing in the indicated environment

[d]) Located on the non-coding strand

n.d: not determined as these clones could not be identified after two sequencing attempts

of *ldh*-specific transcript that did not encompass the *pfk* and *pyk* genes. However, these authors considered an alternative promoter unlikely because they could not identify any promoter-like sequences upstream of the *ldh* gene nor detect transcriptional activity in a promoter fusion construct. Consequently, these authors suggested that the appearance of the *ldh* monocistronic transcript is most likely due to posttranscriptional processing of the polycistronic transcript derived from the *las* operon. However, the data presented here provide additional evidence for the existence of a promoter upstream of the *ldh* gene of the *las* operon, which is activated during the later stages of cheese ripening. The expression of *ldh* but not the other genes in the *las*-operon in combination with the observation that lactate dehydrogenase enzymes may be important for the conversion of lactate to pyruvate (8, 9) may explain why the expression of this gene is enhanced under energy starvation conditions that probably occur during later stages of cheese ripening. The pyruvate generated might in turn be used for the production of acetate, thereby generating additional energy in the form of ATP (9). Intriguingly, accumulation of acetate in cheese is observed between 1 and 6 weeks of cheese ripening (Chapter 3 of this thesis), suggesting that this pathway may be operational in cheese.

A number of genes involved in replication, recombination and repair were identified, including the excision nuclease subunit A. Various excision nuclease subunits were isolated in previous IVET screening procedures (19), indicating their importance under environmental conditions. Transposases and their inactivated derivatives (llmg_0703, llmg_1073 and llmg_pseudo_27) as well as phage related proteins (llmg_2076) were also identified. Phage and transposon related genes were described previously to be down regulated at higher growth rates (6), which would be consistent with their induction at the lower growth rates in cheese as compared to the counter-selection medium M17. However, the absolute expression levels indicate that the putative transposase llmg_0703 is the lowest when cells are grown in MPC, which supports only very low growth rates as a consequence of a lack of peptides or free amino acids. This suggests that factors other than growth rate must be responsible for the induction of that particular transposase.

A promoter located on the antisense strand of a putative serine kinase (*ptsK*) is strongly up-regulated in cheese and in MPC supplemented with Bos as compared to measurements in M17 (10.8- and 5.4-fold, respectively). However, this same promoter appears to be slightly down-regulated in MPC and MPC supplemented with casiton (-1.6- and -3.6-fold, respectively). Similar patterns were seen for the promoters associated with the pseudogene *malQ*, the competence regulator *comX*, as well as the promoters located on the non-coding strands in the regions coding for 16 S rRNA, tRNA_Ala and 23 S rRNA and hypothetical protein llmg_0328. These results suggest that these promoters are activated by bacterial interactions, since their activity appears to depend on the presence of a mixed starter culture. However, the molecular basis of this interaction remains to be determined.

Taken together we present the use of an advanced R-IVET system in combination with a high throughput cheese manufacturing protocol, which provides a clear view on the physiological response during cheese production. It allowed the identification and subsequent validation of genes specifically induced at specific moments of the cheese manufacturing and ripening process. The approach presented allows the selection of in

situ activated promoters and their corresponding induced genes followed by the real time in situ monitoring of the identified promoters activity patterns in a dairy product environment manufactured with a mixed strain starter culture. Next to a number of genes known to be important during dairy fermentation this enabled the identification of several genes with specific expression patterns in the dairy environment as compared to control conditions as well as genes possibly involved in the interaction between the different bacterial strains used for cheese manufacturing.

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Chapter

7

Microbial adaptation to environmental niches can be reproduced by experimental evolution

Herwig Bachmann, Marjo J.C. Starrenburg, Douwe Molenaar,
Michiel Kleerebezem, Johan E.T. van Hylckama Vlieg

Abstract

The field of experimental microbial evolution is rapidly growing but to date in most studies strains are adapted to artificial conditions that are specific for the laboratory environment. The lactic acid bacterium *Lactococcus lactis* naturally occurs on plants and in the dairy environment and it is generally believed, that dairy strains originate from the plant niche. In addition, it is suggested that the serial propagation used in early dairy processing shaped "domesticated" strains that are highly adapted to the milk environment. Phenotypic changes that are described to be linked to this adaptive process are the appearance of numerous amino acid auxotrophies and the utilization of milk casein via efficient casein degradation and peptide uptake systems. Here we investigated the adaptive process from the plant to the dairy niche and show that during the experimental evolution of a *L. lactis* plant isolate in milk, several mutations are selected that affect amino acid metabolism and transport. Three independently evolved strains were characterized by whole genome re-sequencing, revealing 6 to 27 mutations in the individual strains. Two of the adapted strains showed clearly increased acidification rates and yields in milk, and contained three identical point mutations indicating deterministic evolutionary changes. Transcriptome profiling and extensive phenotyping of the wild-type plant isolate compared to the evolved mutants, and a natural dairy isolate confirmed that major physiological changes associated with improved performance in the dairy environment relate to nitrogen metabolism. The results specify the adaptation of *L. lactis* to growth in milk and they demonstrate that niche specific adaptations found in environmental microbes can be reproduced by experimental evolution.

Introduction

The fermentation of feed and food products belong probably to the first processes in which humans unknowingly but actively used and controlled microbes for their benefit. The domestication of cattle started 8000 – 10000 years ago (11) and the consumption of fermented dairy products is documented as early as 3000 BC (2). Fermentation of dairy products is mainly carried out by lactic acid bacteria (LAB) and today these microbes play an important role in global food supply as they are used in the fermentation of dairy products, vegetables and meat (19). It is suggested that the continuous propagation of cultures resulted in today's "domesticated" strains that are highly optimized to perform rapid fermentation under applied process conditions. The emergence of genomics technologies has boosted LAB research and comparative genomics of LAB established the extensive loss of genes and the acquisition of crucial properties via horizontal gene transfer (19, 20).

The lactic acid bacterium *Lactococcus lactis* can be isolated from the plant and dairy environment and it is believed, that the dairy isolates have evolved from the plant isolates (28, 29, 31). The genome sequence of a *L. lactis* plant isolate revealed a number of properties related to the utilization of typical plant polymers, properties that have not been described for *L. lactis* dairy isolates (29). Adaptations described to be dairy specific are an increased number of amino acid auxotrophies (10, 12) and the acquisition of properties allowing the utilization of extracellular proteins (19, 28). In dairy strains the utilization of extracellular proteins like milk casein is facilitated by a cell wall bound extracellular protease that degrades casein into peptides which subsequently are transported into the cell by a dedicated oligopeptide transport system to be further degraded by intracellular peptidases (17).

Niche-specific adaptations lead to the evolution of a "specialist" as compared to the ancestral "generalist", but it remains to be established if such environmental niche adaptation can be repeated under laboratory conditions. The experimental evolution of bacterial strains is increasingly being studied, but in most cases strains are adapted to specific laboratory conditions (5). Here we describe the adaptive evolution of a *L. lactis* strain that was isolated from the plant habitat, to growth in milk. For this purpose three independent cultures were propagated for 1000 generations in milk. Subsequently single colony isolates from each adaptation experiment were extensively characterized by phenotypic testing, transcript profiling and whole genome re-sequencing. The results were compared to a dairy strain. Evolved strains showed clearly increased acidification rates, biomass yield and improved fitness when compared to their ancestral strain, which was mainly associated with prominent changes in amino acid metabolism and transport.

Material and Methods

Bacterial strains and DNA techniques

The strain used for experimental evolution was *L. lactis* KF147 (29) which was originally isolated from mung bean sprouts (Table 1). Laboratory propagation was kept to a minimum, leaving the strain with a limited laboratory history. Strains were grown either in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% lactose or in reconstituted skimmed milk (RSM) (Promex Spray 1% skimmed milk powder; Friesland Foods Butter, Lochem, The Netherlands). The plasmid pNZ521(9) was introduced into strain KF147 by electroporation (34) and transformants were selected by plating on medium supplemented with 5 µg/ml chloramphenicol. Incubation of KF147 and all evolved variants was done at 30°C. Rifampicin was used at a concentration 50 µg/ml. DNA techniques were performed as described elsewhere (26).

Table 1: Strains used in this study

strain	description	reference
KF147	*L. lactis* plant isolate with limited laboroatory history	Siezen et al. 2008
KF147 pNZ521	KF147 harbouring plasmid pNZ521	this study
KF147^{RIF}	Spontaneous rifampicin resistant mutant of KF147	this study
KF147_del_aga	Spontaneous alpha-galactosidase negative mutant of KF147	this study
NZ5521	*L. lactis* KF147-1000g evolved in milk (single colony isolate from Culture 1)	this study
NZ5522	*L. lactis* KF147-1000g evolved in milk (single colony isolate from Culture 2)	this study
NZ5523	*L. lactis* KF147-1000g evolved in milk (single colony isolate from Culture 3)	this study
IL594	*L. lactis* IL594 - dairy isolate (parent strain of IL1403)	Chopin et al. 1984

Experimental evolution

KF147 harboring pNZ521 was used to inoculate 10 ml skimmed milk and grown until the milk coagulated (~ pH 5.1). After coagulation a 10^{-5} dilution was inoculated into 10 ml of milk again. This protocol resulted in 16.6 generations per propagation step. The experiment was carried out with three independent cultures in parallel named Culture 1, Culture 2 and Culture 3 throughout this paper. After 300 generations a sample of the culture was plated on medium containing 5 µg chloramphenicol/ml to select for strains harboring plasmid pNZ521. For each parallel culture ~100 colonies were washed off the plates and the suspension was used for further propagation. The propagation process was continued as described above until the cultures grew for 1000 generations in milk (~ 5 months). Throughout the experiment stock solutions were prepared and frozen in regular intervals.

Phenotypic characterization

The amount of colony forming units (CFU) was determined by plating dilutions on LM17 or indirectly after clearing milk by incubating a solution of 0.2% sodium-hydroxide and 0.2% EDTA with cultured milk in a ratio of 9:1 for 5 minutes and the subsequent measurement of the absorption at 600 nm.

Acidification profiles were determined in milk either by using an automated pH electrode system to follow the pH (CINAC, Ysebaert, Frepillon, France) or by using microplates containing optical pH indicators (Precision Sensing GmbH, Regensburg, Germany).
After Cultures 1, 2 and 3 had been propagated for 1000 generations in milk 5 single colony isolates were obtained from each individually adapted culture. Each isolate was tested for several phenotypic properties. After determination of CFUs, acidification rates and fitness in milk, one single colony isolate of each independently evolved cultures was chosen for further evaluation. These single colony isolates were assigned the names NZ5521, NZ5522 and NZ5523, and they were isolated from Culture 1, 2, and 3 respectively. For each strain the mutation rate was determined by plating either 10^7 or10^8 CFUs of a fully-grown overnight culture on LM17 supplemented with rifampicin (50 µg/ml). The plates were incubated for 3 days and the amount of colonies on each plate were determined. Colonies growing on plates supplemented with rifampicin were considered spontaneous mutants and the mutation rate was calculated as the fraction of growing colonies per the number of cells plated.
Spontaneous α-galactosidase negative mutants of KF147, as identified by their white phenotype on medium containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal), occurred at a rate of approximately 0.2 – 0.5 % and an isolated mutant was designated KF147_del_aga.

Determination of relative fitness

A spontaneous rifampicin resistant mutant of strain KF147 was isolated by plating 10^8 cells on LM17 medium, supplemented with 50 µg rifampicin per ml. After 3 days of incubation single colonies were isolated. A colony that showed no growth difference throughout the growth curve was designated $KF147^{RIF}$ and used for further experiments. The relative fitness of the evolved strains was determined in competition assays of the adapted strains and the rifampicin resistant mutant $KF147^{RIF}$. The two competitors were inoculated in an approximately 1:1 ratio. The amount of CFUs was determined immediately after inoculation and one and three days after inoculation by plating on either LM17 or LM17 supplemented with rifampicin. The amount of CFUs for each adapted strain in the competition experiment was determined by subtracting the amount of rifampicin resistant colonies from the total amount of colonies found. Relative fitness (W) was calculated according to the following formula (8), where N_j and N_i represent the abundance of the two competing strains directly after inoculation (0) and after competition (1):

$$W_{(i,j)} = \frac{\ln \frac{N_j(1)}{N_j(0)}}{\ln \frac{N_i(1)}{N_i(0)}}$$

Genotypic characterization

Full genome re-sequencing using Solexa technology and the identification of insertions/deletions (INDELS) and single nucleotide polymorphisms (SNPs) of strains NZ5521, NZ5522 and NZ5523 was performed by GATC-Biotech (Konstanz, Germany). In addition the wild type strain KF147 was re-sequenced with the same technology to ensure a reference genome with a high sequencing coverage. Quality scores of identified SNPs indicated the fraction of reads consistent with the new consensus sequence and the sequence coverage for each position was determined. Only

SNPs with a sequence coverage of >20-fold and a quality score > 0.97 were used for further analysis. Twenty-four SNPs and INDELs were amplified by PCR amplification of the region of interest in the wild type and adapted strains followed by amplicon sequence determination. The amplification of genomic DNA was performed using a Taq-polymerase (Promega, Madison, USA) and the sequencing of the amplicons was carried out by BaseClear (Leiden, The Netherlands). This strategy, allowed the confirmation of all except one INDEL mutation that was predicted incorrectly. The deletion of a 51.3 kb genomic fragment was confirmed by the amplification across the deletion junction using genomic DNA as a template in combination with primers HB03delFW (5'-gtcatttaagaagcctttcgcatagag-3') and HB03delREV (5'-gaacatagaactccctgctcttggag-3'), and the resulting amplicon was subjected to sequence determination.

Design of microarrays

Microarrays containing in situ-synthesized 60-mer oligomers were produced by Agilent Technologies, according to a custom probe design based on the complete genome sequence of *L. lactis* subsp. *lactis* IL1403 (NCBI Reference Sequence: NC_002662.1, (4)) and the incomplete genome sequence of *L. lactis* subsp. *lactis* KF147 (29). A total of 13392 unique 60-mers having a theoretical melting temperature of approximately 82°C were selected. The melting temperature was calculated using nearest neighbor calculations (23), a Na^+ concentration of 1 M, and an oligonucleotide concentration of 10^{-12} M. Since the sequencing and annotation of strain KF147 was an ongoing process, during the current microarray analysis the probes were re-mapped to the latest sequence and annotation of KF147. The 2721 putative genes were represented by 1 (5.4%), 2 (6.7%), 3 to 6 (68%) or 7 or more probes (11.7%). A total of 218 putative genes were not represented on the array because no unique probe satisfying the selection criteria could be selected. Many of the putative genes not represented on the array were relatively short: 55% had less than 50 amino acids, and 80% had less than 100 amino acids, indicating that not all of them might be coding genes. The array design has been deposited in GEO (Acc. Nr. GPL7410).

RNA isolation, labeling and hybridization

RNA isolation from milk was performed either during logarithmic growth or from stationary phase cells as described by Sieuwerts et al. (Manuscript in preparation) with the deviation that the first step (re-suspension in 60% glycerol) was omitted . Labeling, hybridization of mRNA, and scanning of the microarrays was performed as described before (27). The microarray hybridization scheme for the for transciptome analysis after growth in milk consisted of a compound loop design with 16 arrays (Table S1).

Array data analysis

After blank spots had been removed, array measurements were normalized by local fitting of an M-A plot using the Loess algorithm and the Limma package (30) in R (http://www.r-project.org). Normalized intensities were used for further analysis. The regulation ratios between the different samples were calculated per probe from the hybridizations using

linear modeling functions from the Limma package. The statistical significance of regulation ratios was calculated from variation in biological duplicates, using the eBayes function in Limma (cross-probe variance estimation) and false discovery rate (FDR) adjustment of the *p*-values (30). Subsequently, the probe regulation ratios and the p-values were averaged per gene over all probes targeting the gene. Although clearly the averaged p-values do not represent an FDR-corrected p-value anymore, low average *p*-values for a gene are still indicative of highly significant regulation ratios, and in fact they are likely to underestimate the significance of the observed regulation.

Results

Experimental evolution

The wild type strain KF147 grew relatively poorly in milk as compared to a typical dairy strain and it was shown that the introduction of a plasmid pNZ521 carrying the gene encoding for the extracellular protease had a significant growth enhancing effect (Fig. 1). To work with a reasonably well growing strain as well as for the investigation of the co-evolution

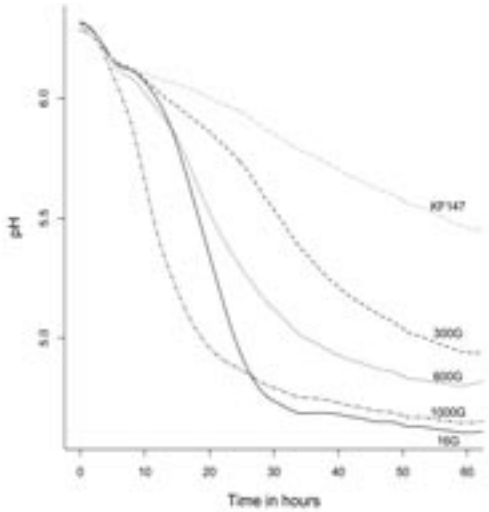

Figure 1: Acidification of milk by the wild type strain KF 147 (longdashed gray line) and Culture 1 adapted to milk for 16 (solid line), 300 (dashed line), 600 (dotted line) and 1000 (dash-dot line) generations. The acidification of milk is closely linked to bacterial growth (24). In comparison to KF147 the data indicates increased growth after 16 generations of adaptation, which is caused by the introduction of the protease plasmid. After 300 generations the plasmid carrying fraction decreased to 0.02% (see Fig. S1), which was accompanied by a decreased growth rate. Populations propagated for up to 1000 generations show increasing acidification (growth) rates, which were probably caused by adaptation. Acidification profiles are the average of 3 biological replicates. Standard deviations of the maximum slopes of each curve (calculated over the consecutive measurements of 3 hours) were 3.3%, 1.5%, 2.1%, 8.1% and 6.6% for cultures KF147, 16-, 300-, 600- and 1000-generations respectively.

of the usually unstable protease plasmid with its host *L. lactis* strain KF147 harboring pNZ521 was used for 3 parallel adaptive evolution experiments in milk (Cultures 1, 2 and 3). Initially it took about 1-2 days to acidify the milk to coagulation. This period increased in all 3 parallel cultures over the first 300 generations. Determination of the fraction of proteolytic positive strains in each culture revealed for all of them that the proportion of cells containing the plasmid carrying the protease gene had drastically decreased during the propagation experiment (Fig. S1). This counterintuitive phenomenon is related to plasmid instability and has been described in several studies previously (14, 21, 22). In order to prolong the host/plasmid co-evolution plasmid harboring strains of each evolved culture were collectively isolated on selective medium, pooled and used for further serial propagations. The results revealed again that the proteolytic trait could not be stabilized in any of the cultures and that it was lost at a similar rate as determined during the first 300 generations. This is illustrated by the acidification rates of the cultures. When acidification rates of Culture 1 at different stages throughout the whole experiment are compared, a relatively rapid acidification at the beginning (16 generations) can be noted whereas the acidification rate clearly decreased after 300 generations. Subsequently only proteolytic positive strains were selected for further propagation which increased the acidification rate that then again dropped as a result of plasmid loss (Fig. 1). After 1000 generations no proteolytic positive strains could be detected in any of the parallel cultures. Nevertheless especially the evolved Cultures 1 and 2 showed a clearly increased acidification rate as compared to the ancestral proteolytic negative KF147 wild type strain (Fig. 1). From each of the 3 independently evolved cultures 5 single colony isolates were isolated and their acidification profiles, yield and fitness were determined in milk. The results of all tests were similar for the 5 investigated colonies from each culture, and one isolate from each culture was chosen for further characterization. Single colony isolates originating from Cultures 1, 2 and 3 were designated NZ5521, NZ5522 and NZ5523, respectively.

Mutant performance in milk

The acidification rates of NZ5521 and NZ5522 were clearly increased as compared to KF147, whereas the acidification rate of NZ5523 was only slightly increased (Fig. 2). The maximum biomass yield of the evolved strains in milk was 2.0 ($p=0.006$), 2.8 ($p<0.001$) and 1.41 ($p=0.31$) times higher for strains NZ5521, NZ5522 and NZ5523, respectively as compared to the ancestral strain KF147. It should be noted that strains NZ5521 and NZ5522 reached the maximum yield within 24 hours of incubation whereas KF147 took 2-3 days to reach it's maximum yield (Fig. 3). The survival if cultured in milk was decreased for strains NZ5521 and NZ5522 (Fig. 3), which is likely to be caused by the lower pH throughout the culture (Fig. 2) (24). The yield of strains NZ5521 and NZ5522 ($\sim 2\times10^9$ CFU/ml is similar to the CFUs reached in milk by typical dairy strains (15), indicating a clear adaptation of the original strain KF147 towards that niche.
The relative fitness of the adapted strains was determined after one day of growth, which for well-adapted strains is approximately the time required to reach the maximum cell density. Moreover, the fitness was also determined after 3 days of incubation, which was on average the time between serial transfers throughout the 1000 generations of experimental evolution.

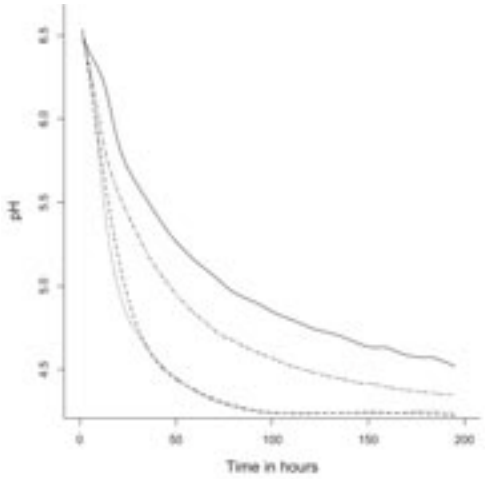

Figure 2: Acidification profiles of the wild type strain KF147 (solid line) and milk adapted single colony isolates NZ5521 (dashed line), NZ5522 (dotted line) and NZ5523 (dash-dot line) if grown in milk. The single colony isolates NZ5521 and NZ5522 show clearly increased acidification rates as compared to the ancestral strain KF147 or the adapted strain NZ5523.

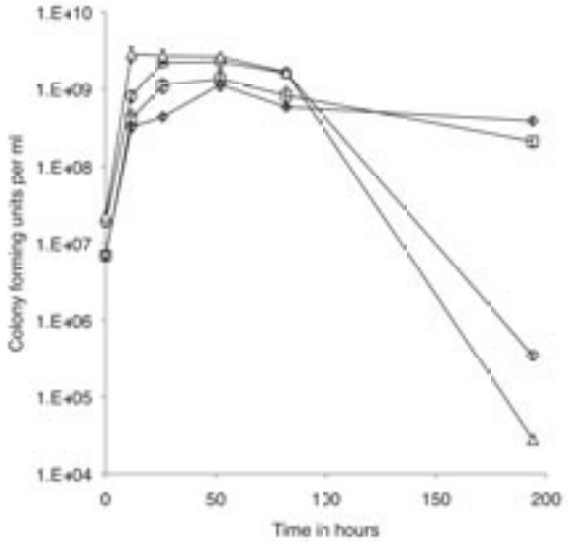

Figure 3: Biomass yield and viability of the wild type and the adapted isolates if grown in milk. The amount of colony forming units (y-axis) was determined for the wild type KF147 (◇) and the adapted isolates NZ5521 (△), NZ5522 (O) and NZ5523 (□) up to 194 hours of incubation. The amount of colony forming units is clearly increased but the long term survival seems much lower for strains NZ5521 and NZ5522 as compared to KF147 and NZ5523. The decreased survival is most likely caused by the lower pH of these cultures throughout stationary phase (see also Fig. 2). Error bars represent standard deviation (n=4).

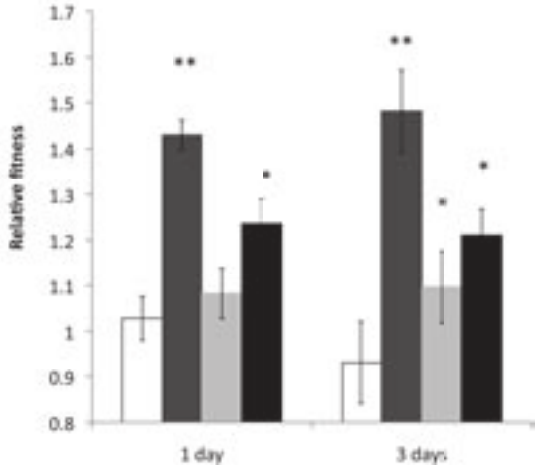

Figure 4: Relative fitness of the wild type strain KF147 (white), NZ5521 (dark grey), NZ5522 (light grey) and NZ5523 (black) if grown in co-culture with strain KF147^{RIF}. Relative fitness (W) (y-axis) was determined 1 and 3 days (x-axis) after inoculation of the co-cultures. Experimental evolution lead to increased fitness of all adapted strains. Error bars show standard deviation (n=4). Significance was calculated using a two-tailed t-test comparing each sample of both time points to the wild type strain KF147; * $p < 0.05$, ** $p<0.01$.

The competition assays revealed that especially strain NZ5521 gained a remarkable fitness advantage over strain KF147 after one day of competition and all strains showed an increased relative fitness after 3 days of incubation (Fig. 4). However, the fitness of NZ5522 is only very slightly increased as compared to strain NZ5521, although these 2 strains show similar acidification profiles and biomass yield.

Genome re-sequencing and mutation frequencies

Whole genome re-sequencing revealed 24, 6 and 6 single nucleotide polymorphisms (SNPs) for strains NZ5521, NZ5522 and NZ5523, respectively (Table 2) and 7 and 2 insertions/deletions (INDEL) in strains NZ5521 and NZ5522, respectively (Table 3). Furthermore the re-sequencing revealed that the strains NZ5522 and NZ5523 had lost an identical 51.3 kb genomic fragment that displays similarity with a lactococcal sucrose transposon identified by Kelly et al. (16). It encodes genes related to conjugative transposons, sugar transport, nucleotide metabolism, cell division an alpha-galoctosidase and others (Table S1). Since this genomic fragment contained the only α-galactosidase gene present in the wild type strain we determined the relative fraction of α-galactosidase negative strains in the adapting cultures throughout the evolution experiment. The results showed that the fraction of α-galactosidase positive strains declined drastically in Culture 2 and Culture 3 between generation 400 and generation 650 (Fig. 5). In contrast, Culture 1 contained only ~8% α-galactosidase negative cells after 650 generations of propagation, and this subpopulation appeared to vanish again during subsequent propagations to 1000 generations (Fig. 5). We interpret the results of Culture 1 as the appearance of α-galactosidase negative mutants with increased fitness, which is subsequently displaced by α-galactosidase positive derivatives with even higher fitness.

Table 2: Identified SNPs in adapted strains as determined by whole genome re-seqeuncing.

SNP position	NZ5521[1]	NZ5522[1]	NZ5523[1]	Base change	Feature[2]	Description	AA change[3]
5959	x			A>G	ORF3	ATP-dependent nuclease, subunit B	E871E
54203	x			G>A	ORF43	Fatty acid/phospholipid biosynthesis enzyme	A129V
59433	x			G>A	ORF2384	Predicted transcriptional regulators	
59488	x	x		A>T	ORF2384	Predicted transcriptional regulators	
163854	x			A>G	ORF134	Na+-driven multidrug efflux pump	I259T
341619			x	C>A	ORF287	ABC-type phosphate/phosphonate transport system, permease comp	S260STOP
393298			x	G>T	ORF334	ABC-type oligopeptide transport system, periplasmic component	A347S
489158	x			A>T	ORF418	Histone acetyltransferase HPA2 and related acetyltransferases	
615870	x			T>C	ORF536	K+ transporter	V252A
736565	x			C>T	ORF623	Cytochrome bd-type quinol oxidase, subunit 2	R97W
828730			x	T>A	ORF703[4]	Transcriptional regulators of sugar metabolism	R34N
828731			x	C>T			
985410	x			G>A	ORF833	Lysophospholipase L1 and related esterases	V87V
995567	x			T>C	ORF843	Uncharacterized protein conserved in bacteria	
1176622	x			C>A	ORF1013	Predicted phosphoadenosine phosphosulfate sulfotransferase	Q365K
1308468		x		G>A	ORF1090	Isopropylmalate/homocitrate/citramalate synthases	
1321438	x			G>T	ORF1101	Alpha-acetolactate decarboxylase	E226STOP
1396392	x			C>T	ORF1152	Predicted membrane protein	
1711438	x			C>A	ORF2527	NO COG	
1876316	x			T>C	ORF2488	Predicted hydrolases of the HAD superfamily	I55T
1934471	x			G>T	ORF1601	Phosphotransferase system, mannose/fructose/N-acetylgalactosam	
1963554	x			T>C	ORF1624	Uncharacterized conserved protein	Y186C
1969963	x			C>T	ORF1629	Uncharacterized conserved protein containing a ferredoxin-like	P375S
2025011			x	G>T	ORF1686	NifU homolog involved in Fe-S cluster formation	T102N
2026207		x		G>A	ORF1688	Selenocysteine lyase	P309L
2081398	x			T>C	--	--	
2205289	x			G>A	ORF1841	Predicted flavin-nucleotide-binding protein	D153D
2358817		x		G>A	ORF1969	ABC-type amino acid transport/signal transduction systems, per	G213D
2409424	x			A>G	ORF2030	ABC-type metal ion transport system, periplasmic component/sur	T278T
2471531			x	C>T	ORF2078	Glucose-6-phosphate isomerase	
total	**21**	**4**	**6**				

[1]) indicates in which strain the mutation was detected

[2]) open reading frames identified in the region of the SNP (Siezen et al. Manuscript in preparation)

[3]) indicates the amino acid change caused by the point mutation as compared to the wild type strain; if no change is indicated the mutation was found upstream of the predicted open reading frame

[4]) two point mutations were detected within one codon

The deletion of this fragment was found to occur spontaneously with a frequency of approximately 0.2 – 0.5% and a spontaneous α-galactosidase-negative mutant could readily be obtained (designated KF147_del_aga). The rapid loss of the 51.3 kb genomic fragment in Cultures 2 and 3 indicates that this fragment might pose a burden under the propagation conditions used.

Table 3: Identified insertions and deletions in the adapted strains as determined by whole genome re-sequencing.

Strain	Feature	INDEL Type	INDEL Base	Relative position in coding sequence	Position relative to start codon	Feature size in number of bases	Description
NZ5521	orf2461	deletion	T	upstream	-116	144	no description
NZ5521 and NZ5522	orf1744	deletion	T	upstream	-64	883	ABC-type dipeptide/oligopeptide/nickel transport systems, perm
NZ5521 and NZ5522	orf1747	deletion	A	upstream	-36	1026	ABC-type dipeptide/oligopeptide/nickel transport system, ATPas
NZ5521	orf1963	insertion	A	upstream	-33	984	Beta-lactamase class C and other penicillin binding proteins
NZ5521	orf1969	deletion	T	downstream			between the convergent features orf1969 and orf1970
NZ5521	orf2129	deletion	T	in coding region	1163	1968	DNA mismatch repair enzyme (predicted ATPase)
NZ5521	orf227	deletion	T	in coding region	131	1875	ABC-type multidrug transport system, ATPase and permease

The higher number of SNPs found in strain NZ5521 may suggest that this strain exhibits a higher mutation rate. Therefore, the mutation rates were determined by detection of the spontaneous appearance of rifampicin resistant mutants. The results revealed that the mutation rates in KF147 and NZ5521 did not differ significantly, while the mutation rates of strains NZ5522 and NZ5523 were 30 (p=0.012) and 26 (p=0.013) times reduced as compared to KF147. Intriguingly, the mutation rate of the spontaneous α-galactosidase negative mutant (KF147_del_aga) appeared also to be significantly reduced compared

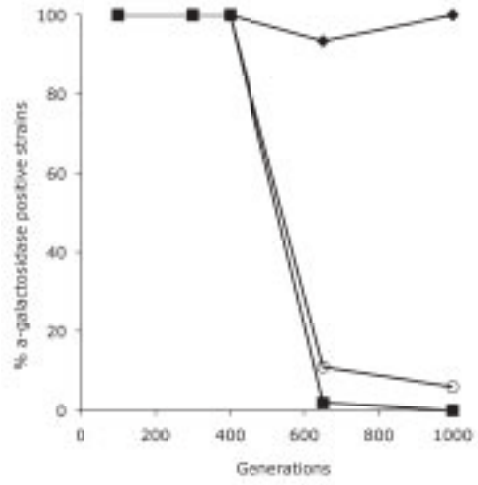

Figure 5: Fraction of α-galactosidase positive strains (y-axis) in the adapting cultures throughout the evolution experiment (x-axis). Culture 2 (■) and Culture 3 (O) revealed a drastic decrease of α-galactosidase positive strains between 400 and 650 generations of adaptation. Culture 1 (◆) showed a decrease of the α-galactosidase positive strains after 650 generations, but the α-galactosidase negative subpopulation was subsequently replaced by α-galactosidase positive strains during the following 350 generations.

to its parental strain (16-fold reduction; p=0.014), indicating that deletion of the 51.3 kb genomic fragment directly leads to a decreased mutation rate of the host strain.

Transcriptome analysis

Transcriptome analysis in milk was performed and compared to the *L. lactis* dairy strain IL594. IL594 is an ancestral strain of the fully sequenced strain IL1403 (4, 6) and in comparison to IL1403 it harbors plasmids coding for traits that are important in the dairy niche, like lactose utilization and an extracellular protease (4, 6). Transcriptome analysis in milk revealed prominent and similar significant transcriptional changes in strains NZ5521 and NZ5522 as compared to KF147, while only very few significant transcriptional changes were observed when comparing strain NZ5523 with the ancestral strain (Fig. S2). These findings are consistent with the observed differential phenotypes, showing a more significant improvement of performance in milk media for strains NZ5521 and NZ5522 as compared to strain NZ5523.

An operon coding for an ABC-type peptide transport system showed the highest differential expression level (~ 350-fold higher) in strain IL594 in comparison to strain KF147. The same operon appeared also highly expressed in strains NZ5521 and NZ5522 (Fig. 6), and remarkably an identical single base-pair deletion of an adenine residue was found 36 bases upstream of the start codon of the first gene in this operon (ORF1747) (Table 3) in both these strains. Additionally, identical deletions (thymidine residue deleted at position -64 in relation to ORF1744; Table 3), were identified in strains NZ5521 and NZ5522 in the upstream region of the 4th gene in this operon (ORF1747-ATPase), resulting in the appearance of an alternative start-codon (Fig. 7). Detailed analysis of KF147 parental sequence in comparison of the mutant sequences in strains NZ5521 and NZ5522 established that the alternative start codon in the latter two strains is preceded by a typical lactococcal ribosomal binding site (RBS), while an appropriate RBS appeared to be absent upstream of the start codon of this gene in the original strain (KF147) (Fig. 7). These two mutations thereby seem to explain the observed increased transcription of this operon in NZ5521 and 5522, and also suggest a more effective translation of the ATPase. The observation that 2 identical mutations were found in two independently evolved strains indicates the important selective advantage generated by these mutations and their consequences (higher expression of the encoded oligopeptide transport system) for growth in milk. This is further corroborated by the observed high expression levels of this same genomic locus in the dairy isolate IL594, and is in agreement with the fact that the function of this oligopeptide transport system can not be taken over by an alternative transport system in *L. lactis* (18).

The transcription level of a genomic locus coding mainly for genes involved in leucine and isoleucine biosynthesis was significantly increased in strains NZ5521, NZ5523 and the dairy isolate IL594 as compared to strain KF147, but was slightly reduced in strain NZ5522 and in strain KF147 harboring pNZ521 (Fig. S3). The re-sequencing results revealed a point mutation in the upstream region of the putative leucine operon (position 1308468, G→A; Table 2) in strain NZ5522. The leucine operon is regulated by transcription attenuation (1) and the identified point mutation appeared to be located in the regulatory terminator/ antiterminator region, which may explain the observed decreased expression level in strain

NZ5522. The reduced expression level of this locus in strain KF147 harboring pNZ521 may be explained by the protease expression of this strain, which supplies sufficient amounts of peptides and free amino acids to repress this operon. Although the leucine-operon and the downstream located isoleucine biosynthesis genes appeared to be transcribed at elevated levels in IL594 it should be noted that he leucine operon contains pseudogenes in all three sequenced *L. lactis* dairy isolates and these strains are known to be auxotroph for this amino acid.

Furthermore an ABC-type metal ion transport system is transcribed at a reduced level in NZ5521 and NZ5522, while in the same strains stress related genes, transcriptional regulators, transport systems and putative secreted and cell surface proteins are expressed at elevated levels. Notably, the same genes are not differentially regulated in NZ5523 (Fig S4), suggesting that these expression alterations in NZ5521 and NZ5522 contributes to the observed improved performance in these strains.

A putative transcriptional repressor of the AcrR/TetR family revealed two point mutations in its promoter region in strain NZ5521, of which one was also found in strain NZ5523. This gene shares similarities with *arl5* in *L. lactis* MG1363. Arl5 mutants have been shown to display 100-fold increased survival after acid challenge as well as increased resistance to heat shock and carbon starvation stress (24). Importantly, the same regulator is highly up-regulated in IL1403 during heme-dependent respiration (32), clearly indicating its involvement in stress resistance.

Among the SNPs and INDELs other than the ones described above the identification of mutations within and around genes coding for transport functions is very prominent (Table 2 and Table 3). Some of these are anticipated to influence growth in milk, like the permease unit of an ABC-type amino acid transporter.

It is also notable that competition assays of the strains KF147, NZ5521, NZ5522 and NZ5523 in co-culture with the proteolytic positive dairy isolate IL594 resulted in no differences of the relative fitness for any of the tested combinations (data not shown). This result can be explained by the generation of high amounts of free peptides by the proteolytic-positive strain in these co-cultures, which supports sufficient growth of both populations. This explanation was further corroborated by the observation that the supplementation of milk with a casein-hydrolysate overcomes the growth limitations of the wild type strain KF147 (Fig. S5), exemplifying the notion that nitrogen metabolism is a key player during the adaptation to the dairy environment.

Discussion

The experimental evolution of *L. lactis* plant isolates clearly resulted in phenotypes with improved acidification rates, biomass yields and fitness in milk. The identification of SNPs and INDELs as well as transcriptome analysis in milk established the importance of nitrogen metabolism throughout this adaptation. In view of the importance of the nitrogen metabolism, it is intriguing that the co-evolution of the bacterial strain with a protease encoding plasmid that provides a clear growth advantage, did not lead to increased plasmid stability. The fine-tuning of expression levels and, therefore, the optimization

of the cost/benefit ratios during host/plasmid co-evolution was earlier demonstrated to be successful (7, 13). The reason that this strategy did not work for the stabilization of the protease plasmid might be explained by the extracellular localization of the protease activity. This characteristic implies a burden for the proteolytic positive strains, while proteolytic negative strains can invade the population and thrive on the free peptides in the culture generated by the proteolytic-positive strains. It was recently shown that the stabilization of this cooperative proteolytic trait can be explained by the high localized availability of the generated peptides (Chapter 8 of this thesis). The identified connection between the presence and absence of a putative transposable element, with the mutation rate, suggests an evolutionary strategy that balances benefits of population stability versus high mutation frequency (25). One of the adapted populations revealed the succession of

Figure 6: Expression profiles of a putative operon encoding a dipeptide/oligopeptide transport system (ORF1747 is the first gene in the operon). Strains NZ5521 and NZ5522 have an identical deletion in the promoter sequence of the operon, which is located 36 bases upstream of the start codon of ORF1747. Both strains containing this deletion showed an elevated expression level of the operon during the logarithmic (log) growth phase as well as during stationary (stat) phase as compared to strain KF147. ORFs 1747-1744 displayed the highest increased expression level in strain IL594, which was observed in both growth phases. The heat-map at the top of the figure displays the Log_2 transformed differential expression levels. All experiments are compared to strain KF147 and they are shown from left to right. Experiments are labeled by strain, growth phase (logarithmic – log, stationary – stat) and culture medium (reconstituted skimmed milk – RSM). The feature names and annotation as well as mutational changes are given on the right. Black fields indicate no significant expression changes $p>0.05$.

Figure 7: The deletion of an adenine residue (blue) in the upstream region of ORF1744 leads to an alternative translational start 78 bases upstream of the original (wild type) start codon (green). The alternative start codon (purple) is preceded by a typical lactococcal ribosomal binding site (red), while the original start codon is not preceded by a recognizable RBS. This deletion was found in strains NZ5521 and NZ5522.

such evolutionary strategies, where eventually strain(s) with elevated mutation rates were able to outcompete the stabilized strain(s) that have lower mutation rates. Transcriptional changes with a putative link to SNPs or INDELs mainly concern genes involved in nitrogen metabolism. When combined with the phenotypic data, these adaptations represent critical events during the adaptation in this new habitat. The loss of a genomic fragment coding for carbohydrate utilization genes typical for plant isolates in 2 of the characterized adapted strains as well as a mutation leading to the silencing of the leucine biosynthesis operon (which is non-functional in any of the known dairy isolates (3, 19, 33)) and the identical mutations in the promoter region of a highly up-regulated oligopeptide transport system in two adapted strains show astonishing parallels to dairy isolates. The oligopeptide transport system of *L. lactis* is indispensable for the transport of casein derived peptides (18) and the acquisition of genes related to amino acid degradation and transport as well as the loss of genes involved in the utilization of carbohydrates that do not occur in milk, have been described to be dairy specific adaptations (19, 20, 28). In combination with the observation that amino acid limitation forms one of the main growth restrictions of KF147, adaptation of the oligopeptide transport system seems a logical consequence during the adaptation to the dairy niche. Within the limited possibilities of in vitro evolution of a pure culture, especially strains NZ5521 and NZ5522 achieved phenotypic changes relative to their ancestral strain that resemble those of typical dairy isolates. Thereby, this study establishes that niche specific adaptations, as they can be found in natural isolates, can be reproduced in a laboratory environment by experimental evolution.

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Supplementary Information for Chapter 7

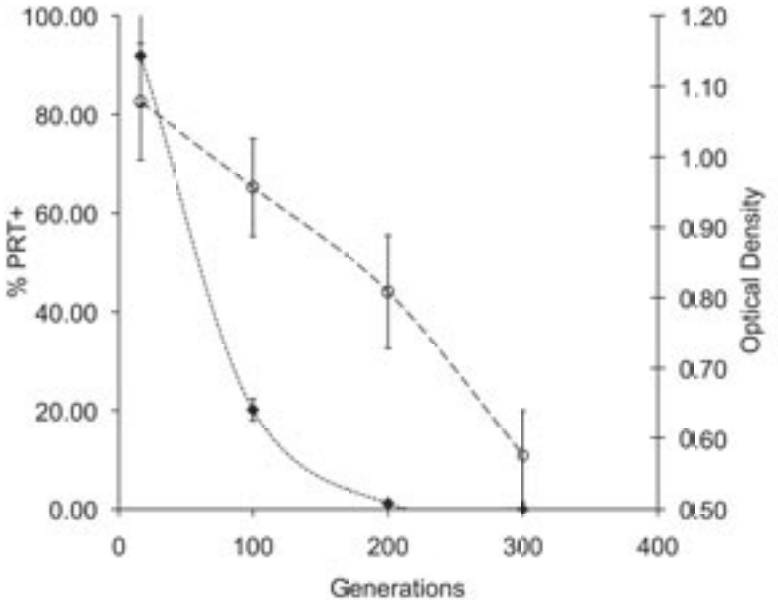

Figure S1: Decrease of proteolytic positive strains during serial propatations of KF147(pNZ521). The fraction of proteolytic positive strains ◆ (left y-axis) and the final cell density reached O (right y-axis) during growth in milk decreases throughout the serial propagation of the culture (x-axis). The draft sequence of L. lactis plant isolate KF147 did not reveal the existence of an extracellular protease (29), which is described to be essential for good growth of L. lactis in milk (21). Strain KF147 took 3-4 days to acidify milk to the point of coagulation, which is significantly longer in comparison to a typical dairy isolate that takes about 1 day to acidify the milk to coagulation, given the same inoculation density. The supplementation of KF147 with the protease carrying plasmid pNZ521 (9) increased the maximum acidification rate in milk from -0.11± 0.005 pH/h to -0.47 ±0.007 pH/hour and the yield almost doubled, indicating the growth enhancing effect of the protease on strain KF147. Despite this positive effect on growth the protease encoding strain disappeared from the culture upon serial propagation. The averages of three independently propagated cultures are shown. Error bars show standard deviation.

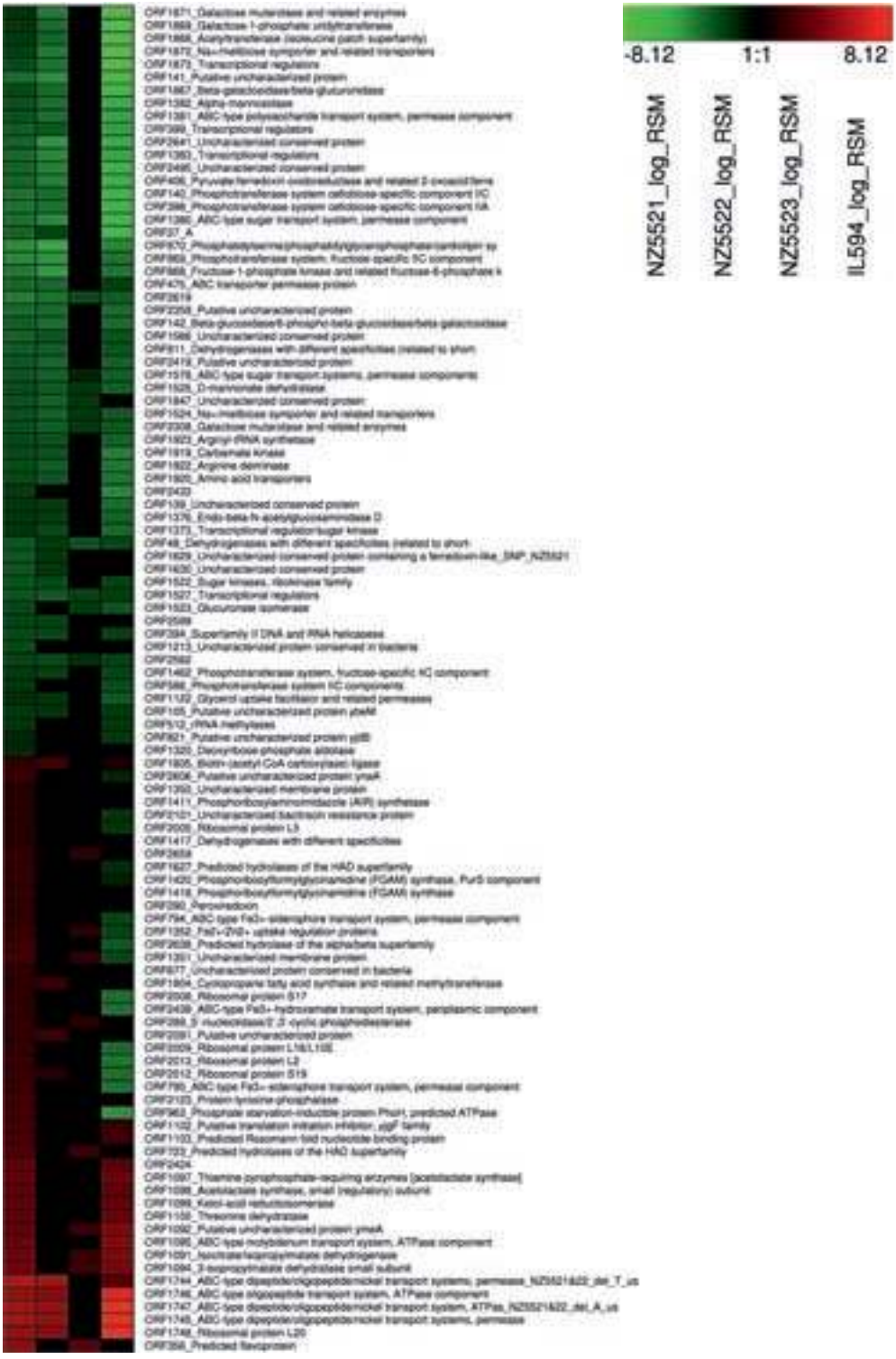
-8.12
1:1
8.12
NZ5521_log_RSM
NZ5522_log_RSM
NZ5523_log_RSM
IL594_log_RSM
ORF1871_Galactose mutarotase and related enzymes
ORF1869_Galactose-1-phosphate uridylyltransferase
ORF1868_Acetyltransferase (isoleucine patch superfamily)
ORF1872_Na+-melibiose symporter and related transporters
ORF1873_Transcriptional regulators
ORF141_Putative uncharacterized protein
ORF1867_Beta-galactosidase/beta-glucuronidase
ORF1382_Alpha-mannosidase
ORF1381_ABC-type polysaccharide transport system, permease component
ORF399_Transcriptional regulators
ORF2641_Uncharacterized conserved protein
ORF1383_Transcriptional regulators
ORF2495_Uncharacterized conserved protein
ORF406_Pyruvate ferredoxin oxidoreductase and related 2-oxoacid:ferre
ORF142_Phosphotransferase system cellobiose-specific component IIC
ORF396_Phosphotransferase system cellobiose-specific component IIA
ORF1380_ABC-type sugar transport system, permease component
ORF37_A
ORF670_Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin sy
ORF869_Phosphotransferase system, fructose-specific IIC component
ORF868_Fructose-1-phosphate kinase and related fructose-6-phosphate k
ORF475_ABC transporter permease protein
ORF2619
ORF2258_Putative uncharacterized protein
ORF142_Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase
ORF1586_Uncharacterized conserved protein
ORF811_Dehydrogenases with different specificities (related to short
ORF2419_Putative uncharacterized protein
ORF1519_ABC-type sugar transport systems, permease components
ORF1525_D-mannonate dehydratase
ORF1847_Uncharacterized conserved protein
ORF1524_Na+-melibiose symporter and related transporters
ORF2308_Galactose mutarotase and related enzymes
ORF1923_Arginyl-tRNA synthetase
ORF1919_Carbamate kinase
ORF1922_Arginine deiminase
ORF1920_Amino acid transporters
ORF2433
ORF139_Uncharacterized conserved protein
ORF1376_Endo-beta-N-acetylglucosaminidase D
ORF1373_Transcriptional regulator/sugar kinase
ORF48_Dehydrogenases with different specificities (related to short
ORF1829_Uncharacterized conserved protein containing a ferredoxin-like SNP_NZ5521
ORF1430_Uncharacterized conserved protein
ORF1522_Sugar kinases, ribokinase family
ORF1527_Transcriptional regulators
ORF1523_Glucuronate isomerase
ORF2589
ORF394_Superfamily II DNA and RNA helicases
ORF1213_Uncharacterized protein conserved in bacteria
ORF2582
ORF1462_Phosphotransferase system, fructose-specific IIC component
ORF586_Phosphotransferase system IIC components
ORF1122_Glycerol uptake facilitator and related permeases
ORF105_Putative uncharacterized protein ybeM
ORF510_rRNA methylases
ORF821_Putative uncharacterized protein yjiB
ORF1320_Deoxyribose-phosphate aldolase
ORF1805_Biotin-(acetyl-CoA carboxylase) ligase
ORF2606_Putative uncharacterized protein ynaA
ORF1350_Uncharacterized membrane protein
ORF1411_Phosphoribosylaminoimidazole (AIR) synthetase
ORF2101_Uncharacterized bacitracin resistance protein
ORF2005_Ribosomal protein L3
ORF1417_Dehydrogenases with different specificities
ORF2658
ORF1627_Predicted hydrolases of the HAD superfamily
ORF1420_Phosphoribosylformylglycinamidine (FGAM) synthase, PurS component
ORF1418_Phosphoribosylformylglycinamidine (FGAM) synthase
ORF290_Peroxiredoxin
ORF794_ABC-type Fe3+-siderophore transport system, permease component
ORF1352_Fe2+/Zn2+ uptake regulation proteins
ORF2639_Predicted hydrolase of the alpha/beta superfamily
ORF1351_Uncharacterized membrane protein
ORF877_Uncharacterized protein conserved in bacteria
ORF1804_Cyclopropane fatty acid synthase and related methyltransferase
ORF2006_Ribosomal protein S17
ORF2439_ABC-type Fe3+-hydroxamate transport system, periplasmic component
ORF288_5'-nucleotidase/2',3'-cyclic phosphodiesterase
ORF2091_Putative uncharacterized protein
ORF2009_Ribosomal protein L16/L10E
ORF2013_Ribosomal protein L2
ORF2012_Ribosomal protein S19
ORF790_ABC-type Fe3+-siderophore transport system, permease component
ORF2120_Protein-lysine-phosphatase
ORF963_Phosphate starvation-inducible protein PhoH, predicted ATPase
ORF1102_Putative translation initiation inhibitor, yjgF family
ORF1103_Predicted Rossmann fold nucleotide-binding protein
ORF723_Predicted hydrolases of the HAD superfamily
ORF2424
ORF1097_Thiamine pyrophosphate-requiring enzymes [acetolactate synthase]
ORF1098_Acetolactate synthase, small (regulatory) subunit
ORF1099_Ketol-acid reductoisomerase
ORF1100_Threonine dehydratase
ORF1092_Putative uncharacterized protein ynaA
ORF1096_ABC-type molybdenum transport system, ATPase component
ORF1091_Isocitrate/isopropylmalate dehydrogenase
ORF1094_3-isopropylmalate dehydratase small subunit
ORF1744_ABC-type dipeptide/oligopeptide/nickel transport systems, permease_NZ5521&22_del_T_us
ORF1746_ABC-type oligopeptide transport system, ATPase component
ORF1747_ABC-type dipeptide/oligopeptide/nickel transport system, ATPase_NZ5521&22_del_A_us
ORF1745_ABC-type dipeptide/oligopeptide/nickel transport systems, permease
ORF1748_Ribosomal protein L20
ORF358_Predicted flavoprotein

Figure S2: Genes differentially expressed (>2-fold difference) in NZ5521 relative to KF147 were compared to the expression level of the same genes in strains NZ5522, NZ5523 and IL594. Annotation as described for Figure 6. All comparisons are from exponentially growing cells isolated from milk and compared to strain KF147. Green indicates down-regulation, red up-regulation. Black fields indicate no significant expression difference, p>0.05. Most genes with decreased expression levels in NZ5521 were also expressed at reduced levels in NZ5522, while only few genes that were expressed at elevated levels in NZ5521 appeared to be differentially expressed in NZ5522. Overall, the transcriptional pattern of NZ5523 differed only slightly from that of KF147, while the adapted strains NZ5521 and NZ5522 displayed more similarities in their expression profiles with the dairy isolate IL594.

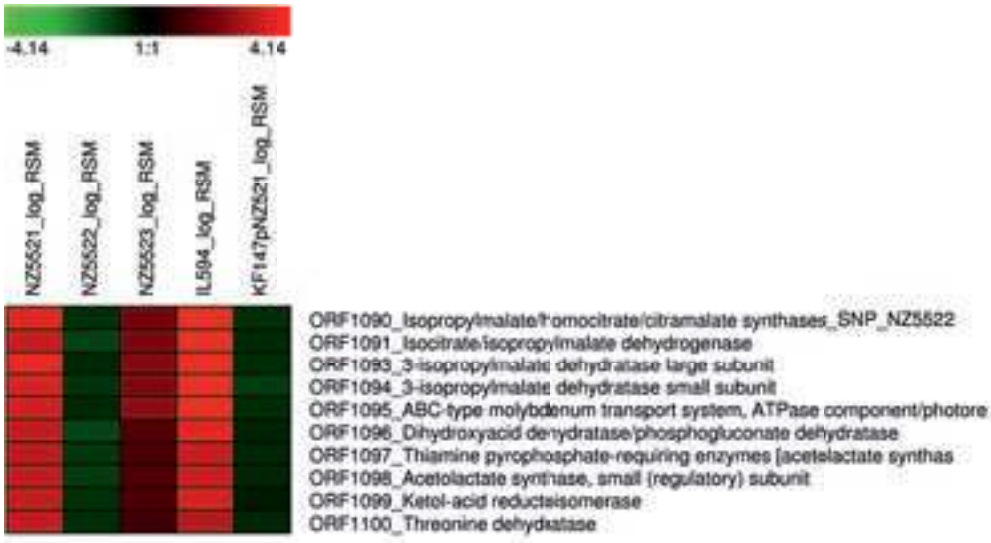

Figure S3: Differential gene expression of a locus encoding genes involved in leucine and isoleucine biosynthesis (relative to KF147). Annotation as described for Figure 6. During the logarithmic growth phase the whole locus was expressed at elevated levels in strains NZ5521, NZ5523 and IL594, while it was expressed at reduced levels in strains NZ5522 and KF147 harboring pNZ5521.

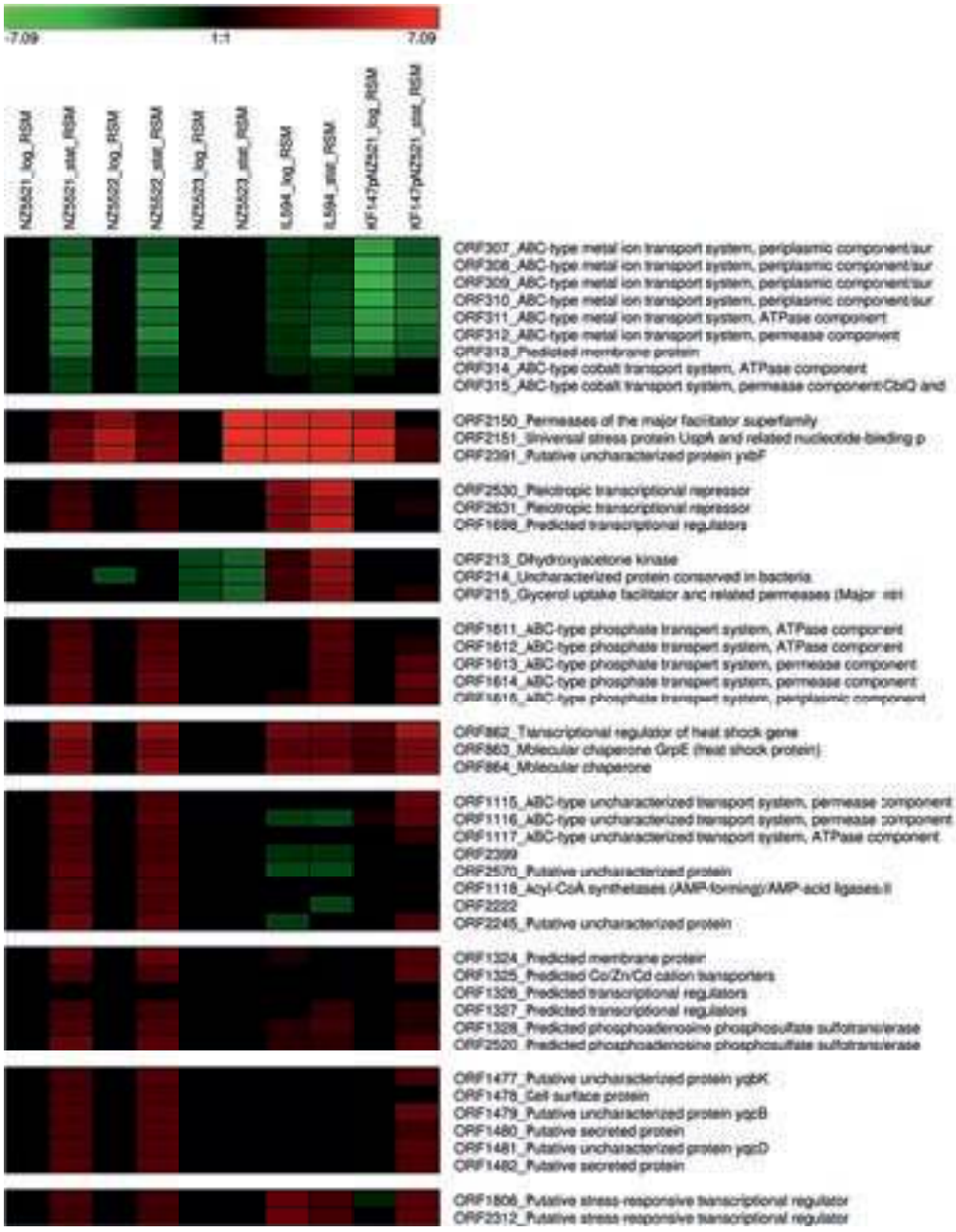

Figure S4: Differential gene expression of distinct regions of the genome (relative to KF147). For each locus the genes are displayed in the order they occur on the genome indicating operonic structures. Annotation as described for Figure 6. Numerous regions are clearly differentially regulated in strains NZ5521 and NZ5522, whereas the same genes are not significantly differentially regulated in strain NZ5523. Black fields indicate no significant expression changes, p>0.05.

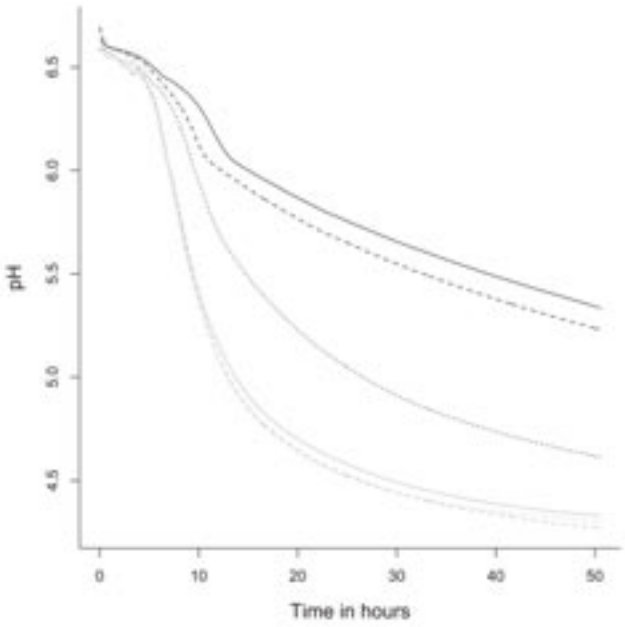

Figure S5: Acidification profiles in milk with and without the supplementation of 0.2% hydrolyzed casein. The curves show KF147 (solid lines), NZ5523 (dashed lines), NZ5521 (dotted lines). Strains were grown in milk (black lines) or in milk supplemented with 0.2% hydrolyzed casein (gray lines). The supplementation of milk with hydrolyzed casein is sufficient to overcome most growth limitations in milk and lead to similar acidification profiles of the adapted and the wild type KF147 strain.

Table S1 Hybridization scheme for transcript analysis of wild type strain KF147, the wild type strain harboring the protease encoding plasmid (KF147(pNZ521)) the adapted strains NZ5521, NZ5522 and NZ5523 and the dairy strain IL594. Cells were grown in RSM and RNA was isolated either during the exponential growth phase (exp) or after cells went into the stationary phase (stat). Strains are described in Table 1.

Array Nr.	Dye			
	Cy3		Cy5	
	Strain	Growth phase	Strain	Growth phase
1	NZ5521	exp	NZ5521	stat
2	NZ5521	stat	KF147(pNZ521)	stat
3	NZ5522	exp	NZ5522	stat
4	NZ5522	stat	KF147(pNZ521)	stat
5	NZ5523	exp	KF147	exp
6	NZ5523	stat	NZ5523	exp
7	IL594	exp	IL594	stat
8	IL594	stat	KF147	stat
9	KF147	exp	IL594	exp
10	KF147	exp	KF147	stat
11	KF147(pNZ521)	exp	NZ5521	exp
12	KF147(pNZ521)	exp	NZ5522	exp
13	KF147(pNZ521)	exp	KF147	exp
14	KF147(pNZ521)	stat	KF147(pNZ521)	exp
15	KF147	stat	NZ5523	stat
16	KF147	stat	KF147(pNZ521)	stat

Table S2 Genes identified on a 51.3 kb fragment, which was deleted in strains NZ5522 and NZ5523 during the adaptation to milk. This fragment encodes primarily genes that were so far not described to occur in dairy isolates. The spontaneous loss of this fragment from the genome lead to a 16-fold decrease of the mutation frequency. The fragment shares similarities with a sucrose-transposon that is described to occur in several plant isolates (16).

Feature	Descripton[1]
ORF1927	Superfamily II DNA/RNA helicases, SNF2 family
ORF2417	Integrase
ORF1928	
ORF1929	Antirestriction protein
ORF1930	
ORF1931	Surface antigen
ORF2401	
ORF1932	Putative conjugative transposon protein
ORF2392	Hypothetical protein
ORF2452	
ORF2456	
ORF2248	
ORF2676	
ORF2314	
ORF2323	
ORF2629	
ORF1933	Putative phage replication protein RstA
ORF1934	DNA segregation ATPase FtsK/SpoIIIE and related proteins
ORF2217	
ORF2624	
ORF2604	
ORF2403	
ORF2297	
ORF2637	
ORF2459	
ORF2459[a]	
ORF1935	Predicted transcriptional regulators
ORF2277	
ORF1936	Transposase and inactivated derivatives
ORF1937	Transposase and inactivated derivatives
ORF1938	Transposase and inactivated derivatives
ORF1939	Glycosidases
ORF1940	ABC-type sugar transport system, permease component
ORF1941	ABC-type sugar transport systems, permease components
ORF1942	ABC-type sugar transport system, periplasmic component
ORF1943	AraC-type DNA-binding domain-containing proteins
ORF1944	AICAR transformylase/IMP cyclohydrolase PurH (only IMP cyclohy
ORF1945	Galactose-1-phosphate uridyltransferase
ORF1946	Galactokinase
ORF1947	Alpha-galactosidase
ORF1948	Transcriptional regulators
ORF1949	Uncharacterized protein conserved in bacteria
ORF2405	
ORF1950	Transposase and inactivated derivatives
ORF1951	Transposase and inactivated derivatives
ORF2612	
ORF2626	
ORF1952	Transposase and inactivated derivatives
ORF1953	Transposase and inactivated derivatives
ORF2220	
ORF1954	Transposase and inactivated derivatives
ORF1954[a]	
ORF1955	Lactate dehydrogenase and related dehydrogenases
ORF1956	Alanine dehydrogenase
ORF1957	Mg2+ and Co2+ transporters
ORF1958	Uncharacterized protein conserved in bacteria
ORF2505	Small integral membrane protein
ORF1959	Putative uncharacterized protein
ORF1960	Predicted membrane protein
ORF2206	Cold shock proteins
ORF2506	Cold shock proteins
ORF2333	Putative uncharacterized protein

1) For open reading frames with no description, no similar proteins could be found in public data bases

Chapter

8

High local substrate availability stabilizes a cooperative trait

Herwig Bachmann, Douwe Molenaar, Michiel Kleerebezem, Johan E.T. van Hylckama Vlieg

The evolution of cooperation is increasingly investigated in microbial systems. An example for cooperative bacterial behavior is the expression of an extracellular protease by the lactic acid bacterium *Lactococcus lactis,* which degrades milk proteins into free utilizable peptides that are essential to grow to high cell densities in milk. Cheating, proteolytic negative strains can invade the population and drive the protease positive strain to extinction. Here we show that the proteolytic trait can persist because a fraction of the generated peptides can be captured by the cell before they diffuse away from it. The described mechanism is likely to be relevant for many extracellular substrate-degrading enzymes and our results demonstrate that it plays an important role in the maintenance of biodiversity.

The application of game theory to microbial systems is increasingly used to explain population dynamics (3, 4, 7, 8, 11). The lactic acid bacterium *Lactococcus lactis* is auxotrophic for a number of amino acids and for growth in one of its preferred niches, milk, it is dependent on a protease, which degrades milk proteins into utilizable peptides (9). The protease is anchored in the cell wall and cleavage products can diffuse away from the cell (Fig. S1). Cheating, proteolytic negative (prt^-) strains can utilize the free peptides generated by the proteolytic positive (prt^+) cooperators without having the burden of protease expression. This allows prt^- strains to invade a prt^+ population (6). The extracellular protease is usually plasmid encoded, which leads to the rapid appearance of such prt^- mutants (12). Upon serial propagations the fraction of prt^+ strains in a mixed culture will decrease, leading to a decreased overall growth rate (Fig. S2), as well as to a decreased biomass yield (Fig. S3). As a result a rapidly growing prt^+ culture can turn into a poorly growing prt^- culture if propagated in milk. This phenomenon, first described in 1931(5), has troubled the dairy starter industry for a long time, and in fact it strongly determines processes, from starter culture production to cheese manufacturing. The dilemma is clearly determined by the extracellular character of the protease. We reasoned that the existence of high local peptide concentrations, generated by the extracellular substrate-degrading enzyme could play a crucial role in the evolution and stable co-existence of the two variant strains. The suggested mechanism would provide an advantage to prt^+ cells over prt^- cells particularly at low cell densities and concomitantly low global peptide concentrations. At high cell densities the prt^+ strain has the burden of protease expression, while the prt^- cheater can thrive on relatively high free peptide concentrations without having the extra burden. A similar mechanism for the capturing of degraded substrate molecules before they diffuse away from the cell, was recently suggested for the invertase dependent sucrose utilization by *Saccharomyces cerevisiae*, which was supported by a modeling approach that encompasses local concentration gradients as a major driving force. Despite the plausibility of such localized substrate concentrations, the experimental evidence presented remained indirect (3).

To directly measure the proposed localized substrate availability around prt^+ cells, we transcriptionally fused the bacterial luciferase reporter genes (*luxAB*) to the lactococcal promoters of the *metE* and *dppA* genes that respond to intracellular peptide and amino acid levels (2), whereby luciferase activity acts as an intracellular amino acid and peptide sensor. The *luxAB* reporter constructs were transformed into *L. lactis* MG1363 a prt^- strain and MG610, a derivative of MG1363 which contains 2 copies of the protease genes *prtMP* stably integrated into the genome (10). Subsequently, it was established in pure cultures that

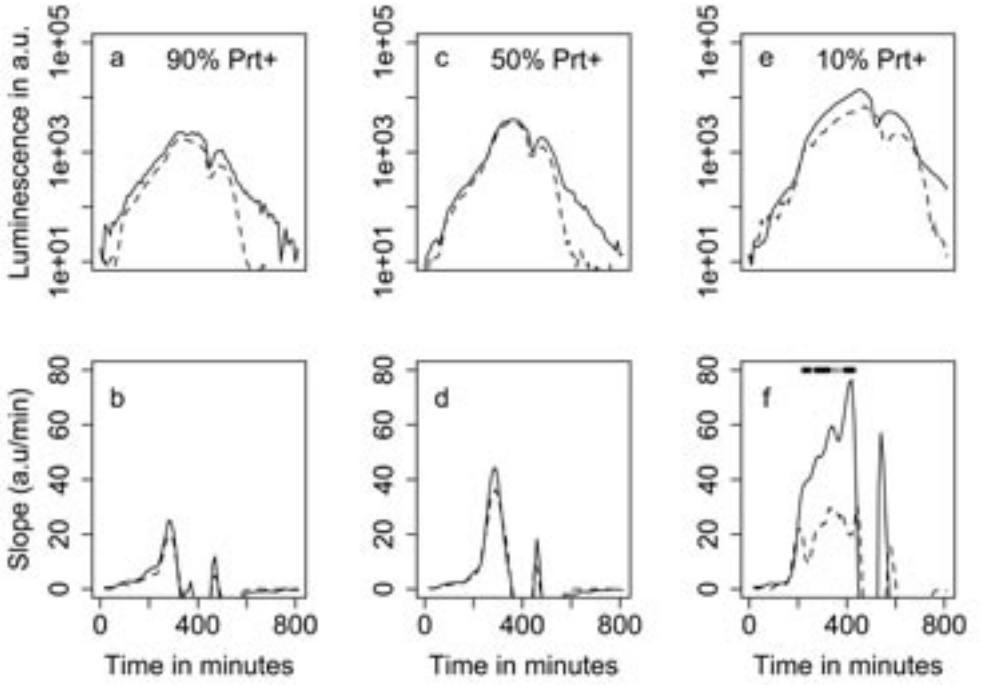

Figure 1: Localized peptide concentrations measured by intracellular luciferase-based peptide sensing. Mixed batch cultures of prt^+ and prt^- strains were grown in reconstituted skimmed milk. The fraction of prt^+ strains in the co-culture were 90% (a,b), 50% (c,d) and 10% (e,f), respectively. Intracellular peptide concentrations were measured via the *dppA* controlled luminescence signal and are given in arbitrary units (y-axis, top panel). The *dppA* reporter construct resides either in the prt^+ (dashed line) or the prt^- (solid line) host strain. The presented data is corrected for the relative abundance of the strain carrying the luciferase reporter. The slopes of the luminescence traces are given in the lower panels. Each curve represents the average of 4 biological replicates. The results show that at a high relative abundance of the prt^+ strain the *dppA* expression levels do not differ, indicating little or no difference in peptide availability for the two strains (panels a-d). At low frequencies of the prt^+ strain the intracellular amino acid levels are higher in prt^+ strains (panel e and f) – which is detected by the down-regulation of *dppA* expression in that strain. For panel f a two-tailed t-test was used to calculate p-values for each individual time point having a more than 1.5-fold difference between the presented slopes (220-430 min on x-axis). The results for each comparison are given in squares above the curves; $p<0.05$ light grey; $p<0.01$ dark grey; $p<0.001$ black. The luminescence dip between 400 and 500 minutes on the x-axis is an intrinsic property of the luminescence reporter, and coincides with cells going into stationary phase.

both reporter constructs are down-regulated when residing in the prt^+ strain as compared to the prt^- strain (Fig. S4). Mixed cultures with different prt^+/prt^- ratios contained the reporters for intracellular amino acid availability in either of the two host strains (see Fig. S1 and Table S2 for experimental design). If the proposed model of a host-dependent localized peptide availability is correct, a cell density dependent and host-specific response of the peptide and amino acid sensor system would be expected throughout the growth of a batch culture. This host-specific response can be measured as the slope of a sliding window for each consecutive measurement of the luminescence time series. In cultures

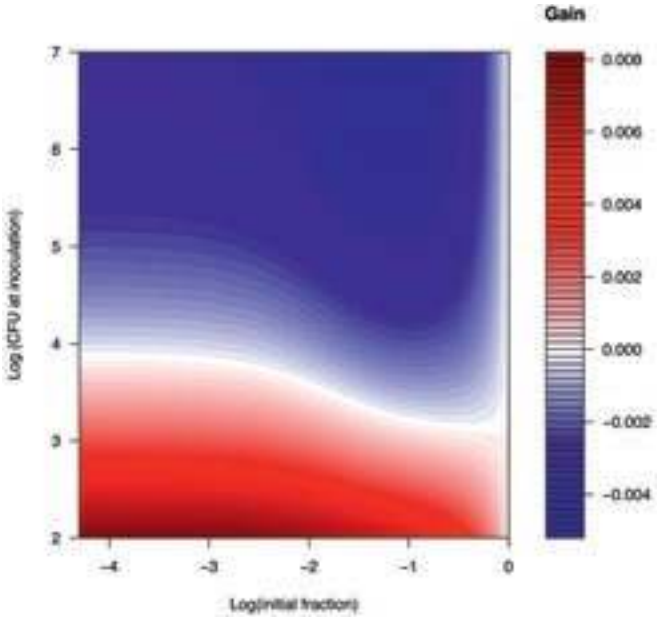

Figure 2: Modeling population dynamics of prt$^+$ and prt$^-$ mixed strain cultures. The heat map displays the fractional gain of the prt$^+$ strain after one culturing step. The dependency of this fractional gain on the initial fraction of prt$^+$ cells in the culture (x-axis) and the inoculation density in colony forming units (CFU) (y-axis) is shown. With increasing inoculation densities and/or an increasing fraction of the prt$^+$ strains in the culture, the overall advantage of the prt$^+$ strain vanishes. Model and parameters are given in the supplementory information.

with a relatively high proportion of prt$^+$ cells (90% and 50% prt$^+$) the luminescence signals differed very little, irrespective whether the reporter was in the prt$^+$ or the prt$^-$ strain (Fig. 1a-1d). This indicated that in these mixed cultures the level of extracellular protease activity is apparently sufficient to generate peptide levels that allow repression of the *dppA* and *metE* promoters, also in the prt$^-$ strain of a co-culture. In contrast, if the culture contained only 10% prt$^+$ cells, a distinct difference could be measured between the *dppA*- (Fig. 1e and 1f) or *metE*- (Fig. S5) sensor activity in prt$^+$ and prt$^-$ strains. Initially a rapid increase of the luciferase signal, which coincides with depletion of the free amino acids and peptides in milk, is observed in both strains. However, the subsequent phase is characterized by a continued increase of the luminescence signal when the reporter resides in the prt$^-$ strain, while a decreasing relative expression per cell is observed for the reporter residing in the prt$^+$ strain. The higher *dppA*- and *metE*- sensor activity in the prt$^-$ host establishes the response to the lower intracellular levels of peptides, which is not encountered by the prt$^+$ host, illustrating the proposed high local peptide availability.

Population dynamics of the two competitors were described in a simple mathematical model describing the influence of cell density and population composition on the growth rates of the two host strains in a mixed culture (Fig. 2). Model predictions were confirmed with serial dilution experiments, propagated at different inoculation densities. The relative fitness of the prt$^+$ strain was significantly increased if cells were propagated at low cell densities (Fig. 3).

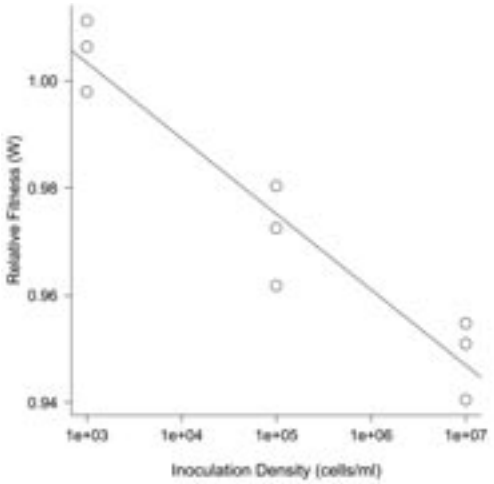

Figure 3: Relative fitness (y-axis) of prt^+ strains if propagated at different cell densities. Equal amounts of prt^+ and prt^- strains were inoculated in milk and propagated for about 100 generations. The inoculation densities at each propagation event are indicated (x-axis). For each condition, three biological replicates were propagated (o). Linear regression shows a highly significant correlation between the fitness of prt^+ strains and the inoculation density (R^2=0.91, $p<0.0001$). The prt^+ strain could be stabilized in the culture when propagated a low cell densities (W~1) and their abundance in the culture decreased when propagated at high cell densities (W<1).

The recent study on snowdrift game dynamics in yeast(3) is a good example for the described phenomenon and the same game theoretical approach is very likely to be applicable for the proteolytic trait of lactococci. The appearance of cheating prt^- lactococci is frequently observed, and has been investigated in great detail based on its importance for starter culture performance in the dairy industry (6, 12, 13). The presented data established a direct effect on cell metabolism caused by the illustrated quasi-spatial-separation between lactococci in suspension. The maintenance of biodiversity is influenced by spatial structures (1) and our example demonstrates that such structure can exist on a very small scale, between individual bacteria in a suspension. We expect the described phenomenon to influence the properties of numerous microorganisms expressing extracellular substrate-degrading enzymes and that it plays and important role in the maintenance of biodiversity in microbial ecosystems with high cell densities like in soil or the gastrointestinal tract. The fact that localized substrate availability can impact on intracellular regulatory mechanisms has also direct implications on culture heterogeneity, far beyond the difference of only the expression of the substrate-degrading enzyme.

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Supplementary information for chapter 8

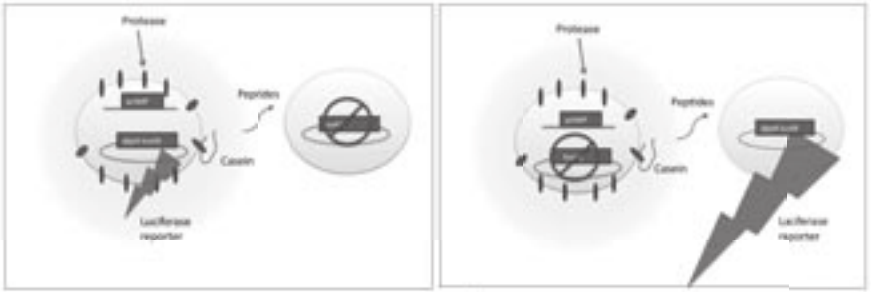

Figure S1: Peptide cross-feeding and intracellular peptide and amino acid monitoring in mixed cultures of *L. lactis*. The prt$^+$ strain expresses an extracellular cell wall bound protease PrtMP. Milk proteins such as casein are degraded by the protease into utilizable peptides that can diffuse away from the cell and be used by neighboring cells. Cheating prt$^-$ variants or strains can invade the population and drive the co-operating prt$^+$ strains to extinction. The prt$^-$ strain *L. lactis* MG1363 and its prt$^+$ derivative MG610 (harboring two copies of *prtMP* integrated into the genome) were used for competition experiments. For the measurement of peptide cross-feeding the bacterial luciferase genes *luxAB* were transcribed under control of lactococcal promoter *dppA* which is known to sense short peptides (2). This plasmid encoded construct was located in the prt$^+$ strain of the mixed culture, and a promoterless *luxAB* construct was located in the prt$^-$ strain (panel a). The same experiment was performed with the *dppA*-luciferase and the promoterless reporter constructs in the opposite strains (panel b). Analogously the experiment was also performed with a *metE*-luciferase reporter construct.

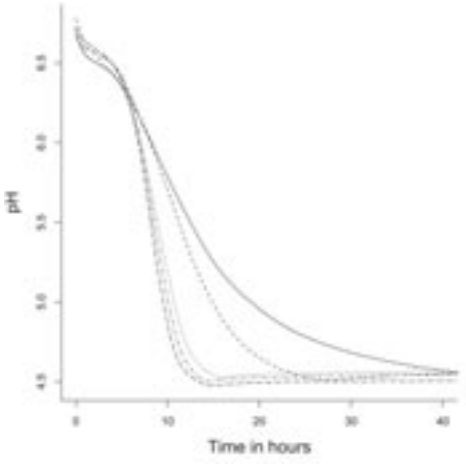

Figure S2: Enhancement of acidification by increasing the prt$^+$ fraction of a mixed culture. Acidification profiles of *L. lactis* in milk are strongly correlated to bacterial growth (4). Mixed cultures of prt$^+$ and prt$^-$ cells showed increasing acidification rates (and therefore increasing growth rates) as the prt$^+$ fraction increased. The illustrated fractions of prt$^+$ strains are 0% (solid line), 1% (dashed line), 5% (dotted line), 20% (dot-dash line) and 100% (long-dash line) and they display the slowest to the fastest acidification rate respectively. Naturally occurring lactococcal strains often encode the extracellular protease on a plasmid that displays segregation instability (6). To prevent experimental artifacts due to plasmid instability, the prt$^+$ strain *L. lactis* MG610, which has two protease copies stably integrated into the genome (5), was used in all competition experiments.

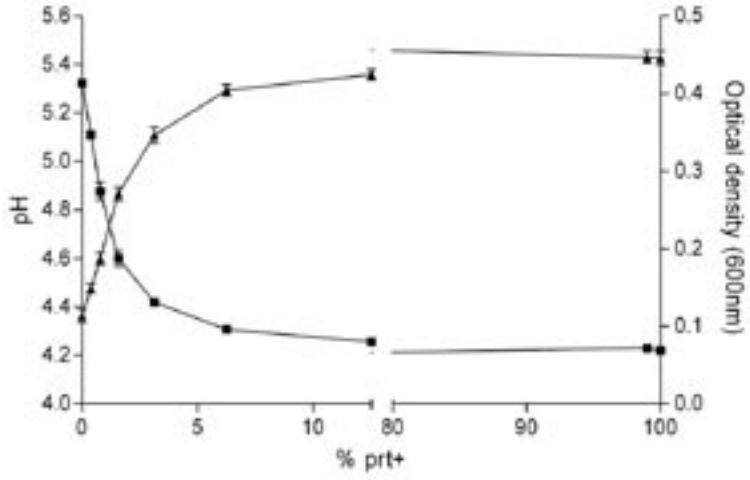

Figure S3: Influence of the fraction prt$^+$ strains on culture yield (▲)(n=8) and the culture pH (■) (n=3) when grown in milk for 24 hours (error bars show standard deviation). With an increasing fraction of prt$^+$ strain, the culture reaches higher cell densities and lower pH values after 24 hours of growth. Please note that normal milk contains a low concentration of free amino acids that sustains some growth of the prt$^-$ strains. Cultures of lactic acid bacteria eventually stop growing (stationary phase) as a consequence of the acidification of the medium. Nevertheless, stationary phase cells continue to acidify by uncoupled fermentation of the available carbon source. A pure culture of the prt$^-$ strain MG1363 typically reaches a maximum cell density between 3×10^8 and 7×10^8 cells per ml in milk (milk batch dependent), whereas a pure prt$^+$ culture of strain MG610 in milk typically reached a maximum cell density of 2×10^9 cells per ml (5).

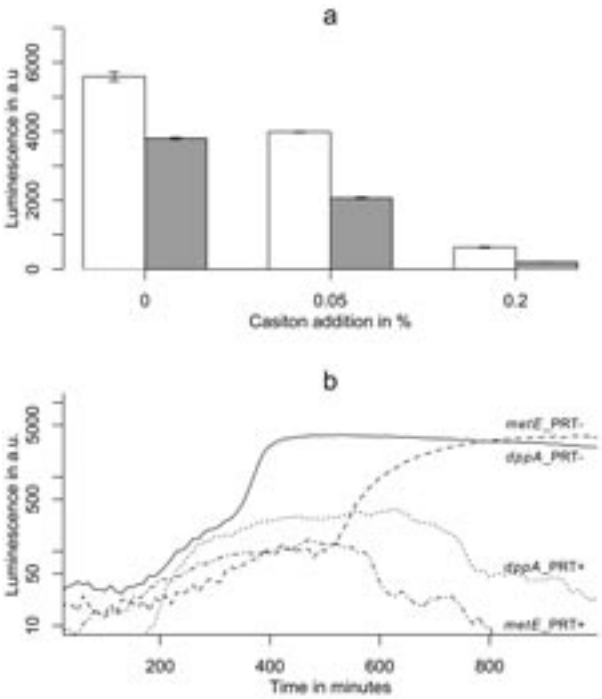

Figure S4: Luminescence reporter dependency on free peptides. The peak luminescence measurement (y-axis) of the *metE* (gray bars in panel a) reporter construct in the prt$^-$ host MG1363 recorded throughout batch cultures in milk or in milk supplemented with either 0.05% or 0.2% hydrolyzed casein (casiton) (x-axis) demonstrates the dependency of the reporter on the availability of free peptides in the medium. Similarly the dependency of the *dppA* (white bars in panel a) reporter construct on hydrolyzed casein is demonstrated. The data shown in panel a are average values of 3 biological replicates. Error bars show standard deviation. Panel b: The promoters of *metE* and *dppA* fused to the *luxAB* genes were used as intracellular sensors for amino acid ava lability in different host strains, prt$^+$ MG610 or prt$^-$ MG1363 (labeling see chart). Each point is the average of 3 biological replicates. Error bars for individual time points are similar to the ones shown in panel a. In the prt$^-$ strain MG1363, both reporter constructs show an exponential increase of the luminescence signal throughout early growth in milk, which corresponds to the increase in cell density. At cell densities between $1x10^7$ and $1x10^8$ cells/ml the luciferase signal of both reporter genes increases disproportionally in relation to the cell density. This disproportional increase is not, or only partially, observed when the *metE* and *dppA* reporter constructs are present in the prt$^+$ strain MG610. The disproportionate reporter activity increase in the prt$^-$ strain can be explained by the depletion of free amino acids and peptides in milk, which is supported by the supplementation of milk with a casein hydrolysate that down-regulates the reporter constructs in a dose-response like manner in both the *metE* construct and the *dppA* construct (panel a). If the maximum luminescence signals in pure prt$^+$ and prt$^-$ cultures are corrected for CFUs the luciferase activity levels are decreased approximately by the factors 40 and 100 for the P*dppA* and P*metE* reporter constructs, respectively, in the prt$^+$ host strain as compared to its prt$^-$ counterpart. These results confirm the dependency of the reporter signal on extracellular peptides, irrespective whether they originate from an external source (casiton supplementation) or from the in situ production by the prt$^+$ strain MG610. These results establish the suitability of the P*dppA* and P*metE* reporter constructs for the in situ measurement of intracellular amino acid availability. Plasmid pNZ5520 (1), which contains a promoterless copy of the *luxAB* genes, showed no detectable luminescence signal in milk and was used as a control construct.

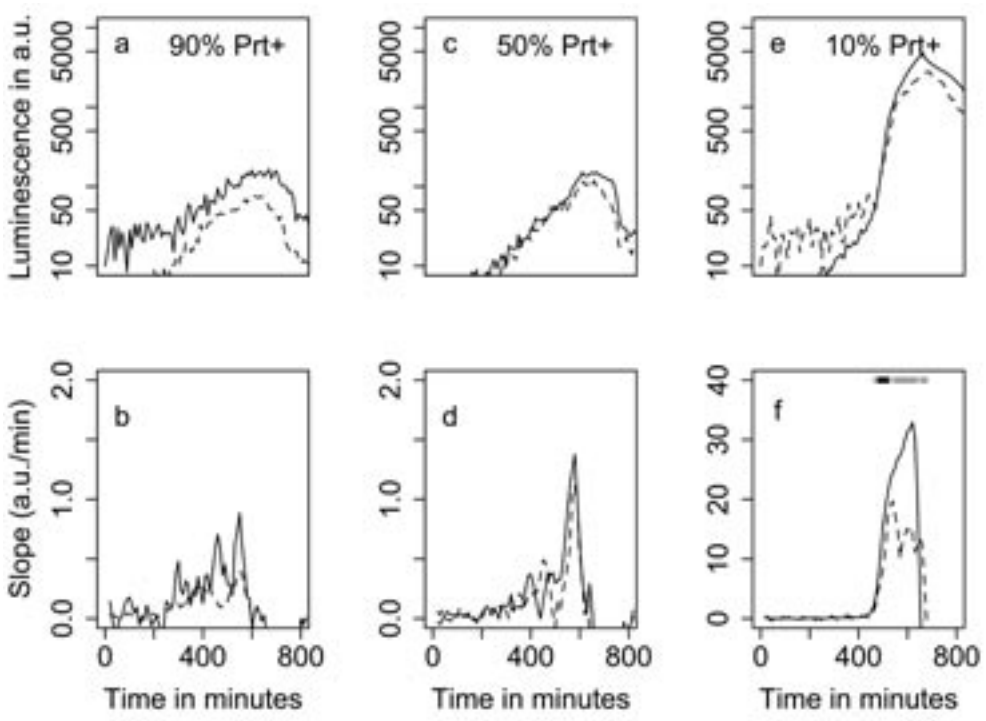

Figure S5: Intracellular peptide sensing in mixed batch cultures of prt$^+$ and prt$^-$ strains using the *metE*-luciferase reporter construct. The experiment is analogous to the experiment shown in Figure 1 but here the lactococcal promoter sequence *metE* was used as a reporter for intracellular amino acid availability. Luminescence signals of the *metE* reporter construct either in the prt$^+$ (dashed line) or the prt$^-$ (solid line) host strain (top panels) are shown. The slopes of the luminescence traces, calculated using a sliding window over 6 consecutive measurements for each point in the time series, are shown in the lower panels (y-axis). The fraction of prt$^+$ strains in the co-culture were 90% (panels a and b), 50% (panels c and d), 10% (panels e and f). Each curve represents the average of 4 biological replicates. For panel f a two-tailed t-test was used to calculate p-values for each individual time point having a more than 1.5-fold difference between the presented slopes (220-430 min on x-axis). The results for each comparison are given in squares above the curves; $p<0.05$ light gray; $p<0.01$ dark gray; $p<0.001$ black.

Bacterial strains and plasmids

Lactococcus lactis MG1363 (3) and MG610 (5), and their plasmid carrying derivatives (Table S1) were grown either in M17 medium (Merck, Darmstadt, Germany) or in sterilized reconstituted skimmed milk (Promex Spray 1% skimmed milk powder; Friesland Foods Butter, Lochem, The Netherlands) – referred to as milk throughout the paper - supplemented with 0.5% glucose. The pre-culture medium for the competition experiments with the luciferase reporter genes was a chemically defined medium for *L. lactis* containing eight amino acids(1). All experiments were carried out at 30°C.

Table S1 Strains and plasmids used in this study

StraStrain/plasmid	Description	Reference
MG1363	*L. lactis* MG1363; proteolytic negative	Gasson (1983)
MG610	EmR *L. lactis* MG1363 derivative with 2 copies of *prtMP* integrated into the genome	Leenhouts (1991)
pNZ5520	CmR, plasmid without promoter upstream of *luxAB*	Bachmann (2008)
pNZ5530	CmR, pNZ5520 derivative with *dppA* (llmg_0362) promoter upstream of *luxAB*	Bachmann (2008)
pNZ5531	CmR, pNZ5520 derivative with *metE* (llmg_1225) promoter upstream of luxAB	Bachmann (2008)

Intracellular availability of amino acids

To assess the intracellular availability of amino acids, the bacterial luciferase genes *luxAB* were transcriptionally coupled to the lactococcal promoters of the genes *dppA* and *metE*, using the *luxAB* reporter vector pNZ5520 (1). The generated plasmids and pNZ5520 were introduced into strains MG1363(3) and MG610 (5) by electroporation as previously described (7) (Table S1). Based on optical density, co-cultures of prt^+:prt^- with the ratios 10:90, 50:50 and 90:10 were prepared in reconstituted skimmed milk supplemented with 0.5% glucose and 5 µg/ml chloramphenicol. The obtained data is corrected for the relative abundance of the strain carrying the luciferase reporter. The peptide sensing reporter constructs in co-cultures were either in the prt^+ or in the prt^- host (Table S2). 200 µl aliquots were distributed to 96 well microplates, and luciferase activity was measured every 10 minutes as previously described(1). Each presented curve represents the average of 4 biological replicates. The slopes of the luminescence traces were calculated using a sliding window over 6 consecutive measurements for each point in the time series. The sequences of *dppA* and *metE* of the reporter constructs originate from *L. lactis* MG1363 (accession number: NC_009004) locus tag llmg_0362 and llmg_1225 respectively.

prt⁺/prt⁻ ratio	**10% prt⁺ : 90% prt⁻**	**50% prt⁺ : 50% prt⁻**	**90% prt⁺ : 10% prt⁻**
Reporter in prt⁺	*dppA* : contr.	*dppA* : contr.	*dppA* : contr.
Reporter in prt⁻	contr : *dppA*	contr : *dppA*	contr : *dppA*
Reporter in prt⁺	*metE* : contr.	*metE* : contr.	*metE* : contr.
Reporter in prt⁻	contr : *metE*	contr : *metE*	contr : *metE*

Table S2: Experiments carried out to measure localized peptide concentrations in mixed cultures of prt⁺ and prt⁻ strains. The first row of the table indicates the percentages of the prt⁺ and the prt⁻ host strains in the mixed populations at the beginning of the batch fermentations. *dppA* and *metE* indicate strains harboring the reporter constructs with the corresponding promoter fusions. A control plasmid (contr.) without a promoter upstream of *luxAB* (pNZ5520) was used in the second host strain of a mixed culture. The peptides/amino acid-sensing reporter constructs were either in the prt⁺ of the prt⁻ host strain.

Serial dilution experiments and calculation of relative fitness

Serial dilution experiments with *L. lactis* in milk were carried out in 10 ml tubes. Strains were precultured in GM17 medium and washed once in a physiological salt solution. Based on optical density, equal amounts of the two competing strains, MG1363 and MG610, were mixed. Each of these mixed cultures was then diluted to cell densities of approximately 10^7, 10^5, and 10^3 cells/ml. Subsequently they were incubated at 30°C and propagated every 36 (10^{-6} dilution), 24 (10^{-4} dilution) and 12 (10^{-2} dilution) hours. The propagation steps were performed 5, 8 and 16 times respectively, resulting in approximately 100 generations for each of the propagation experiments. At the time of propagation all cultures had acidified the milk to the stage of coagulation (pH<5.1), indicating full growth of the culture. The ratios of MG1363:MG610 were determined by plating serial dilutions on GM17 and GM17 supplemented with 4 µg erythromycin per ml, immediately after mixing at the beginning of the experiment, and at the end of growth. The erythromycin resistant colony forming units (CFU) represent MG610 cells, and the MG1363 CFU were deduced by subtracting the MG610 CFU from the total CFU determined on non-selective GM17 plates. The complete experiment was carried out in triplicate (Fig. 3).

Calculations

During a serial dilution experiment growth of the competing strains (E^+ and E^- - enzyme producing and non enzyme producing respectively) is described by equations (1) and (2). $D_{[x]}$ is the dilution factor upon inoculation from culture (x-1) to culture x, and T is the time from inoculation to when the culture stops growing. The number of transfers is given by n.

$$E^+_n = D_1 \cdot D_2 \cdot \ldots D_{[n-1]} \cdot E^+_0 \cdot \exp^{(n \cdot \mu^+ \cdot T)} \qquad (1)$$

$$E^-_n = D_1 \cdot D_2 \cdot \ldots D_{[n-1]} \cdot E^-_0 \cdot \exp^{(n \cdot \mu^- \cdot T)} \qquad (2)$$

Relative fitness is calculated as the ratio of cell doublings of the two competitors throughout the experiment (*3*)

$$W = \frac{\ln(\frac{E^{+}_{n}}{E^{+}_{0}}) - \ln(D_1 \cdot D_2 \ldots D_{n-1})}{\ln(\frac{E^{-}_{n}}{E^{-}_{0}}) - \ln(D_1 \cdot D_2 \ldots D_{n-1})} \qquad (3)$$

Modeling population dynamics of serial dilution experiments with varying inoculation densities

Basic modeling parameters

Variable	Description
t	Time
E^+, E^-	Enzyme producing and non enzyme producing strains
A_0, A_0^+, A_0^-	Total cell density and cell densities of E^+ and E^- at t=0
A(t), $A(t)^+$, $A(t)^-$	Total cell density and cell densities of E^+ and E^- at time t
A_{sp}	Cell density at which advantage of E^+ disappears
A_{min}	Carrying capacity of a pure E^- culture
A_{max}	Carrying capacity of a pure E^+ culture
A_{fin}	Carrying capacity of the culture as determined by α
k	Constant determining the rate of increase of A_{fin} with α
μ_b	Basic growth rate of cells
γ^+, γ^-	Fractional increase of growth rate of E^+ and E^- at low or high cell density respectively
α_0, $\alpha(t)$	Fraction of E^+ at time 0 and at time t

The growth rate of the enzyme producing strains (E^+) and non-enzyme producing cells (E^-) is a function of the total cell density. Above a defined cell density A_{sp} the growth advantage due to localized peptide availability to E^+ cells disappears and the growth advantage of the E^- cells increases with increasing cell densities. Between the initial cell density A_0 and A_{sp} the growth advantage of the E^+ cells decreases linearly, whereas for E^- it increases linearly between A_{sp} and A_{max}. In a batch culture in milk growth is eventually limited by the low pH and accumulation of lactate. The bacterial yield (carrying capacity) obtained at various fractions of prt^+ strains in culture was determined experimentally (Fig. S3) and is described as a hyperbolic function *(4)*. The constant k is equivalent to the fraction of prt^+ strains in a culture that allows growth to 50% of the maximum cell density of a pure prt^+ culture.

$$A_{fin} = A_{min} + \frac{\alpha \cdot (1+k) \cdot (A_{max} - A_{min})}{\alpha + k} \qquad (4)$$

The dynamics of the total cell density (A) and the cell densities of the individual prt$^+$ and prt$^-$ strains (A^+ and A^-, having growth rates μ^+ and μ^-, respectively) are described by the equations (*5*) to (*10*):

$$A(t) = A^+(t) + A^-(t) \qquad (5)$$

$$\alpha(t) = \frac{A^+(t)}{A^+(t) + A^-(t)} \qquad (6)$$

$$\mu^+(A) = \begin{cases} \mu_b \cdot (1 + \gamma^+ \cdot \frac{A_{sp} - A}{A_{sp}}) & A_{sp} < A_{fin} \;\&\; A \le A_{sp} \\ \mu_b & A > A_{sp} \;\&\; A < A_{fin} \\ 0 & A \ge A_{fin} \end{cases} \qquad (7)$$

$$\mu^-(A) = \begin{cases} \mu_b & A_{sp} < A_{fin} \;\&\; A \le A_{sp} \\ \mu_b \cdot (1 + \gamma^- \cdot \frac{A - A_{sp}}{A_{fin} - A_{sp}}) & A > A_{sp} \;\&\; A < A_{fin} \\ 0 & A \ge A_{fin} \end{cases} \qquad (8)$$

$$\frac{dA^+}{dt} = \mu^+(A^+ + A^-) \cdot A^+ \qquad (9)$$

$$\frac{dA^-}{dt} = \mu^-(A^+ + A^-) \cdot A^- \qquad (10)$$

Growth rate $\mu^+(A)$ is increased by γ^+ if the cell density is lower than the density at which the advantage of protease expression disappears (A_{sp}), it is equal to μ_b if the cell densities $A_{sp}<A<A_{fin}$ and growth stops if $A \ge A_{fin}$ *(7)*. The cell density dependent fractional increase of the growth rate γ^+ is modeled as a linear relationship between the actual cell density and A_{sp}. Growth rate $\mu^-(A)$ *(8)* is calculated analogously to equation *(7)*. In the model the average distance between microbial cells (cell density) plays a crucial role on the outcome of competition experiments. The burden of enzyme expression in mixed cultures was estimated from the outcomes of serial dilution experiments, which showed the loss of the prt$^+$ strain at a rate of 1-2% per generation (Chapter 7 of this thesis). The cell density at which the advantage of prt$^+$ cells disappears was based on the fact that a pure prt$^-$ culture was unable to reach cell densities higher than 7×10^8 colony forming units per ml and on the fact that a luciferase reporter driven by the lactococcal *dppA* and *metE* promoter showed a disproportionally high increase in luminescence at a cell densities between 1×10^7 and 1×10^8 cells per ml respectively. Simulation shows the dependency of population dynamics on the inoculation density and the fraction of prt$^+$ strains in the culture (Figure 2; Modeling parameters: $A_{min}=8.5$, $A_{max}=9.2$, $\mu_b=0.63$, $A_{sp}=7.5$, $\gamma^+=0.02$, $\gamma^-=0.03$, $k=0.02$) suggesting that low inoculation densities should allow the stabilization of the prt$^+$ cooperators in a mixed population.

Supplementary References:

1. **Bachmann, H., M. Kleerebezem, and J. E. van Hylckama Vlieg.** 2008. High-throughput identification and validation of in situ-expressed genes of *Lactococcus lactis*. Appl Environ Microbiol **74:**4727-36.
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Chapter 9

Summary, Future Perspectives and Concluding Remarks

This thesis describes the investigation of the industrially important lactic acid bacterium *Lactococcus lactis* in one of its natural niches, the dairy environment. Regulatory and adaptive responses to the dairy environment were studied using various approaches from transcription analysis with sophisticated molecular biological tools to experimental evolution and the detailed phenotypic and genotypic description of dairy adapted strains. Below a short summary is given on the novel tools that were developed to study physiological responses in the dairy environment as well as the key results that were obtained throughout this thesis.

In situ analysis

That the analysis of a bacterial strain should be ideally performed under in situ conditions seems a plausible notion, since the final goal is often the understanding of an organism in its natural habitat. Nevertheless, most studies are performed with pure cultures in laboratory media, mainly because analyses are easier to perform and better defined conditions allow targeted interventions and are more likely to generate unambiguous results. Unarguably, many of the experiments performed with such pure cultures in a laboratory medium yielded very valuable insight in the molecular responses of bacteria to various environmental conditions such as different types of stress. Moreover, these studies have also allowed the functional description of many genes through e.g. the characterization of over-expression or deletion mutants. Despite these advantages of in vitro experiments there is an increasing appreciation for the investigation of an organism in its natural or application environment. Specific enzyme activities of bacterial strains might differ depending on the environmental conditions the cells reside in, which is important for the mining of biodiversity for strains with industrially relevant properties. To explore the influence of culturing conditions the specific activities of 5 cheese-flavor related enzymes were measured in crude extracts of 84 lactococcal strains grown in either a nutritionally rich or a nutritionally poor medium. The outcome not only showed large differences in activities of the target enzymes in the two media for each strain but also revealed large regulatory variations between closely related strains (Chapter 2). The data demonstrate that environment-to-environment variation is large and highly strain-specific. We conclude that these enzyme measurements are not likely to be useful in predicting the strain-specific influence on e.g. cheese flavor formation when such strains would be used as adjunct/secondary cultures for cheese manufacturing. This example exemplifies the need for in situ analysis in the field of functional fermentation.

Tool development

At the beginning of this PhD project the number of tools to investigate the in situ response in complex environments of *L. lactis* were limited. We specifically aimed to increase the understanding of the physiological responses in cheese but a method for the manufacturing of individual cheeses in a cost effective and high throughput manner was not available. Chapter 3 describes the development of a protocol that allows an experienced person to manufacture nearly 600 individual cheeses simultaneously. These cheeses, designated MicroCheeses, were benchmarked to industrial cheese and in many aspects the miniature cheeses closely resembled the industrial

product. Key parameters like moisture and salt content, proteolysis or flavor profiles were very similar in MicroCheese and industrial (pilot) scale cheese products. Within the work presented in this thesis, the MicroCheese tool was employed for real-time gene expression measurements during cheese ripening (Chapter 6), but the MicroCheese protocol is also highly suitable for the high throughput assessment of numerous other questions concerning microbiological and technological aspects of the cheese-making process.

To identify the specific transcriptional responses of *L. lactis* in cheese, a dedicated tool was developed that includes the bacterial luciferase genes *luxAB*. The expression of luciferase is readily detectable by the emission of visible light upon the addition of a (long chain) aldehyde, making this enzyme a highly suitable reporter gene for high-throughput measurements. Luciferase expression in lactococci was described earlier (6, 23) but as demonstrated in Chapter 4 riboflavin was limiting the luminescence signal in early stationary phase. Riboflavin is a precursor of the co-factor $FMNH_2$, which is needed for the luminescence reaction. Riboflavin was also clearly limiting the luminescence signal throughout growth in chemically defined medium. This finding was crucial for the successful application of this reporter in the research described in the rest of this thesis and may facilitate broader application of luciferase as a reporter in *L. lactis*.

For the investigation of the transcriptional response a Recombinase-based In Vivo Expression Technology (R-IVET) tool was improved and implemented in *L. lactis* (Chapter 5). R-IVET uses a recombinase (*cre*) as a primary reporter gene, which upon expression leads to the irreversible excision of marker genes located between two recombination sites (*loxP)* on the chromosome. In comparison to earlier versions (4) we included two additional reporter genes, of which one was a chromosomally located α-galactosidase (*melA*) that allowed positive primary clone selection. The second reporter was a transcriptional fusion of the luciferase genes to the *cre* reporter gene, allowing the real-time semi-quantitative analysis of transcriptional activity under in-situ conditions. Initially, the system was used to identify promoters that were induced in a nutritionally poor medium as compared to a nutritionally rich medium. Subsequently, the quantitative analysis of luciferase activity reporter in different media allowed the identification of several molecules and conditions modulating activation of the identified promoter sequences, serving as a proof of principle for this promoter-trapping system.

Regulatory responses to the dairy environment

To investigate the regulatory response of *L. lactis* to the dairy environment we used the *L. lactis* R-IVET library as described in Chapter 5 as an adjunct/secondary culture for Gouda-type cheese manufacturing. We identified 75 genes that were specifically induced during the first two days of cheese manufacturing as well as 24 genes that were specifically induced during the later phases of cheese ripening. Of the identified clones 95 were used as single strain adjunct cultures with the high-throughput cheese making protocol developed in Chapter 3 and gene expression in cheese could be followed for up to 200 hours. The same clones were also characterized in the laboratory medium M17 and three different milk- and cheese-like conditions, allowing the identification of the environmental signals involved in activation of promoter activity for some of the identified promoter sequences. The identified promoters control expression of several genes belonging to the CodY regulon, but also several other genes

involved in amino acid transport and metabolism. The results demonstrate that lactococci experience amino acid limitation during cheese manufacturing and ripening. Other important functional categories identified were transcription, carbohydrate transport and metabolism, and energy metabolism.

Adaptive responses to the dairy environment and the stabilization of a cooperative trait

Next to the investigation of the regulatory response the adaptive responses to the dairy niche were also investigated. *L. lactis* can be isolated from plant material and the dairy environment and it is generally believed that dairy isolates evolved from plant isolates. The adaptations described to be specific for the dairy environment are e.g. an increased number of amino acid auxotrophies (7, 8) as well as the acquisition of a sophisticated casein degradation and a peptide transport system (11). In Chapter 7 it was investigated if the suggested evolutionary changes can be reproduced in the laboratory. This was achieved by experimentally evolving a *L. lactis* plant isolate to improve its growth-performance in milk by propagation for 1000 generations in this environment. A single colony isolate from each of three independently evolved cultures was phenotypically characterized, including the determination of their growth characteristics in milk, as well as transcriptome profiling. Moreover, the genomes of these three evolved isolates were fully re-sequenced. Two of the three isolates displayed clearly improved acidification rates and yields when cultured in milk. Re-sequencing allowed the identification of 6-27 mutational changes in the individual strains and transcriptional profiling revealed a major influence of these adaptive mutations on amino acid transport and metabolism. These results are consistent with described adaptations to the dairy niche (7, 8, 16). To the best of our knowledge, this is the first study that comprehensively shows that adaptive changes as they are found in natural isolates can be reproduced by experimental evolution. An interesting phenomenon encountered during these studies was the instability of a plasmid carrying an extracellular protease. The introduction of such a plasmid into a protease negative plant isolate clearly led to increased acidification (growth) rates and yields in milk. However, upon serial propagations in milk, protease negative (cheating) strains appeared in the culture and they could thrive - on the peptides released into the medium by the protease positive (cooperating) strains - without having the burden of protease expression. As a consequence, such efficiently growing protease positive culture eventually converted into poorly growing protease negative cultures after serial propagation in milk. This paradoxical behavior is a classical dilemma in the dairy research field, and has been described as early as 1931 (9). Because of its industrial relevance to starter culture stability it has been investigated in numerous studies. Despite all efforts no satisfactory explanation could be given as to how such a cooperative trait can evolve and be stabilized. We hypothesized that high local substrate concentrations around the proteolytic positive strain could explain the stabilization of such a cooperative trait (Chapter 8). Based on this hypothesis a model was constructed in which cell density forms a major determinant of the population dynamics between protease positive and protease negative cells. To directly show that high localized substrate concentrations exist promoter-luciferase constructs were used that functioned as intracellular peptide and amino acid sensors (suitable promoters were identified in Chapter 6). Luminescence screening with mixed cultures consisting of a protease

positive and protease negative strains established the existence of the proposed mechanism and serial propagation experiments further confirmed the validity of the model. The results are likely to be highly relevant for many other extracellular substrate degrading enzymes and as described in Chapter 8 for the maintenance of biodiversity.

Possible applications

The different chapters described in this thesis have a number of potential applications. The high throughput cheese making protocol offers for the first time a cost-effective way for systematic screening of e.g. culture collections for strains with particular flavor forming capacities. Such an approach was taken by using a set of 38 *L. lactis* plant isolates as adjunct culture to Gouda-type MicroCheese. The cheeses were subjected to volatile flavor profiling after 6 weeks of cheese ripening and the largest strain-to-strain differences were observed for the flavor compound 3-methyl-butanal (Fig. 1). The results indicate that potential adjunct cultures can be screened for differences in flavor compound production. The MicroCheese system may find additional application in investigating and screening the influence of a broad range of process specifications e.g. the influence of temperature regimes, varying salt concentrations or the addition of specific ingredients. Read-outs that can be assessed include fore-mentioned

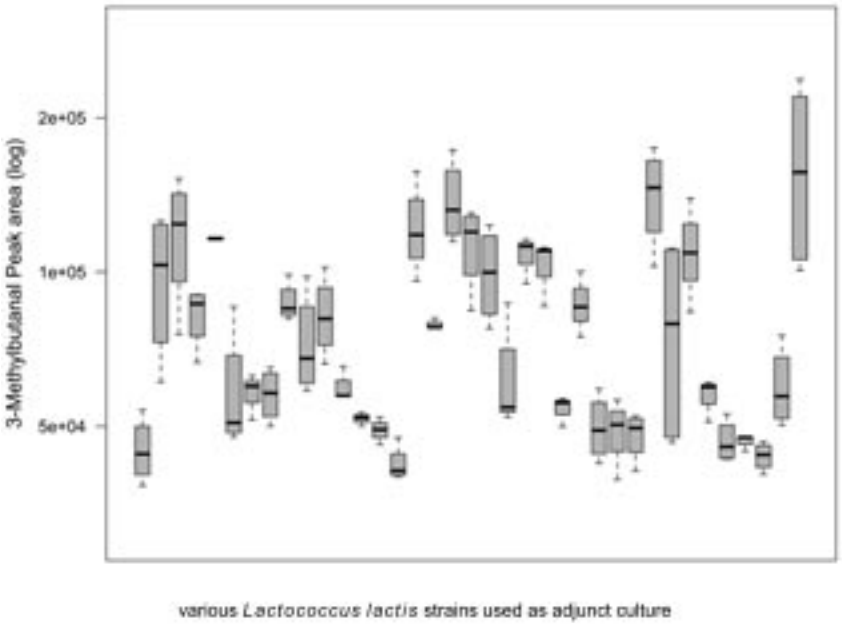

Figure 1: Screening of flavor compound production by various *L. lactis* adjunct cultures in Gouda-type MicroCheeses (x-axis) revealed largest strain-to-strain variation for 3-methylbutanal concentrations as measured by GC-MS (y-axis). The MicroCheeses were manufactured with industrial and environmental isolates of *L. lactis* as adjunct cultures. This limited biodiversity screen shows that the addition of various natural isolates as adjunct cultures can clearly impact on the flavor profile of cheese. Each box represents the measurements from 3-8 individual MicroCheeses manufactured with a particular adjunct culture (boxes show the median and the 25th and 75th percentiles, whiskers show the total range of measured values).

flavor compound profiles, proteolysis, rheological properties or the survival of starter bacteria or pathogenic bacteria. A number of such screening efforts have already been carried out, including some industrial innovation projects (personal communication W. Engels). Because of the interest and the commercial value of this method it was also protected in patent WO2008153387. There are several prospects for further improvement. As the introduction of the MicroCheese protocol effectively means that cheese production is not a bottleneck in most screening applications anymore, attention is likely to shift to increasing the range and sensitivity of read-outs. Current challenges are therefore the high throughput analysis of GC-MS profiles and the automated data analysis as well as the downscaling of numerous cheese related analyses to a high throughput format. Exploiting the possibilities that this protocol offers could lead to the development of new products, safer products or lower production costs. Next to the possibilities in product development the MicroCheese protocol will be useful to further investigate the complex biology of cheese manufacture and ripening.

The identification of promoter regions that are specifically induced during cheese manufacturing and ripening may also find its way to specific applications. The promoters identified could be used for targeted (recombinant) gene expression in cheese. Such an expression system could be obtained by exclusive self-cloning strategies, which may be regarded as food grade. The types of functional interventions that could be envisioned range from the over-expression of flavor producing enzymes to the expression of lytic gene cassettes or lethal genes that may accelerate cheese ripening via induced cell lysis (2, 5, 14).
Finally, the stabilization of the proteolytic trait in mixed strain starter cultures is still troubling the starter culture and dairy industry. Our experiments were carried out with engineered strains and under very well defined conditions. In natural systems the protease is often encoded on a plasmid that also carries the lactose operon, amino acid transporters and other genes that are important for growth in milk (16). It remains to be shown whether varying inoculation densities in natural starter cultures allows the stabilization of the proteolytic positive strains. When such experiments proof to be successful, only simple protocol changes during sub-culturing steps could be sufficient to improve the stability of mixed dairy starter cultures.

Future directions

The R-IVET screening performed revealed transcriptional activity deriving from the non-coding or antisense strand in several of the identified clones. Such transcriptional activity has been detected previously in many (R)-IVET studies (15). In this thesis approximately 10% of all identified R-IVET clones showed transcriptional activity from the antisense strand, while a R-IVET screen with *X. campestries* revealed more than 35% of all identified clones that were located on the antisense strand (19). Various IVET systems are plasmid based, which might not always reflect the transcriptional activity of the same sequence on its chromosomal location. In one study the transcription of identified antisense clones from the genomic locus was confirmed and it was shown that this region contained overlapping reading frames (transcribed from both strands of this locus) that were coding for functional proteins. The authors proposed that the coding density of bacterial genomes is therefore much higher than currently believed (17). It was also suggested that antisense transcripts might be involved in the down-regulation of

genes (15). Tiled microarray technology as well as proteomics approaches should assist the functional characterization of this insufficiently understood phenomenon.
The use of the improved R-IVET screening technology in cheese, including the subsequent validation of the identified clones, led to the detection of several genes that may be involved in bacterial interactions. This was concluded from the observation that these genes were only induced in the presence of the mixed strain starter culture Bos. The nature of these interactions and their impact on, or control of the population dynamics of mixed strain starter cultures remains to be determined.
The adaptation of a *L. lactis* plant isolate to the dairy niche underlined the importance of amino acid metabolism in this niche and exemplified that this adaption is at least in part deterministic. Next to these findings it was also demonstrated that increased mutation rates can be beneficial during the adaptive process. One of the mutations found was the deletion of a thymidine residue (T) in a poly-T stretch of the coding region of a mismatch repair protein. The deletion of this thymidine residue causes a frame shift, which most likely inactivates this gene-function. It is important to note that most INDELs found in the adapted *L. lactis* strains were either deletions or insertions of adenine (A) or thymidine residues that were predominantly located in poly-A or poly-T stretches of 5 nucleotides or more. Although, no effect of the deletion in the mismatch repair protein on the mutation rate was experimentally established, it is tempting to speculate that poly-nucleotide stretches in a functional protein involved in DNA mismatch repair would provide an elegant mechanism where error-prone replication leads to the variation of mutation rates within a population. In changing environments a mutant with an increased mutation rate could adapt more rapidly by accumulating beneficial mutations that eventually allow it to dominate the population. The frequent occurrence of INDELs in poly-nucleotide stretches could revert the mutated mismatch repair protein to a functional protein and thereby create a well adapted strain with a lower (wild type) mutation rate that might become dominant in the new environment.
Another intriguing finding that was not further pursued is the ability of *L. lactis* MG1363 to grow on lactose. Previously, strain MG1363 has been described to be unable to utilize lactose (24), but our growth experiments clearly established the opposite, albeit that growth was observed only after prolonged incubation times. The analysis of 96 individual MG1363 cultures grown in M17 medium supplemented with lactose as the only carbon source indicated that growth occurred in all cultures after 40 (±2) hours of incubation. The almost identical length of the lag phases in the individual cultures seem to exclude the possibility that alternative lactose utilization pathways are activated by e.g. the insertion of IS elements as shown for the activation of an alternative lactate dehydrogenase in *L. lactis* (3). Similar lactose-growth characteristics have been described for *L. lactis* IL1403 and the authors found that an alternative lactose utilization pathway is inducible by the addition of small amounts of cellobiose and that this pathway is always de-repressed in a *ccpA* mutant (1). In contrast, a *ccpA* mutant of MG1363 appeared to be unable to utilize lactose even after prolonged incubation, suggesting that *ccpA* is involved in lactose catabolism in MG1363 but that its role differs from that described in IL1403. A similar phenomenon of prolonged lag-phases caused by regulatory responses of carbohydrate metabolism was described for *L. plantarum*. If transferred from glucose containing medium to galactose containing medium *L. plantarum* showed a lag-phase of 20-30 hours. However, a *L. plantarum ccpA* mutant or mutants that do not express the mannose PTS did not display this

prolonged lag-phase on galactose-media. Analogously, relieving catabolite repression in wild-type *L. plantarum* cells either by pre-culturing in maltose or by spiking the media with trace amounts of glucose abolished the lag-phase on galactose-media (18). Such examples of (pre-culture or mutation dependent) accelerated initiation of carbohydrate utilization are highly interesting, not only from an academic point of view, but also for industrial fermentations which continuously strive to shorten their production processes.

Since DNA sequencing of metagenomes became affordable a new dimension was added to the investigation of environmental samples. It allows the detection of un-culturable species as well as species present at very low frequencies. Furthermore, it is possible to detect new metabolic capabilities in the total gene pool of environmental samples (13). Mixed dairy starter cultures but also the bacterial communities in raw milk are still relatively poorly described and the population dynamics in such cultures throughout dairy fermentation are not well understood. Sequencing efforts could clearly accelerate the investigation of such complex cultures, but to eventually unravel for example the bacterial interactions in such ecosystems would still require targeted strategies.

In recent years it has also become increasingly evident that clonal bacterial populations can be relatively heterogeneous (10, 20-22). The analysis of single cells revealed several interesting insights into molecular mechanisms that could not be detected by studying a population of cells (12). Such clonal heterogeneity is probably a broadly occurring phenomenon and may be more widespread than one would assume based on currently available literature. To which extent population heterogeneity plays a role on the functional properties of starter cultures during dairy fermentation remains to be determined.

Concluding remarks

In the presented thesis the in situ response of *L. lactis* was investigated with a number of different approaches. Amino acid metabolism and transport were identified as key characteristics of efficient lactococcal growth in milk and cheese. This was established by the transcriptional analysis of *L. lactis* during cheese ripening as well as by the adaptive evolution of a *L. lactis* plant isolate to growth in milk. In view of the importance of amino acid metabolism and transport the described protease-paradox is a very counter-intuitive phenomenon. However, this paradoxical behavior might be magnified by laboratory/industrial propagation schemes where cell densities of individual strains are usually much higher as compared to their natural environment.

Next to the value of the results obtained throughout this thesis it will be interesting to see some of the raised questions being addressed in future research.

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Samenvatting

Lactococcus lactis is een van de belangrijkste bacteriën tijdens de productie van zuivelproducten zoals karnemelk en kaas. Tijdens de fermentatie van kaas bepaalt *L. Lactis* eigenschappen als smaak, textuur en houdbaarheid. Gezien de relevantie voor de zuivelindustrie is er al veel onderzoek gedaan naar de fysiologie en de moleculaire biologie van *L. lactis*. Het overgrote deel van dit onderzoek werd echter uitgevoerd onder gecontroleerde laboratoriumomstandigheden. Een van de huidige uitdagingen in de microbiologie is om het gedrag van een organisme te begrijpen in zijn natuurlijke of toepassings-milieu: voor *L. lactis* dus in kaas.

Om *L. lactis* in een zuivelomgeving te kunnen onderzoeken , was het nodig om een aantal technieken te ontwikkelen. Met het nieuwe high throughput protocol voor het maken van kaas- 'Microcheese' genaamd- werd het mogelijk om gelijktijdig circa 600 individuele kazen te bestuderen. Om genen te kunnen identificeren die specifiek in kaas tot expressie komen werd een Recombinase based In Vivo Expression Technology (R-IVET) systeem verbeterd en aangepast voor *L. lactis*. Voor het eerst kon met de combinatie van R-IVET en Microcheese technologie bestudeerd worden welke genen belangrijk zijn in kaas tijdens de kaasrijping.

Naast de zuivelvarianten van de lactococen bestaan er varianten die in de natuur op planten voorkomen. Het lijkt aannemelijk dat, evolutionair gezien, de zuivelvarianten zijn voortgekomen uit de plantvarianten. Om te kunnen begrijpen welke aanpassingen belangrijk zijn om goed te kunnen groeien in melk werd deze evolutie nagebootst door een plantenstam 1000 generaties in melk te laten groeien (~5 maanden). Drie onafhankelijk van elkaar geëvolueerde stammen werden fenotypisch en genotypisch gekarakteriseerd onder andere door het hele genoom te re-sequencen. Zowel de korte termijn aanpassingen die *L. lactis* doet (zoals aangetoond in de R-IVET analyse) als de lange termijn aanpassingen (zoals aangetoond in het evolutieexperiment) onderstrepen het belang van het aminozuur metabolisme en transport voor groei in melk en kaas.

Tijdens de bovengenoemde experimenten kwam een merkwaardige eigenschap van *L. lactis* aan het licht. Sommige *L. lactis* stammen kunnen melkeiwit buiten de cel afbreken. De hele populatie profiteert hiervan, want deze kan de afgebroken eiwitten gebruiken voor de groei. Ondanks dit voordeel verdwijnen stammen met deze eigenschap tijdens de groei in melk. Dit verschijnsel was al jaren een wetenschappelijke paradox en een probleem voor kaasproducenten. In dit proefschrift wordt een verklaring gepresenteerd voor dit fenomeen.

Dit proefschrift laat zien dat het belangrijk is om processen in zuivel ('in situ') te bestuderen om te begrijpen hoe smaak en kraak van het eindproduct tot stand komen. Ook presenteert dit proefschrift een aantal nieuwe methoden die in situ onderzoek mogelijk maken. Hiermee wordt een bijdrage geleverd aan de bestaande kennis over lactococcen in melk en kaas, en zouden industriële processen verbeterd kunnen worden, zoals bijvoorbeeld door stabilisatie van de populatiesamenstelling van gemengde starterculturen in de zuivel.

Populärwissenschaftliche Zusammenfassung

Lactococcus lactis ist ein Milchsäurebakterium, das hauptsächlich auf Pflanzen und in Milchprodukten vorkommt. Es ist eines der wichtigsten Bakterien für die Produktion von Buttermilch und halbharten Käsesorten wie zum Beispiel Gouda-Käse. Durch die industrielle Relevanz von *L. lactis* ist es eines der am besten charakterisierten Milchsäurebakterien und stellt einen wichtigen Vertreter der Gram-positiven Bakterien dar. Viele der mit *L. lactis* durchgeführten Studien untersuchten den Einfluss von verschiedenen Stress-Faktoren, wie sie bei der Erzeugung von Milchprodukten vorkommen, auf das Verhalten der Bakterien. Zum Beispiel wurden der Einfluss von Temperaturschwankungen, hohen Salzkonzentrationen, niedrigem pH-Wert, Limitierung der Kohlenhydratzufuhr oder oxidativer Stress sehr detailliert untersucht. Da ein Großteil dieser Studien mit reinen Kulturen in einem künstlichen Medium ausgeführt wurde, sind die Resultate nicht unbedingt repräsentativ für das Verhalten der Bakterien in einem Milchprodukt. Eine der aktuellen Herausforderungen besteht deshalb darin, die bakterielle Physiologie und Molekularbiologie auch in der natürlichen Umgebung des Organismus (in situ) zu erforschen und zu verstehen.

In der vorliegenden Dissertation wurden verschiedene Methoden entwickelt bzw. angewandt, um regulatorische sowie evolutionäre Anpassungen von *L. lactis* in fermentierter Milch bzw. Käse zu erforschen. Zu diesem Zweck war es nötig, ein Produktionssystem für Käse zu entwickeln, das den Vergleich vieler experimenteller Variationen erlaubt. Es wurde ein Modellsystem entwickelt, das es einer einzigen Person ermöglicht, bis zu ~600 individuellen Käsen im Kleinformat (170 mg) parallel zu erzeugen. Weiters wurde eine Methode zur Messung der Genaktivität von *L. lactis* während der Produktion und Reifung von Käse entwickelt. Dieses System erlaubt neben der Identifikation von in Käse induzierten Genen die Messung der Genaktivität mittels einer enzymatischen Reaktion, die zur Emission von sichtbarem Licht führt. Anders ausgedrückt bedeutet dies, dass die Aktivität von bestimmten *L. lactis* Genen in Käse daran gemessen werden kann, wie viel Licht der Käse ausstrahlt. Durch die Kombination der zwei genannten Systeme wurde es möglich, die Genaktivität von *L. lactis* während der Käsereifung zu verfolgen. Die Ergebnisse zeigten, dass relativ viele verschiedene funktionelle Klassen an Genen während der Reifung von Käse aktiv sind, aber vor allem, dass *L. lactis* trotz der hohen Eiweißkonzentration in Käse an einem Mangel an Aminosäuren - den Bausteinen von Eiweiß - leidet.

Neben der Erforschung der regulatorischen Anpassungen von *L. lactis* an Milch bzw. Käse, wurden auch evolutionäre Anpassungen an diese ökologische Nische untersucht. Dies wurde durch die kontinuierliche Sub-Kultivierung eines von Pflanzen isolierten *L. lactis* Stammes in Milch erreicht. Diese Sub-Kultivierung dauerte 1000 Generationen (~ 5 Monate) und die an Milch angepassten Bakterien wurden detailliert auf phänotypische (Wachstumsrate, Versäuerungsrate von Milch, Biomasse, Transkriptom Profiling und Fitness) sowie genotypische (Mutationen) Merkmale untersucht und mit dem Mutterstamm verglichen. Zwei von drei adaptierten Stämmen zeigten signifikante Verbesserungen der Wachstums- und Versäuerungsrate sowie der maximal erreichbaren bakteriellen Biomasse in Milch. Das Sequenzieren der gesamten Genomsequenz zeigte, dass vor allem Mutationen, die den Transport und die Biosynthese von Aminosäuren beeinflussen, für die Veränderungen der adaptierten Stämme in Milch verantwortlich sind. Die Resultate bestätigen die allgemeine Annahme, dass *L. lactis* Stämme,

die von Milch oder Milchprodukten isoliert wurden, von Stämmen abstammen, die ursprünglich auf Pflanzenmaterial zu finden waren. Die Anpassung an die ökologische Nische Milch konnte somit durch experimentelle Evolution reproduziert werden.
Weiters wurde die Stabilisierung von protease-positiven Lactococcen in Mischkulturen mit protease-negativen Lactococcen untersucht. Protease Aktivität führt zur Spaltung von Milcheiweiß in Peptide, die dann in die Zelle transprotiert werden, wo sie als Bausteine für Zellwachstum dienen. Die Protease ist nur außerhalb der Zelle aktiv, wodurch die freigesetzten Peptide durch das Medium diffundieren und auch durch Stämme aufgenommen werden können, die selbst keine Protease produzieren. Da die Produktion der Protease zu einer Verlangsamung des Wachstums führt, werden nach längeren Phasen der Sub-Kultivierung protease-positive Stämme aus Mischkulturen verdrängt. Proteolytische Aktivität ist allerdings essenziell für ein gutes Wachstum von Lactococcen in Milch. Durch Sub-Kultivierung in Milch wird dadurch aus einer gut wachsenden Kultur stets eine schlechter wachsende Kultur. Dieses Problem wurde im Jahr 1931 zum ersten Mal beschrieben und war wegen seiner großen Relevanz für die Milchindustrie seitdem Gegenstand vieler Studien. Jedoch konnte das Phänomen nie vollständig erklärt werden. In der vorliegenden Arbeit wurde gezeigt, dass ein Konzentrationsgradient von Peptiden zwischen protease-positiven und protease-negativen Bakterien in Milch besteht. In einem Modell, das auf diesem Konzentrationsgradienten beruht, konnte die Populationsdynamik zwischen den protease-positiven und den protease-negativen Zellen beschrieben werden, was schlussendlich auch experimentell bestätigt werden konnte.
Die Resultate dieser Dissertation heben die Wichtigkeit von Studien hervor, die das Verhalten von Bakterien in ihrer natürlichen/angewandten Umgebung untersuchen und geben Einsicht in molekularbiologische und physiologische Anpassungen von *L. lactis* an Milch und Milchprodukte. Neben der breiten Anwendbarkeit einiger der entwickelten Methoden können die Ergebnisse zu Verbesserungen von industriellen Prozessen, wie der Stabilisierung der Populationszusammensetzung von gemischten Käse-Starterkulturen, beitragen.

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One thing that becomes evident during a PhD project is that co-operation with other people and scientific discussions are crucial to move forward. The approaches and technologies used throughout my thesis are varied. This made joint efforts essential in solving some of the encountered challenges. The help I received from various people was therefore highly appreciated. I am grateful to the following people for their contributions - which ranged from direct co-operation on certain chapters described in this thesis via technical assistance and scientific discussions to nice chats next to the coffee machine (which often ended up with tips that saved me days of work). Thanks a lot Eva D., Hans T., Roellie, Jan K., Wim v. G., Charles, Durrita, Jaqueline, Henk, Jean, Wim E., Peter d. K., Jan v. R., Jildert, Catrienus, Fedde, Marja T., Jeroen W., Jan H., Jan d. W., Jolanda, Bert, Zeger, Rita, Patrick, Marjo, Saskia, Tanja, Peter B., Ronnie, Marke, Anne, Roger, Ingrid, Jeroen H., Eddie, Douwe, Bas, Rob, Marieke, Sander, Arno, Mark S., Matthé, Frank d. B., Philippe, Igor M., Stijn, Jan R., Annereinou, Eline, Mariela, Veszela, Maria, Mark v. d. G., Leonie, Michiel W., Roland, Juma, Kerstin, Cindy, Sacha, Christof, Frank W. and Petra. People are not listed in a particular order and I apologize to anyone I forgot.

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About the Author

Herwig Bachmann (1973) was born in Lienz, Austria and grew up in a small village on the Austrian-Italian border. After finishing his High-school/Agricultural College education he spent over two years travelling and doing various jobs including the seasonal self employment in a small scale alpine cheese making business. In October 1995 he started studying Food Sciences and Biotechnology at the University of Agricultural Sciences (BOKU) in Vienna. Internships took him to a dairy company in Australia as well as to a DLO institute in the Netherlands where he investigated the microbiological degradation of feedstuff in the rumen. The last year of his degree he spent on his master thesis, which was carried out at the Centre of Applied Genetics (BOKU, Vienna). Within a plant-pathogen interaction group he developed a yeast-based bioassay for the detection of mycotoxins that have estrogenic activity. After obtaining his university degree in January 2002 he moved to the Netherlands where he started as a Junior Scientist in the biotechnology startup company Galapagos Genomics. There he worked on the discovery of novel drug targets within the disease areas psoriasis and Alzheimer's disease. In February 2004 he started working for NIZO Food Research. The project he worked on was aimed at better understanding the behavior of *Lactococcus lactis* during the fermentation of milk, which resulted in this thesis. Currently he is working at the Centre for Integrative Bioinformatics (Faculty of Earth and Life Sciences, VU University of Amsterdam). Herwig is married to Rebecca and has three children – Jacob (5), Samuel (3) and Juno (1).

List of Publications

Bachmann H, Molenaar, Kleerebezem M, van Hylckama Vlieg JE. High localized substrate availability stabilizes a cooperative trait. Submitted for publication

Bachmann H, Starrenburg MJC, Molenaar D, Kleerebezem M, van Hylckama Vlieg JE. Microbial adaptation to environmental niches can be reproduced by experimental evolution. Submitted for publication

Bachmann H, de Wilt L, Kleerebezem M, van Hylckama Vlieg JE. Gene expression analysis of *Lactococcus lactis* during the manufacturing and ripening of cheese. Submitted for publication

Bachmann H, Kruijswijk Z, Molenaar D, Kleerebezem M, van Hylckama Vlieg JE. A high throughput cheese-manufacturing model for effective cheese starter culture screening. Submitted for publication

Bachmann H, Starrenburg MJC, Dijkstra A, Molenaar D, Kleerebezem M, Rademaker JLW, van Hylckama Vlieg JE. Regulatory phenotyping reveals important diversity within the species *Lactococcus lactis*. Appl Environ Microbiol. In Press.

Siezen R and Bachmann H, Genomics of dairy fermentation.
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Bachmann H, Kleerebezem M, van Hylckama Vlieg JE.
High throughput identification and validation of in situ expressed genes in *Lactococcus lactis*. Appl Environ Microbiol. 2008 Aug;74(15):4727-36

Bachmann H, Kruijswijk Z, van Hylckama Vlieg JET. Method for high-throughput cheese making. Patent WO2008153387;

Bachmann H, Santos F, Kleerebezem M, van Hylckama Vlieg JE. Luciferase detection during stationary phase in *Lactococcus lactis*. Appl Environ Microbiol. 2007 Jul;73(14):4704-6.

Poppenberger B, Berthiller F, Bachmann H, Lucyshyn D, Peterbauer C, Mitterbauer R, Schuhmacher R, Krska R, Glössl J, Adam G.; Heterologous expression of Arabidopsis UDP-glucosyltransferases in *Saccharomyces cerevisiae* for production of zearalenone-4-O-glucoside. Appl Environ Microbiol. 2006 Jun;72(6):4404-10.

van Hylckama Vlieg JE, Rademaker JL, Bachmann H, Molenaar D, Kelly WJ, Siezen RJ. Natural diversity and adaptive responses of *Lactococcus lactis*. Curr Opin Biotechnol. 2006 Apr;17(2):183-90.

Cone, JW, Van Gelder, AH, Bachmann, H., Hindle, VA. (2002) Comparison of organic matter degradation in several feedstuffs in the rumen as determined with the nylon bag and gas production techniques. Anim. Feed Sci. Technol. 96, 55-67.

Cone JW, Van Gelder AH, Bachmann, H. (2002) Influence of inoculum source on gas production profiles. Anim Feed Sci Technol 99, 221-231

Overview of Completed Training Activities

Discipline specific activities

- Genetics and physiology of food associated microorganisms (VLAG), Wageningen, The Netherlands, 2004
- Nutracells meeting, Wageningen, The Netherlands, 2005
- 1st FEBS Advanced Lecture Course: "Systems Biology: From Molecules and Modelling to Cells",
  Gosau, Austria, 2005
- LAB 8 congress, Egmond aan Zee, The Netherlands, 2005
- Netherlands bioinformatic congress (NBIC), Ede, The Netherlands, 2006
- International symposium of microbial ecology 2006 , Vienna, Austria, 2006
- Benelux Bioinformatics Congress (BBC), Wageningen, The Netherlands, 2006
- Food summit WCFS, Arnheim, The Netherlands, 2006
- GUT Microbiota in Health and Disease, 2nd International Workshop , Amsterdam, The Netherlands, 2007
- Gordon Research Conference on Microbial Population Biology, Andover, USA, 2007
- 5th NIZO dairy conference, Papendal, The Netherlands, 2007
- NBC congress, Ede, The Netherlands, 2008
- LAB 9 congress, Egmond aan Zee, The Netherlands, 2009
- 1st- 6th Kluyver symposium, 2004-2009

General activities

- Patent course, Delft, 2006
- Project leader course, Ede, 2006
- Commercial skills, Ede, 2008
- Masterclass Marine Microbial Diversity, Amsterdam, The Netherlands, 2008

Optionals

- Preparation PhD research proposal
- Genomics Momentum, NGI, Amsterdam, The Netherlands, 2007
- Weekly group meetings, Molecular biology group, NIZO Food Research, 2004-2009
- Monthly group meeting, Division Flavor, NIZO food research, 2004-2009

Cover design
Herwig Bachmann

The cover shows the emission of light by 96 MicroCheeses manufactured with genetically engineered lactococci. Each square field within the image represents one MicroCheese. Individual cultures used for the manufacturing of each MicroCheese harbor a genetic construct in which the luciferase genes (*luxAB*) are controlled by various lactococcal promoter regions (DNA regions that are responsible for the activity of a particular gene). These constructs lead to the emission of visible light upon promoter activity. High levels of promoter activity can be recognized by higher light intensities. The image was taken ~12 hours after the cheese making process was started (~6 hours after pressing the curds). The 16 panels of the cover page show the identical image, which was digitally manipulated to show different color schemes/contrasts. See Chapter 6 of this thesis for more details on gene expression analysis in cheese.

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