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Genomics update

Genomics of dairy fermentations

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Introduction

Microbes are used in the process of making industrial and artisanal fermented dairy products, such as cheese, yogurt, sour cream and fermented milks (Fig. 1). These microbes are predominately lactic acid bacteria (LAB), such as lactococci, lactobacilli and streptococci. For quality and consistency, industrial production requires the use of starter cultures, which are very carefully created, cultivated and maintained (Fig. 2). What happens in the fermentation process? Milk sugars (mainly lactose) are fermented with the major final product being lactic acid. Lactic acid not only inhibits the out-growth of other organisms but also lowers the pH of the food product. Taste and texture, the feeling of food in your mouth, is also important. Lactic acid bacteria make the specific end-products that impart flavour and modify the texture of the final product. Cheese production makes use of predominantly *Lactococcus lactis*. It is the major component of cheese starter cultures and, as the worldwide cheese market is huge, it is one of the most important microbes for the food industry. Several of the important functions for fermentation are encoded on conjugative plasmids in these bacteria, among them lactose metabolism and the breakdown of milk proteins during cheese production (Siezen *et al*., 2005; Shearman *et al*., 2008). The lactobacilli are also important players in dairy fermentations with *Lactobacillus bulgaricus* mainly used in yoghurt manufacture, together with *Streptococcus thermophilus*. This use of microbial consortia adds yet another degree of complexity to an already complex production process.

There are now over 20 genomes of LAB published and annotated, providing insight into their metabolic capabilities, as reviewed in (Pfeiler and Klaenhammer, 2007; Mayo *et al*., 2008). Comparing these genomes for shared or unique genotypes is a start, but the world of dairy fermentation is not content just with comparison. The real questions that are being asked are: what makes my yogurt or cheese different, and how can I develop new flavours, textures and products? This is increasingly being investigated by natural diversity analysis of microbes, and *in situ* omics measurements in dairy products. These and many other studies were reported at the 9th Symposium on LAB: Health, Evolution and Systems Biology, held in September 2008 (http://www.lab9.nl). Here we highlight some of the latest developments in genomics in these areas.

Fig. 1. From milk to fermented dairy product.

Genome sequencing and diversity

An overview of genome sequences of some of the microbes used in dairy fermentations is given in Table 1. The most recent additions are *Lactobacillus helveticus* DPC4571, a starter/adjunct culture with traits that are extremely desirable in Swiss cheese production, which include autolysis, reduced bitterness and enhanced flavour development (Callanan *et al*., 2008), and also the industrially important plasmid pLP712 of *L. lactis*, encoding lactose catabolism and proteolytic enzymes (Shearman *et al*., 2008). *Propionibacterium freudenreichii*

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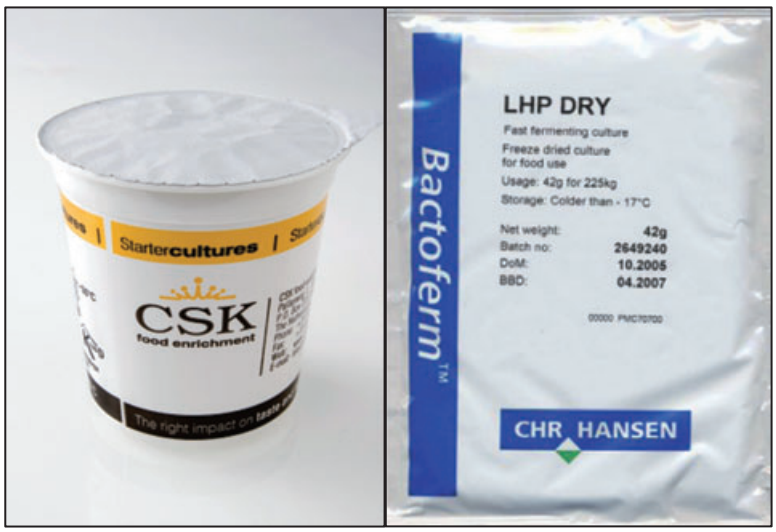

Fig. 2. Starter cultures for dairy fermentations.

ssp. *shermanii* CIP 103027 is a member of the dairy propionibacteria, commonly isolated from cheese and other dairy products, and is important for the development of flavour and the characteristic holes formed by CO_2 in Emmental (Swiss-type) cheese (Dherbecourt *et al.*, 2008). *Brevibacterium linens* BL2 is used as an adjunct culture in the ripening stage of soft cheddar-type cheese production. It produces an enzyme that converts L-methionine into methanethiol, an important aromatic component of the cheddar cheese aroma. Multiple strains have now been sequenced of *L. lactis*, *S. thermophilus*, *Lb. delbrueckii*, *Lb. helveticus* and *Lb. casei* (Table 1), providing deeper insight into their genomic diversity. A pangenome sequencing analysis of 11 strains of *S. thermophilus* has identified 65 kb DNA in regions > 1.5 kb not previously found in the three sequenced genomes (Danielsen and Rasmussen, 2008).

The diversity of LAB natural isolates is being studied by comparative genome hybridization (CGH) using microarrays based on a single reference genome, e.g. for *Lb. casei* (Cai *et al.*, 2008) and *Lb. helveticus* (Broadbent *et al.*, 2008), or multiple reference genomes, e.g. *S. thermophilus* (Rasmussen *et al.*, 2008) and *L. lactis* (Ganesan *et al.*, 2008) (G. Felis, pers. comm.). A novel genotype-calling algorithm PanCGH has been developed to analyse these pangenome arrays, and this has been applied to *L. lactis* strains (Bayjanov *et al.*, 2008).

Genome mining

Genome sequence analysis can provide the first insight into metabolic potential. An excellent, albeit older, example is the prediction that *L. casei*, a non-starter LAB that increases in later stages of cheese ripening, has the potential to use citrate as an alternative energy source when lactose has been depleted (Diaz-Muniz *et al.*, 2006).

A putative complete citric acid cycle (TCA) was reconstructed from the genome sequence, and experimentally shown to be active under simulated cheese ripening conditions, converting citrate mostly to acetic acid instead of lactic acid, yielding 2 ATP per molecule of citric acid.

The potential to form flavours from amino acids was compared in all sequenced LAB by searching their genomes for enzymes involved in proteolysis and amino acid conversions (Liu and Siezen, 2006; Liu *et al.*, 2008). Focusing on enzymes involved in metabolism of the sulfur-containing amino acids methionine and cysteine, which are known precursors of many dairy flavours, the largest set of enzymes was found in typical dairy LAB such as *L. lactis*, *S. thermophilus* and *Lb. casei*. The genome sequence of *Lactobacillus helveticus* DPC4571 (Callanan *et al.*, 2008) revealed a number of formerly unknown endopeptidases with potential roles in hydrolysis of proline-rich caseins and bitter peptides. These peptidases were cloned, overexpressed and further characterized with synthetic peptide substrates and in a cheese model system (Slattery *et al.*, 2008). Amino acid auxotrophy in *Lb. helveticus* CNRZ32 was predicted from its genome sequence, and agreed well with phenotypic amino acid requirements (Christiansen *et al.*, 2008).

Lipolysis of milk fat also contributes to flavour formation in cheese. By combining several bioinformatics methods, 23 putative esterases for lipolysis were identified in the genome of *Propionobacterium freudenreichii* CIP103027, the main agent for lipolysis in Emmental cheese (Dherbecourt *et al.*, 2008). Twelve of these putative esterases were selected and expressed in *E. coli*, of which six showed esterase activity on short-chain napthyl esters, thereby confirming the efficiency of genome mining.

The putative transport capabilities of eleven Gram-positive bacteria, including the dairy LAB *Lb. casei, L. lactis, Lc. mesenteroides, Lb. delbrueckii* and *S.*

Table 1. Microbial genome sequencing relevant to dairy fermentations.

Species and strain	Subspecies/strain	GenBank code	Size (kb)	GC%	Origin/use	Reference
Lactococcus lactis	*lactis*/IL1403	NC_002662	2365	35	Cheese	Bolotin *et al.* (2001)
	cremoris/SK11	NC_008527	2641	35	Cheese	Makarova *et al.* (2006)
	cremoris/MG1363	NC_009004	2530	35	Cheese	Wegmann *et al.* (2007)
Streptococcus thermophilus	CNRZ1066	NC_006449	1796	39	Yoghurt, cheese	Bolotin *et al.* (2004)
	LMG18311	NC_006448	1797	39	Yoghurt, cheese	Bolotin *et al.* (2004)
	LMD9	NC_008532	1864	39	Yoghurt, cheese	Makarova *et al.* (2006)
	11 strains					Danielsen and Rasmussen (2008)
Lactobacillus delbrueckii	*bulgaricus*/ATCC11842	NC_008054	1865	49	Yoghurt	van de Guchte *et al.* (2006)
	bulgaricus/ATCC-BAA365	NC_008529	1857	49	Yoghurt	Makarova *et al.* (2006)
Lactobacillus helveticus	DPC4571	NC_010080	2081	38	Cheese	Callanan *et al.* (2008)
	CNRZ32	n.a.	~2280	37	Cheese	J.Broadbent *et al.*, Utah State University, USA
	R0052	n.a.	n.a.	n.a.	Dairy	J.Broadbent *et al.*, Utah State University, USA
	CM4	n.a.	2028	n.a.	n.a.	Calpis; Kitasato University, Japan
Lactobacillus casei	BL23	NC_010999	3079	n.a.	Cheese	J Deutscher *et al.*, Centre National de la Recherche Scientifique, France
	ATCC334	NC_008526	2924	46	Cheese	Makarova *et al.* (2006)
	casei/ATCC393	n.a.	~3000	n.a.	Cheese	M. Hatori, University of Tokyo, Japan
Leuconostoc mesenteroides	*mesenteroides*/ATTC8293	NC_008531	2075	37	Olives	DOE Joint Genome Institute, USA
Brevibacterium linens	BL2	AAGP01000000	4366	63	Cheese	DOE Joint Genome Institute, USA
Propionibacterium freudenreichii	*shermanii*/CIP103027	n.a.	~2500	67	Cheese	Dherbecourt *et al.* (2008)
	ATCC6207	n.a.	~2640	67	Cheese	DSM and Friesland Foods, the Netherlands

n.a., not available, either proprietary or incomplete.

thermophilus, has been predicted using extensive comparative genome analysis (Lorca *et al*., 2007). This study has provided detailed information of the potential uptake systems for carbohydrates, peptides and amino acids in each species, as classified according to TCDB, the membrane transport protein classification database (http://tcdb.ucsd.edu).

One of the most exciting and useful aspects of having full-genome sequences is the ability to construct genome-scale metabolic models. They enable input and output fluxes, ATP production, growth rate, biomass yields and product formation to be predicted, and then experimentally tested (for LAB examples see Smid *et al*., 2005; Notebaart *et al*., 2006; Teusink *et al*., 2006). New genome-scale models have now been made for *S. thermophilus* (Pastink *et al*., 2008), and the pangenome (multiple strains) of *L. lactis* (Wels *et al*., 2008). Individual genome-scale models of the three sequenced *L. lactis* strains have been reconstructed using Pathway Tools and the BioCyc database (Ganesan *et al*., 2008) (http://www.biosystems.usu.edu/cibcyc).

Experimental omics

In situ *transcriptome analysis*

Most of the omics data related to dairy fermentations has been obtained from *in vitro* experiments, which were designed to mimic a dairy product environment (Kok *et al*., 2005; Neves *et al*., 2005; Kilstrup, 2006). Experimental data obtained from the product environment are limited. The major problem is that dairy environments such as fermented milk and especially cheese have a very rich protein and fat content. This makes the isolation of bacterial RNA, proteins or metabolites extremely difficult. In a recent study, the transcriptome profile of *L. helveticus* CNRZ32 grown in milk was compared with growth in a defined medium (Smeianov *et al*., 2007). The milk isolate had 42 upregulated genes, encoding cell-envelope proteinases, oligopeptide transporters, endopeptidases and enzymes involved in lactose, cysteine and purine metabolism. A DNA microarray time series was analysed during the first 20 h of a batch fermentation of *L. lactis* in milk (De Jong *et al*., 2008). The data were used to reconstruct gene regulatory networks and revealed a number of unknown regulons and DNA motifs in the genome of *L. lactis*.

Recently, the first methodological studies on the extraction of RNA directly from cheese (Monnet *et al*., 2008a), or by separation of bacterial cells from cheese before RNA isolation were reported (Makhzami *et al*., 2008; Ulvé *et al*., 2008). An alternative approach was developed to follow gene expression directly in cheese using recombinant *in vivo* expression technology (R-IVET). R-IVET is not dependent on RNA isolation but it rather 'records' *in situ* promoter activity throughout the incubation period by the irreversible excision of a marker fragment from the genome. Genome-scale analysis of *in situ* gene expression was developed for *L. lactis*, and allowed the identification and validation of positively regulated promoters in a product environment (Bachmann *et al*., 2008a). For the evaluation of *in situ* activated target sequences a high-throughput, cheese-manufacturing model, termed Micro-Cheese, was developed (Bachmann *et al*., 2008d). This MicroCheese system in combination with the R-IVET toolbox was used to identify and validate *L. lactis* promoters induced during the manufacturing and ripening of a Gouda-type cheese made with a mixed starter culture (Bachmann *et al*., 2008b).

In situ *proteomics and metabolomics*

Hannon and co-workers described the preparation of an aqueous phase of cheddar cheese and the subsequent separation of bacterial proteins from milk proteins by affinity chromatography and gel filtration (Hannon *et al*., 2008). Proteome analysis identified bacterial proteins from cheese manufactured with pure cultures of either *S. thermophilus* or *L. lactis* but also from cheeses manufactured with a mixed culture of both strains. The analysis showed that many genes involved in stress response and energy generation were upregulated during the cheese fermentation. Yvon and co-workers separated bacterial cells from the cheese matrix, determined the activity of eight flavour-forming enzymes and investigated the proteome and metabolome of the cell extracts (Yvon *et al*., 2008). Minor differences were found in the proteome between 1 and 7 days after cheese manufacturing, but important differences were seen in bacterial metabolites.

Bacterial interactions in dairy consortia

The impact of genomic approaches on the elucidation of microbial interactions was reviewed recently (Sieuwerts *et al*., 2008a). Current developments in the dairy environment include transcriptome and proteome studies on mixed cultures of *S. thermophilus* and *L. bulgaricus* in milk (Monnet *et al*., 2008b; Sieuwerts *et al*., 2008b). This bacterial consortium represents a typical yoghurt culture, and the results reveal new insights into interactions between the two bacteria (Fig. 3). The measurement of volatile bacterial metabolites in mixed-culture dairy fermentations may also permit the identification of bacterial interactions (Janssen *et al*., 2008).

Evolutionary aspects of dairy fermentation

A comparison of nine genome sequences of LAB revealed extensive gene loss and horizontal gene transfer during

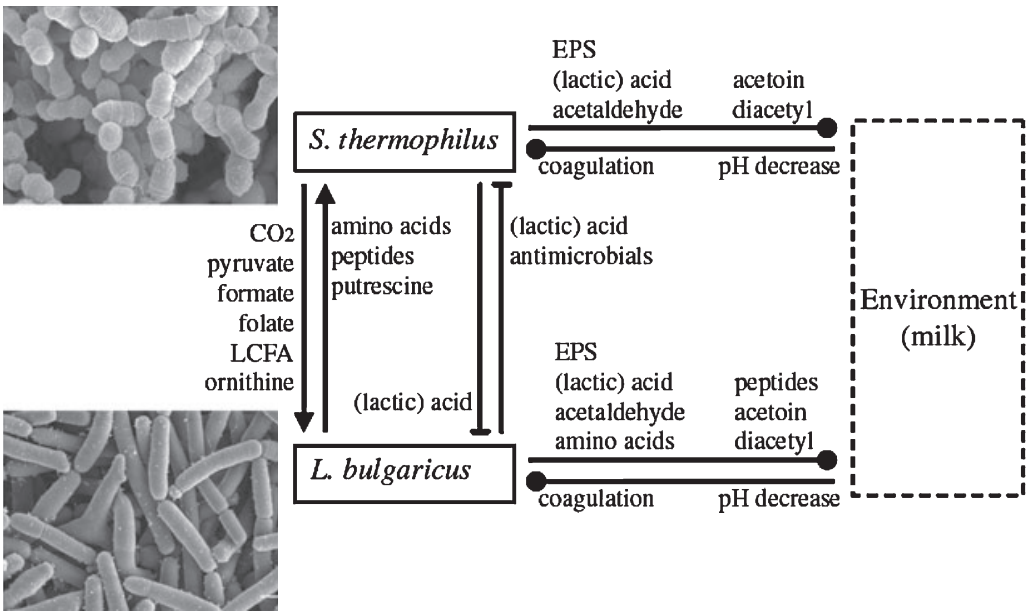

Fig. 3. Microbial interactions in yoghurt (adapted from Sieuwerts *et al*., 2008a). Reprinted with permission from the American Society for Microbiology.

the evolutionary adaptation to their habitat (Makarova *et al*., 2006). Evolutionary genomic studies of LAB pointed to a substantial gene loss especially in the *Lactococcus–Streptococcus* branch (Makarova and Koonin, 2007). Gene loss in relation to dairy niche adaptation was reported for *L. lactis, Lb helveticus* and the yoghurt bacteria (Bolotin *et al*., 2004); van de Guchte *et al*., 2006; Callanan *et al*., 2008; Siezen *et al*., 2008). The genome sequences of *S. thermophilus* and *Lb. bulgaricus* revealed that > 10% of all potential coding sequences are pseudogenes, indicating that evolutionary processes to adapt to the dairy environment are still very actively ongoing (Bolotin *et al*., 2004; van de Guchte *et al*., 2006). Loss of genes for carbohydrate metabolism and amino acid biosynthesis in *Lb. bulgaricus* reflect an adaptation to the protein-rich milk environment.

Most studies with *L. lactis* were carried out with strains isolated from the dairy environment. The diagnostic sequencing of two *L. lactis* plant isolates has now shown that these strains contain many genes never before reported as part of the genome of *L. lactis*. These genes are mainly involved in the utilization of complex carbohydrates, which typically occur in plant material (Siezen *et al*., 2008). In a follow-up study, one of these plant isolates was adapted to growth in milk by propagating it for 1000 generations in milk. Three independently evolved strains were extensively characterized and reveal interesting insights into evolutionary aspects of this adaptation process (Bachmann *et al*., 2008c).

The acquisition of new genes via horizontal gene transfer has been proposed for several dairy specific LAB (Bolotin *et al*., 2004; Siezen *et al*., 2005; Makarova and Koonin, 2007; Callanan *et al*., 2008), and includes transfer between *S. thermophilus*, *L. lactis* and *Lb. bulgaricus* (Bolotin *et al*., 2004). Recently, a genomic island of 100 kb, with deviant GC content and flanked by IS elements, was found in the genome of *L. helveticus* DCP4571, and included fatty acid and amino acid metabolism genes (Callanan *et al*., 2008). One mechanism of horizontal gene transfer is the phage-mediated transduction of DNA. Recently, it was shown for the first time that this mechanism allows the transfer of plasmids from the genus *St reptococcus* to the genus *Lactococcus* (Ammann *et al*., 2008). As bacteriophages can cause cell lysis, they can have a big impact on the performance of starter cultures and they are responsible for substantial financial losses to the dairy industry. Resistance to phage infection can be conferred by CRISPRs (clustered regularly interspaced short palindromic repeats), which are variable repeats separated by DNA spacers found in the genomes of many prokaryotes, including LAB (Barrangou *et al*., 2007). A recent comparative genome analysis identified 66 CRISPR loci in LAB (Horvath *et al*., 2008a). A poor correlation of CRISPR families with bacterial phylogeny supports the notion that CRISPRs are acquired via horizontal gene transfer and have further evolved independently. This evolution is mainly determined by phage predation and it forms an important part of the ecology between phages and their hosts. CRISPR sequences were further studied in *S. thermophilus* (Horvath *et al*., 2008b) and it was shown that they are responsible for increased phage resistance achieved by successive

phage challenges (Deveau *et al*., 2008). It is suggested that the directed evolution of strains with multiple phage resistances should be possible, which forms an attractive approach for stabilizing industrial fermentation processes.

When a new process or product is being developed in an industrial setting, the initial stages involve setting up small-scale experiments and then a small-scale pilot plant to mimic the industrial environment. Intelligent use of genomics data should give a competitive edge as it can provide detailed information on the spatio-temporal aspects of the process. It is no surprise then that the number of omics studies performed in a product-like environment is rapidly increasing. A comparison of the data is difficult as most studies use different bacterial strains or methodologies, but the principle discoveries will form the basis of detailed descriptions as to what is happening in these complex environments. It is beyond doubt that the elucidation of the *in situ* behaviour of bacterial cultures in the post-genomics era will lead to a better insight into dairy fermentations and help to improve industrial fermentation processes.

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