

Chapter 2

Genomics and Functional Role of Actinomycetes on Smear Ripened Cheeses

Christophe Monnet, Nagamani Bora, Françoise Irlinger, and Alan C. Ward

Abstract Cheese organisms are derived from the dairy environment, from humans and from the environment. Milk and its evolution provide the selective environment for growth of cheese organisms but salt is the major additional selective pressure imposed upon the smear cheese surface. Perhaps the evolutionary history of the actinobacterial phylogenetic clade as “Terrabacter” and selection for resistance to desiccation on aerobic surfaces has provided species able to exploit these environments. But human selection over the history of cheese-making may have selected cheese-microorganism consortia adapted to produce desirable cheese properties, including the ability to outgrow contaminating microorganisms on the cheese-surface and modify the organoleptic properties. The properties of some of the characteristic cheese-related organisms relevant to cheese such as iron availability, catabolism, bacteriocins, osmotolerance and proteolysis of casein are described and compared to related species from other environmental habitats from a genomics perspective.

Keywords Actinomycetes • Genomics • Bacteriocins • Functional traits

2.1 Evolution of *Actinomycetes* Influencing Smear Cheeses

Cheese is an ancient food, probably at least 8,000 years, first mentioned by the Sumerians and documented by the ancient Egyptians, Greeks and Romans (Fox and McSweeney 2004). Recent genome sequencing demonstrates vividly how

C. Monnet (✉) • F. Irlinger
UMR782 GMPA, INRA AgroParisTech,
Avenue Lucien Brétignières, 78850 Thiverval-Grignon, France
e-mail: monnet@grignon.inra.fr

N. Bora
School of Pharmacy, Faculty of Health and Life Sciences,
De Montfort University, Leicester, UK
e-mail: n.bora@outlook.com

A.C. Ward
School of Biology, Newcastle University,
Baddiley Clark Building, Newcastle upon Tyne NE17RU, UK

microorganisms have co-evolved with man and his environment. This can be seen in the molecular record, for example in *Mycobacterium tuberculosis* (Hershberg et al. 2008). Although the molecular record for cheese organisms is less compelling, in lactobacilli there is evidence of the same co-evolution but this time between the organism and selective pressure imposed by man through practice, and their evolution in the dairy environment. Oftedal (2012) describes the evolution of milk whereas Nagpal et al. (2011) describes the interaction of milk with the mammalian gut where lactobacilli also play a role.

Phylogenetic analysis of *Lactobacillus casei* indicates three major clusters, splitting about 1.5 million years ago, but with a more recent cheese-related cluster showing gene decay and deletion of carbohydrate utilisation genes (Cai et al. 2007) corresponding to the introduction of cheese approximately 8,000 years ago (Fox and McSweeney 2004). Similarly *Lactobacillus sanfranciscensis* is seen as a 5,000 year adaptation to sourdough production (Vogel et al. 2011). In *Arthrobacter arilaitensis* Re117, which has only been isolated from raw milk (Mallet et al. 2012) and from the surface of cheese, the chromosome is much smaller than in *Arthrobacter* strains of soil origin (3.9 Mbp versus more than 5.0 Mbp), as a consequence of an extensive loss of genes (Monnet et al. 2010). Genes for the transport and catabolism of many substrates are absent, probably owing to the limited substrates present in cheeses.

The identification of cheese-specific organisms implies some continuity of transmission through cheese-making practice and selection by market forces, which operate for the cheese-making organisms and for the secondary flora on smear cheeses. Mounier et al. (2006) document the interaction between cheese, the cheese-workers and the house flora and it was presumably the cheese-workers who would have carried organisms from successful cheese-making enterprises to new locations. The rapidity with which such an unintentional process can take place was highlighted by the rapid cross-contamination of cell cultures across the world with HeLa cells (Lucey et al. 2009).

The surface of smear cheeses is characterised by diverse flora that include yeasts and Gram positive and negative bacteria in a dynamic successional population. However, actinomycetes, such as *Brevibacterium linens*, have been seen as typical, leading, for example, to their inclusion in smear starter cultures.

The actinomycetes are the major clade, with the majority of described species, within the *Actinobacteria*, a major evolutionary line in the 16S rDNA phylogenetic tree (Ward and Bora 2008). They are characterised as Gram positive, high GC bacteria, however, the members of this major taxon are defined phylogenetically (Stackebrandt et al. 1997; Zhi et al. 2009; Gao and Gupta 2012) and are morphologically and physiologically diverse.

Battistuzzi et al. (2004) identified 32 proteins present, in common, across 72 genomes of prokaryotes and eukaryotes and have performed phylogenetic analysis of the concatenated and aligned dataset, using a local clock method, and dated the evolutionary nodes. Their analysis groups three phylogenetic lines, Actinobacteria, Deinococcus-Thermus and Cyanobacteria, into a major lineage –although the

relationships between major clades remain difficult to determine and different whole genome methods derive differing trees (Yu et al. 2005; Henz et al. 2005; Kunin et al. 2005; Wang and Caetano-Anolles 2006). Battistuzzi et al. (2004) identified common features of resistance to desiccation (Potts 1994) and the synthesis of photo-protective compounds (Wynn-Williams et al. 2002) evolved with the first colonisation of the land and the evolution of oxygenic photosynthesis. This exposed environment and the evolution of oxygen in plant-like photosynthesis by the cyanobacteria must have imposed new stresses on these proto-actinobacteria. This analysis puts the common ancestor of actinobacteria as one of the “Terrabacter” (Battistuzzi et al. 2004; Battistuzzi et al. 2009), and the evolutionary forces that have shaped the actinobacterial clade as the invasion of the land perhaps 3 billion years ago – although the evolutionary radiation we perceive as the actinomycetes probably only dates back to half that.

In the Dry Valleys of the Ross Desert, Antarctica, an extreme, dry environment (Nienow and Friedmann 1993) cyanobacteria dominate three of the five cryptoendolithic communities found in the sandstone rocks, also postulated as descendants of the “Terrabacter” (Battistuzzi et al. 2004) and the group most studied for desiccation resistance (Potts 1994). But in the lower zone of the communities, actinomycetes *Micrococcus roseus*, *Brevibacterium* sp., and *Arthrobacter* sp., as well as *Deinococcus radiopugnans*, are present. The phyla Actinobacteria and Bacteroidetes appear to be widespread in Antarctica whereas others, such as Acidobacteria, Proteobacteria, *Deinococcus-Thermus*, Firmicutes and Cyanobacteria, are less widely dispersed (Smith et al. 2006; Yergeau et al. 2010).

Actinomycetes also seem to be common isolates on surfaces of buildings, from deteriorating walls and stone (Scheerer et al. 2009; Schäfera et al. 2010) and, after cyanobacteria and algae, on cave surfaces (Cañveras et al. 2001), including rock paintings. But they are also often isolated from wall paintings (Pepe et al. 2010).

More relevantly, for cheese microbiology, they are common members of the microbial communities found on skin, especially, since it is most studied, human skin (Grice and Segre 2011). Actinobacteria probably predominate on areas of the skin rich in sebaceous glands rather than exposed dry areas of skin. So actinomycetes may be adapted, in some way, to growth in surface exposed biofilms in which oxygen, light and low water availability (desiccation and osmotic stress) impose stress, but clearly not all actinomycetes or only actinomycetes are able to survive under these conditions.

Using 98 actinobacterial genomes, fully assembled in 2010, Gao and Gupta (2012) searched for molecular signatures unique to the actinobacteria: most of these were indels, specific sequences of amino acids inserted or deleted in specific genes. The physiological or evolutionary significance of these conserved changes are not known. In addition they searched for signature proteins; they found 24, 19 of which are hypothetical, the remaining five include *whiB* (Zheng et al. 2012) whose pleiotrophic regulatory effects on growth and differentiation remain with no comprehensive mechanistic explanation, ParJ, involved in chromosome segregation, LpqB, involved in cell wall biosynthesis, and a septation inhibitor protein.

The surface of smear cheeses is a challenging environment, an exposed surface subject to light, oxygen and osmotic stress as well as specific nutritional demands. Such selection pressure may lead to selfish and co-operative behaviour of microflora (Mora et al. 2013). Members of the actinomycetes are found in such environments but genome analysis is not able to identify unique characteristics enabling them to colonise such environments.

2.2 Genomics and Functional Aspects of Some Smear Cheese Bacteria

Dairy bacteria are a heterogeneous group contributing to various dairy industrial applications, such as acid curd production, texture modification, and generation of cheese flavour and colour. The stated qualities are being better understood with a genomics approach. Whole genome sequencing is booming with there being nearly 68 genomes of bacteria isolated from dairy products that are sequenced. Currently, the genomes being sequenced involve mostly Lactic Acid Bacteria (LAB), which represent 80 % of the total number of sequenced dairy bacteria (Table 2.1). These LAB belong to 6 different genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Enterococcus*) and 24 species. The sequencing of these genomes has created unprecedented opportunities for evolutionary genomics of LAB (Makarova and Koonin 2007; Zhu et al. 2009). However, numerous other bacterial genera are present in ripened cheeses (e.g. *Corynebacterium*, *Microbacterium*, *Propionibacterium*, *Brevibacterium*, *Arthrobacter*, *Agrococcus*, *Mycetocola*, *Staphylococcus* ...), for which no or only a few genome sequences are available. Currently, the genome sequences of five bacteria involved in the ripening of smear cheeses are available: *Brevibacterium aurantiacum* ATCC 9174, *Corynebacterium casei* UCMA 3821, *Staphylococcus equorum* ssp. *equorum* Mu2, *Arthrobacter arilaitensis* Re117 and *Corynebacterium variabile* DSM 44702. For the two latter strains, which belong to major species found on the surfaces of smear cheeses, the genome sequences are complete and extensive genomic analyses have been performed (Monnet et al. 2010; Schröder et al. 2011). One of the main outcomes of these analyses was the determination of genetic determinants involved in the adaptation to the cheese habitat by comparing the genome sequences with those of taxonomically related microorganisms present in other habitats. For instance the whole genomes of *C. variabile* and *A. arilaitensis* Re117 that contain six copies of the *rrn* operon are associated with the ability for rapid growth. Rapid growth of both the cheese-making organism and the surface flora is necessary to outcompete potential contaminants that might be pathogens or confer off-flavours. A better knowledge of these determinants may be useful for the screening and selection of strains that can be used as ripening culture components. Indeed, it has been shown that ripening strains that are deliberately inoculated into cheese milk frequently do not establish themselves on the surface of smear cheeses (Brennan et al. 2002; Feurer et al. 2004b; Mounier et al.

Table 2.1 General features of the sequenced genomes of bacteria isolated from dairy products or used in dairy products

Species/strain	Genome size (Mb)	No. of contigs	No. of genes	GC content (%)	Origin or use	Institution	Sequence availability ^a	References
<i>Arthrobacter arilaitensis</i> Re117	3.86	1	3,518	59.2	Smear cheese, France	INRA, Grignon, France	Yes	Monnet et al. (2010)
<i>Bacillus cereus</i> ATCC 10987	5.20	1	6,126	38.0	Cheese spoilage, Canada	TIGR, Rockville, USA	Yes	Rasko et al. (2004)
<i>Brevibacterium aurantiacum</i> ATCC 9174 ^b	4.37	123	3,825	62.8	Smear cheese, Germany	JGI, Utah, USA	Yes	
<i>Corynebacterium bovis</i> DSM 20582	2.52	503	2,339	72.5	Milk	Bielefeld University, Germany	Yes	Schröder et al. (2012)
<i>Corynebacterium casei</i> UCMA 3821	3.11	106	2,844	55.3	Smear cheese, France	INRA, Grignon, France	Yes	Monnet et al. (2012)
<i>Corynebacterium variabile</i> DSM 44702	3.30	142	3,071	67.0	Smear cheese, Ireland	Bielefeld University, Germany	Yes	Schröder et al. (2011)
<i>Enterococcus italicus</i> DSM 15952	2.31	157	2,454	39.0	Cheese	BCM, HGSC, Texas, USA	Yes	
<i>Enterococcus lactis</i> CK1114	2.50				Cheese, Russia	INRA, Jouy en Josas, France		
<i>Hafnia alvei</i> GB001	4.90	137	4,704	48.6	Cheese, France	INRA, Grignon, France		
<i>Lactobacillus casei</i> ATCC 334	2.90	1	2,776	46.7	Hard cheese, Switzerland	JGI, Utah, USA	Yes	Makarova et al. (2006)
<i>Lactobacillus casei</i> BD-II	3.10	1	3,139	46.3	Koumiss, mare's fermented milk, China	State Key Laboratory of Dairy Biotechnology, Shanghai, China	Yes	Ai et al. (2011)
<i>Lactobacillus casei</i> BL23	3.08	13	3,044	46.3	Used in fermented milk, probiotic	INRA, Jouy en Josas, France	Yes	Mazé et al. (2010)
<i>Lactobacillus casei</i> DN-114001	3.14	1		46.3	Used in fermented milk, probiotic	Danone Vitapole, France		

(continued)

Table 2.1 (continued)

Species/strain	Genome size (Mb)	No. of contigs	No. of genes	GC content (%)	Origin or use	Institution	Sequence availability ^a	References
<i>Lactobacillus casei</i> LC2W	3.00	1	3,121	46.3	Traditional dairy products, China	State Key Laboratory of Dairy Biotechnology, Shanghai, China	Yes	Chen et al. (2011a)
<i>Lactobacillus casei</i> Shirota YIT 9029	3.04	1	3,044	46.3	Used in fermented milk, Japan	Yakult, Japan		
<i>Lactobacillus casei</i> Zhang	2.70	1	2,879	46.0	Koumiss, mare's fermented milk, China	School of Food Science and Engineering, Mongolia Agricultural University, China	Yes	Zhang et al. (2010)
<i>Lactobacillus coryniformis</i> subsp. <i>torquens</i> KCTC 3535	2.80	433	2,952	43.0	Air of dairy barn	KRIBB, Korea	Yes	
<i>Lactobacillus cypriacaei</i> KCTC 13900	2.30	760	2,267	39.0	Halloumi cheese, Cyprus	KRIBB, Korea	Yes	Kim et al. (2011a)
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	1.86	1	1,562	49.7	Yogurt	INRA, Jouy en Josas, France	Yes	Van de Guchte et al. (2006)
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC BAA-365	1.86	1	1,721	49.7	Cheese, yogurt	JGO, Utah, USA	Yes	Makarova et al. (2006)
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> DN-100 107	2.13	1			Dairy product, probiotic	Danone Vitapole, France		
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ND02	2.10	1	2,177	49.5	Fermented yak milk, China	State Key Laboratory of Food Science and Technology, Wuxi, China	Yes	Sun et al. (2011a)

<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> 2038	1.87	1	1,790	49.7	Used for yogurt production, Bulgaria	Key Laboratory of Systems Biology, Shanghai, China	Yes	Hao et al. (2011)
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> DSM 20072	1.88		2,048	49.8	Hard cheese	BCM, HGSC, Texas, USA	Yes	
<i>Lactobacillus fermentum</i> F6					Traditional dairy products, China	Key Laboratory of Food Nutrition and Safety, Tianjin, China		
<i>Lactobacillus fermentum</i> IFO 3956	2.10	1	1,915	51.0	Fermented plant material, Japan	School of Veterinary Medicine, Azabu University, Japan	Yes	Morita et al. (2008)
<i>Lactobacillus fructivorans</i> KCTC 3543	1.37	11	1,368	38.9	Air of dairy barn, starter culture	KRIBB, Korea	Yes	Nam et al. (2012)
<i>Lactobacillus helveticus</i> CM4	2.03	1	2,174	37.1	Starter culture of Calpis sour milk, Japan	Calpis, Kitasato University Japan		
<i>Lactobacillus helveticus</i> CNRZ 32	2.20	390		37.1	Cheese, starter culture	University of Wisconsin, USA		
<i>Lactobacillus helveticus</i> DPC 4571	2.08	1	1,830	37.0	Swiss Cheese, starter culture	Teagasc, Cork, Ireland	Yes	Callanan et al. (2008)
<i>Lactobacillus helveticus</i> DSM 20075	1.80	235	2,128	36.0	Hard cheese, Switzerland	BCM, HGSC, Texas, USA	Yes	
<i>Lactobacillus helveticus</i> H10	2.17	1	2,148	36.8	Traditional dairy products, China	Key Laboratory of Food Nutrition and Safety, Tianjin, China	Yes	Zhao et al. (2011)
<i>Lactobacillus helveticus</i> H9					Traditional dairy products, China	Key Laboratory of Food Nutrition and Safety, Tianjin, China		

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Table 2.1 (continued)

Species/strain	Genome size (Mb)	No. of contigs	No. of genes	GC content (%)	Origin or use	Institution	Sequence availability ^a	References
<i>Lactobacillus kefirifaciens</i> ZW3	2.10	1	1,908	37.7	Traditional dairy product Kefir, China	Key Laboratory of Food Nutrition and Safety, Tianjin, China	Yes	Wang et al. (2011)
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> ATCC 25302	2.90	174	3,100	46.0	Dairy product	BCM, HGSC, Texas, USA	Yes	
<i>Lactobacillus plantarum</i> JDM1	3.20	367	2,948	44.7	Starter culture, probiotic, China	Department of Medical Microbiology and Parasitology, Shanghai, China	Yes	Zhang et al. (2009)
<i>Lactobacillus plantarum</i> subsp. <i>plantarum</i> P8					Traditional dairy product, China	Key Laboratory of Food Nutrition and Safety, Tianjin, China		
<i>Lactobacillus rhamnosus</i> HN001	2.87	1	2,811	47.0	Cheddar cheese	Fonterra Research Centre, New Zealand	Yes	
<i>Lactobacillus rhamnosus</i> R0011	2.90	10	2,782	46.7	Dairy starter culture, probiotic	Institut Rosell Lallemand Inc., Genome-Quebec, Canada	Yes	Tompkins et al. (2012)
<i>Lactobacillus zeae</i> ATCC 393	2.92	2	2,865	46.6	Cheese	University of Tokyo, Japan		
<i>Lactobacillus zeae</i> KCTC 3804	3.10	113	2,965	47.8	Raw cow's milk, Korea	KRIBB, Korea	Yes	Kim et al. (2011b)
<i>Lactococcus garviae</i> TB25	2.01	92	1,977	38.1	Cheese, Italy	University of Milan, Italy	Yes	Ricci et al. (2012)
<i>Lactococcus garviae</i> IPLA 31405	2.05	23	1,874	38.5	Spanish raw-milk cheese	IPLA-CSIC, Spain	Yes	Florez et al. (2012)

<i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diacep/lactis</i> DPC 3901					Raw milk cheese	Teagasc, Cork, Ireland		
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> A76	2.40	1	2,719	35.9	Cheese, France	University of Groningen, The Netherlands	Yes	Wegmann et al. (2007)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363	2.53	1	2,517	35.8	Cheese strain NCDO712, plasmid-free	University of Groningen, The Netherlands	Yes	Wegmann et al. (2007) and Linares et al. (2010)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> NZ900	2.50	1	2,594		Strain MG1363 with nisRK genes	University of Groningen, The Netherlands	Yes	Linares et al. (2010)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> QA5	2.51	1		35.0	Dairy product	INRA, Jouy en Josas, France		
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> SK11	2.44	1	2,658	36.8	Cheese	JGI, Utah, USA	Yes	Makarova et al. (2006)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> IL1403	2.36	1	2,310	35.4	Cheese starter culture	INRA, Jouy en Josas, France	Yes	Bolotin et al. (2001)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> S3	2.48	163	2,487	35.0	Cheese, France	INRA, Grignon, France		
<i>Leuconostoc lactis</i> KCTC 3528		1,151			Milk	KRIBB, Korea		
<i>Listeria innocua</i> CLIP 11262	3.00	1	3,196	37.4	Cheese, Morocco	Institut Pasteur, France	Yes	Glaser et al. (2001)
<i>Listeria monocytogenes</i> serovar 4a strain M7	2.97	1	2,977	38.2	Cow's milk, China	Zhejiang University Institute of Preventive Veterinary Medicine, China	Yes	Chen et al. (2011b)
<i>Pediococcus pentosaceus</i> ATCC 25745	1.83	1	1,832	37.4	Fermentation starter culture	JGI, Utah, USA	Yes	Makarova et al. (2006)
<i>Propionibacterium acidipropionici</i> DSM 4900	3.59		3,371	68.8		JGI, Utah, USA		

(continued)

Table 2.1 (continued)

Species/strain	Genome size (Mb)	No. of contigs	No. of genes	GC content (%)	Origin or use	Institution	Sequence availability ^a	References
<i>Propionibacterium freudenreichii</i> ATCC 6207	2.64	12		67.4	Hard cheese, Switzerland	DSM Food Specialities, The Netherlands		
<i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i> CIRM BIA1	2.70	426	2,375	67.0	Hard cheese, Switzerland	INRA, Rennes, France	Yes	Falentin et al. (2010)
<i>Staphylococcus equorum</i> subsp. <i>equorum</i> Mu2	2.95	6	2,988	32.8	Smear cheese, France	INRA, Grignon, France	Yes	Irlinger et al. (2012)
<i>Streptococcus agalactiae</i> FSL S3-026	2.45	8	2,334	36.1	Bovine milk	College of Veterinary Medicine, Cornell University, USA	Yes	Richards et al. (2011)
<i>Streptococcus infantarius</i> subsp. <i>infantarius</i> CJ18	1.99	1	1,964	37.6	Fermented camel milk, Kenya	ETH, Swiss Federal Institute of Technology, Zurich	Yes	Jans et al. (2012)
<i>Streptococcus macedonicus</i> ACA-DC 198	2.14	1	2,297	37.6	Traditional kasseri cheese, Greece	Agricultural University of Athens, Greece	Yes	Papadimitriou et al. (2012)
<i>Streptococcus thermophilus</i> CNRZ 1066	1.80	1	1,915	39.1	Yogurt	INRA, Jouy en Josas, France	Yes	Bolotin et al. (2004)
<i>Streptococcus thermophilus</i> DN-001 147	1.82	1		39.1	Dairy product, probiotic	Danone Vitapole, France		
<i>Streptococcus thermophilus</i> JIM8232	1.90	1	2,145	38.9	Milk	INRA, Jouy en Josas, France	Yes	Delorme et al. (2011)
<i>Streptococcus thermophilus</i> LMD-9	1.91	1	1,715	39.1	Yogurt, starter culture	JGI, Utah, USA	Yes	Makarova et al. (2006)
<i>Streptococcus thermophilus</i> LMG 18311	1.80	1	1,892	39.1	Yogurt	Louvain University, Belgium	Yes	Bolotin et al. (2004)
<i>Streptococcus thermophilus</i> ND03	1.83	1	1,919	39.1	Fermented yak milk, starter culture, China	State Key Laboratory of Food Science and Technology, Wuxi, China	Yes	Sun et al. (2011b)

2006; Goerges et al. 2008). The understanding of genetic determinants specific to the cheese habitat may also provide clues to diminish the occurrence of pathogens or spoilage bacteria on smear cheeses. Whole genome comparisons of smear cheese bacteria are useful in understanding the interesting functional properties such as the production of pigments and aroma compounds or of undesired properties such as biogenic amine production.

2.2.1 *Influence of Thriving Habitat on Loss of Genes*

Genes that have no beneficial function tend to be eliminated, due to the energy required for their maintenance. These genes can be eliminated by recombination, which is favoured by the presence of mobile elements such as Insertion Sequences (ISs). Genes may also be inactivated by spontaneous mutations or by IS insertion. In comparison to soil-originating *Arthrobacter* strains, which are characterized by their ability to metabolize a large variety of substances, the *A. arilaitensis* Re117 chromosome is much smaller (3.9 Mbp *versus* more than 5.0 Mbp), as a consequence of an extensive loss of genes (Monnet et al. 2010). In particular, many genes involved in the transport and catabolism of substrates have been lost, probably as a result of the lower diversity of substrates present in cheeses. This was confirmed by analysis of specific protein families, such as the ABC-type sugar import components, which was three to four times lower in the cheese bacterium *A. arilaitensis* Re117. This strain had also a lower number (9 *versus* 21 to 30) of LacI-family regulators, which are frequently involved in the control of degradative pathways. The reductive evolution of *A. arilaitensis* Re117 from an environmental ancestor was confirmed by synteny plots, which revealed deletions of segments up to 70 kbp. A process of genome reduction has also been suggested for the lactic acid bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus* in the context of adaptation from a plant-associated habitat to the stable protein and lactose-rich milk environment (Van de Guchte et al. 2006).

The size of the chromosomes of the cheese bacteria *C. variable* DSM 44702 (3.4 Mbp) and *C. casei* UCMA 3821 (approximately 3.1 Mbp) is close to that of the soil bacterium *C. glutamicum* R (3.3 Mbp). However, comparative analyses showed that a large part of the *C. variable* DSM 44702 proteins had no homologous counterparts in the proteomes of corynebacteria from other environments (Schröder et al. 2011). The *A. arilaitensis* Re117 chromosome also carries numerous ISs, which represent nearly 5 % of the genome. Such IS expansion has been observed in several species, where it has been suggested that they are a consequence of a population bottleneck associated with a change of environmental habitats (Preston et al. 2004). A similar phenomenon was also observed for another cheese-associated bacterium, *Lactobacillus helveticus* DPC 4571 (Callanan et al. 2008).

2.2.2 Catabolism of Carbon Substrates Present in Cheeses

Genomic analyses showed that both *A. arilaitensis* Re117 and *C. variabile* DSM 44702 are well-equipped in genes involved in the transport and catabolism of substrates present in cheeses, which may be used to produce energy (Monnet et al. 2010; Schröder et al. 2012). Secreted proteolytic enzymes were identified, which probably contribute to the breakdown of caseins. Both strains have a bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase (PutA), which catalyses the oxidation of proline to glutamate using a membrane-bound quinone and NAD as electron acceptor. Since caseins are very rich in proline residues, this amino acid may constitute a prominent substrate. In addition, *A. arilaitensis* Re117 is able to use tyrosine and glutamate as growth substrate, which are two other main amino acids present in cheeses. The genomes of *A. arilaitensis* Re117 and *C. variabile* DSM 44702 encode proteins that convert gamma-aminobutyrate, a four-carbon non-protein amino acid known to occur in cheese (Siragusa et al. 2007), into succinate, an intermediate of the tricarboxylic acid (TCA) cycle. In contrast to *C. casei* UCMA 3821 and *C. variabile* DSM 44702, *A. arilaitensis* Re117 and *Staph. equorum* ssp. *equorum* Mu2 are able to catabolize lactose and galactose. However, it is not clear that this compound is a major substrate as it is exhausted at the beginning of ripening, before the growth of the acido-sensitive bacteria. Lactic acid, which is exhausted later during the ripening of smear cheeses, can be used as substrate by *A. arilaitensis* Re117, *B. aurantiacum* ATCC 9174, *Staph. equorum* ssp. *equorum* Mu2, *C. casei* UCMA 3821 and *C. variabile* DSM 44702, which have lactate transporters and lactate dehydrogenases. According to the genome annotation, *C. variabile* DSM 44702 and *A. arilaitensis* Re117 can channel propionate into the tricarboxylic acid cycle, but this substrate is not present in significant amounts in smear cheeses although recent studies (see Chap. 4) show that members of the genus *Propionibacterium* were detected on Livarot cheese from France. In comparison to *Arthrobacter* strains originating from soils, *A. arilaitensis* Re117 has a cluster of four genes (*dgoKADT*), which encode proteins involved in the catabolism of D-galactonate. This cluster results from a horizontal gene transfer as the closest orthologs are found in strains of Gram-negative species. To our knowledge, the presence of D-galactonate has never been investigated in cheeses. It may be hypothesized that in some varieties of cheeses, D-galactonate is produced transiently by oxidation from residual lactose or galactose, as described in some fungi (Elshafei and Abdel-Fatah 2001). This compound would then be utilized as a growth substrate by *Arthrobacter* species present in cheeses. Genes involved in the catabolism of citrate, a compound naturally occurring in milk, are present in *C. casei* UCMA 3821, *C. variabile* DSM 44702 and *A. arilaitensis* Re117. However, the latter strain is unable to grow with citrate as a carbon source, probably due to mutation of the CitAB two-component system. Genomic analyses of *C. variabile* DSM 44702 and *A. arilaitensis* Re117 also revealed the presence of numerous enzymes involved in the catabolism of lipids, including secretory triacylglycerol lipases, the characteristic enzymes of the beta-oxidation pathway and fatty acid-CoA ligases. The diversity of the acid-CoA ligases

(13 and 11 different genes present in *C. variable* DSM 44702 and *A. arilaitensis* Re117, respectively) may indicate different substrate specificities, which would be necessary for the catabolism of the range of fatty acids present in cheeses. The catabolism of lipids has an impact on the flavour of cheeses. Indeed, fatty acids have a direct effect on flavour and are precursors of compounds having low olfactive thresholds (e.g., alcohols, ketones, lactones and esters).

2.2.3 Iron Acquisition

Bovine milk has a low iron content of 0.2–0.4 mg/l (Gaucheron et al. 1997), and contains lactoferrin, a glycoprotein that is antimicrobial by chelation of iron (Jenssen and Hancock 2009). Lactobacilli require no iron (Archibald 1983; Bruyneel et al. 1989; Weinberg 1997). However, at the surface of the cheese, oxygen ensures iron is ferric, Fe^{III}, which is highly insoluble especially at pH 6 and will diffuse very slowly. What iron is available may be sequestered by the growth of yeast. So the cheese surface is probably even more demanding on iron acquisition than the cheese matrix. Iron availability has been shown to be growth limiting on the surface of model cheeses (Monnet et al. 2012). However, low iron availability is a common feature of habitats, for soil organisms and pathogens.

The genomes of the four cheese-originating *Actinomycetales*, *C. variable* DSM 44702, *C. casei* UCMA 3821, *A. arilaitensis* Re117 and *B. aurantiacum* ATCC 9174 are well equipped with genes involved in iron acquisition. In *A. arilaitensis* Re117, two siderophore biosynthesis gene clusters have been identified, one having no counterpart in the *Arthrobacter* strains from soil (Monnet et al. 2010). These genes are transcribed during the growth in cheese, and when more iron is available, the IdeR regulator decreases their transcription level (Monnet et al. 2012).

The genome of *C. variable* DSM 44702 contains a genomic island with genes for the biosynthesis of a catechol siderophore from chorismate, and the *fes* gene, similar to enterobactin esterase, for the removal of iron from the siderophore (Schröder et al. 2011). With a non-ribosomal peptide synthetase, this is a complete pathway for the synthesis of catechol siderophores from chorismate. In the taxonomic subline in which *C. variable* is located (cluster 3), similar siderophore biosynthesis genes are only present in the genome of the pathogen *C. jeikeium*.

Brevibacteria are variable in siderophore production with little correlation between phylogeny and the ability to produce siderophores. Most strains seem to be able to utilize the hydroxamate siderophore produced by siderophore positive strains. Several cheese-related brevibacteria, including *Brevibacterium linens* strains, are auxotrophic for siderophore biosynthesis (Noordman et al. 2006) and are presumably dependent upon siderophores produced in the cheese surface biofilm by other organisms. MLST analysis of cheese-related brevibacteria suggests they are polyphyletic (Forquin et al. 2009) and the authors suggest strains may have adapted independently several times. But brevibacteria may also be restricted in their contribution in the cheese flora by their iron limitation. However, iron limitation, through

lactoferrin, is clearly an evolutionary strategy to limit contamination in milk and iron restriction may contribute to the ability of the surface flora to outcompete potential spoilage organisms. In fact supplementation with siderophores seems to be even more effective at relieving iron limitation for members of the natural cheese surface flora (Noordman et al. 2006; Monnet et al. 2012) but whether specific siderophores, such as the hydroxamate siderophores taken up by *brevibacteria*, could differentially encourage potentially desirable components of the surface flora without also encouraging contamination is not known.

Examination of the draft genome sequence of *B. linens* ATCC 9174 also revealed the presence of a gene cluster involved in the synthesis of a catechol-type siderophore (locus BlinB01002486 to BlinB01002493). Three of these genes (BlinB01002490 to BlinB01002492) resulted from a horizontal gene transfer as the closest orthologs are found in Gram-negative species.

In *Staph. aureus*, the *sbnABCDEFGHI* operon encodes the proteins involved in the biosynthesis of the siderophore staphylobactin, which enhances virulence. It has been suggested that the *sbn* operon is specific to *Staph. aureus* among the staphylococci, as it is absent in the 13 coagulase-negative staphylococci as investigated by Dale et al. (2004). However, its presence was shown recently in *Staph. equorum* Mu2 (SEQMU02_11830 to SEQMU2_11870) (Irlinger et al. 2012), a coagulase-negative *Staphylococcus* strain isolated from a smear cheese. The number of ABC-type iron-siderophore transport components is higher in *Actinomycetales* originating from cheeses (from 20 to 56 genes) than the mean number (13 genes) found in *Actinomycetales* from other environments (Monnet et al. 2012). It is likely that there is a selective pressure in cheese for strains with efficient iron acquisition systems. In addition, cheese *Actinomycetales* bacteria have fewer proteins with iron-sulfur cluster domains (Monnet et al. 2012). It can be hypothesized that these strains decreased their need for iron by eliminating proteins requiring iron. It is also possible that iron acquisition is at the origin of major interactions between the microorganisms that grow at the surface of cheese. In fact, siderophores excreted by one strain may be utilized by another strain, whose growth would then be stimulated. In contrast, siderophores may also chelate most of the available iron and, as a result, inhibit the growth of strains that are devoid of the corresponding iron-siderophore transport system.

2.2.4 Osmotolerance

Salt is a key element in cheese preservation. In the presumed history of cheese, the early production of cheese in hot climates may have used preservative levels of salt that precluded much microbial activity, however, cheese production in the cooler climate of Europe may have allowed the use of lower levels of salt. These levels of salt, including those used in smear cheeses, are selective rather than prohibitive in restricting microbial growth.

The *Actinomycetales* found on cheese, such as *Arthrobacter*, *Brevibacterium* and *Corynebacterium* are well equipped with genes protecting against osmotic stress and desiccation whether they are from cheese or are found in the skin

microbiome or are soil-related species. The high salt concentrations and exposure to air on the cheese surface during the ripening process impose significant water availability stress as does the wet-dry cycle in soil and surface exposure of organisms on the skin.

Extreme halophiles rely upon the influx of inorganic ions to match the external concentrations, especially accumulation of potassium ions, but that strategy has required significant evolution of all the cell's enzymes to operate at high salinity. Organisms other than these members of the halophilic archaea in the family *Halobacteriaceae* and halophilic bacteria of the order *Haloanaerobiales* accumulate compatible (Brown 1978) organic solutes (da Costa et al. 1998).

Upon osmotic shock the initial response is from mechanosensitive channels (Kung et al. 2010), *MscL* and *MscS* that are found in the genomes of *Arthrobacter* including *A. arilaitensis* Re117, *Corynebacterium* including *C. casei* and *C. variabile*, and *Brevibacterium* including *B. linens* BL2. This is usually followed by a short-term accumulation of potassium ions using a *TrkH* transporter (Corratgé-Faillie et al. 2010); a putative *TrkH* transporter is found in *A. arilaitensis* Re117, *C. casei* and *B. linens* BL2 but is not annotated or found by blastp in the genome of *C. variabile*. A CgIK K⁺ transporter, identified as involved in pH homeostasis in *C. glutamicum* (Follmann et al. 2009) is present in *C. variabile*, although not in the organisms in the other genera, which may correlate with early growth on the cheese surface (Brennan et al. 2002) when the pH is low and the ability to utilize both D- and L-lactate.

Increased concentrations of compatible osmoprotectants (Fig. 2.1) may be synthesized *de novo* or accumulated by uptake systems.

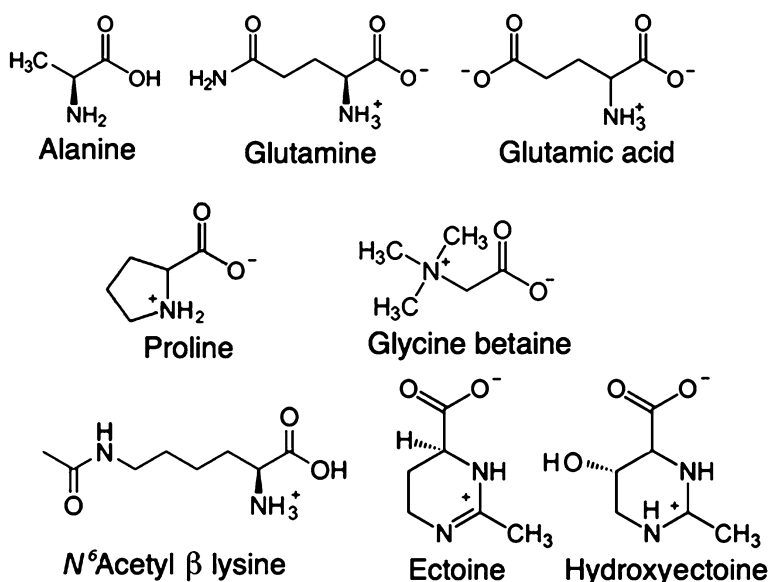


Fig. 2.1 Structure of amino acid and amino acid derived compatible solutes (From Avonce et al. 2006)

Amino acids such as alanine, glutamate, glutamine and proline are used as compatible solutes by different bacteria. All three enzymes (ProA/B/C) for the biosynthesis of proline from glutamate are present in all the cheese-related genomes despite the presence of a lot of proline in the major cheese protein casein. Similarly NADP⁺ dependent glutamate dehydrogenase and glutamate synthase for glutamate biosynthesis are present in all the genomes.

Glycine-betaine is one of the most widely distributed compatible substrates and can be synthesized from choline, an essential nutrient for mammals which is present in milk, if there is an uptake system and choline oxidase and betaine-aldehyde dehydrogenase. All the cheese-related organisms have a high affinity choline transporter, like that identified in *A. arilaitensis* (Monnet et al. 2010), and choline oxidase and betaine-aldehyde dehydrogenase genes. They also have genes related to the Na⁺ glycine betaine symporter BetP in *C. glutamicum*, although the possession of glycine betaine related genes like these are not unique to the cheese-related organisms in these genera. However, there are multiple BCCT and ABC transporters involved in betaine/carnitine/choline transport, and an analysis in *A. arilaitensis* Re117 (Monnet et al. 2010) showed 15 genes involved in transport related to glycine betaine while environmental strains *A. aureus* TC1, *Arthrobacter* sp. FB24 and *A. chlorophenicus* A6 have 6–9 genes and no orthologous gene for 7 of the *A. arilaitensis* genes in any of the environmental genomes.

One mechanism to overcome osmotic stress is the accumulation of osmoprotectants such as ectoine, proline and glycine betaine in the cytoplasm. Ectoine and hydroxyectoine are two other common compatible osmolytes but the genes for biosynthesis are not present in the genomes of the cheese-related organisms (*A. arilaitensis*, *C. casei* and *C. variabile*) and are only present in a few other genomes in these genera. Genes related to the EctP ectoine/glycine betaine/proline transporter of *C. diphtheriae* are widely present including in the cheese-related genomes so these strains can probably scavenge this compatible solute. Ectoine and glutamine are the main compatible solutes in the genus *Brevibacterium* (Frings et al. 1993b) and they synthesize ectoine, and in smaller amounts its hydroxy derivative, for osmoprotection (Onraedt et al. 2004). This compound is present in the rind of smear cheeses (Klein et al. 2007).

One exceptional feature of the *B. aurantiacum* ATCC 9174 genome is the large number of betaine/carnitine/choline family transporters. Indeed, nine different betaine/carnitine/choline family transporters have been identified in this strain (based on the number of proteins matching the pfam PF02028 Hidden Markov Model), whereas the mean number in the other *Actinomycetales* is 1.5 (analysis of the 361 genomes belonging to the order *Actinomycetales* present in August 2012 in the Integrated Microbial Database, <http://img.jgi.doe.gov/>). Only *Saccharomonospora saliphila* YIM 90502, a halophilic actinomycete which is able to grow in the presence of 20 % NaCl (Syed et al. 2008) has more betaine/carnitine/choline family transporters (13 proteins). The *C. variabile* DSM 44702 genome contains the *ectP* gene encoding an ectoine transporter, *proP* which encodes an osmoregulated proline transporter, and six genes encoding proteins of the betaine/carnitine/choline transporter family (Schröder et al. 2011). Glycine betaine can be synthesized from choline,

which is present in milk and cheese. It is likely that the high salt concentration at the surface of smear cheeses favoured the selection of efficient osmotransporter systems. It may also explain the presence, in some smear cheeses, of bacterial species usually present in marine environments, e.g. *Halomonas* sp., *Marinilactibacillus psychrotolerans*, *Pseudoalteromonas* sp., and *Vibrio* sp. (Maoz et al. 2003; Feurer et al. 2004a; El-Baradei et al. 2007; Ishikawa et al. 2007).

2.2.4.1 Osmotolerance and Trehalose

Trehalose is a non-reducing disaccharide formed from two glucose molecules that has a physiological role in energy storage, as an environmental protectant against various stresses such as desiccation or dehydration, external osmolarity fluctuations, heat, cold and oxidation. It is a component of complex cell wall molecules in mycobacteria and related genera (corynebacteria, nocardia, rhodococci) with trehalose-esterified mycolic acids. There are at least five pathways for the biosynthesis of trehalose (Fig. 2.2; Avonce et al. 2006), four of them have been identified in *Rubrobacter xylanophilus* (Empadinhas and da Costa 2008) a deep-seated clade in the actinobacteria, a pattern repeated in mycobacteria (e.g. *M. tuberculosis*, *M. avium*) and streptomycetes (Avonce et al. 2006).

Trehalose biosynthetic genes are widely distributed (Avonce et al. 2006) though it only accumulates to any significant concentration in fewer organisms and in some of those, like *Bacillus subtilis*, it plays a role only as a carbon storage compound.

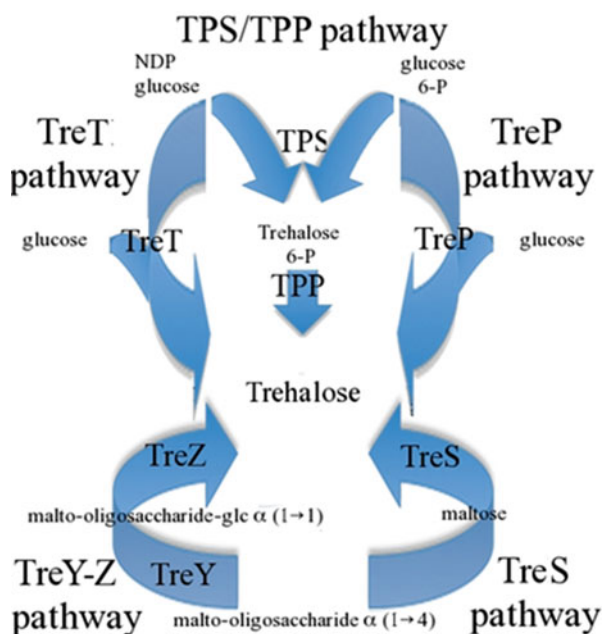
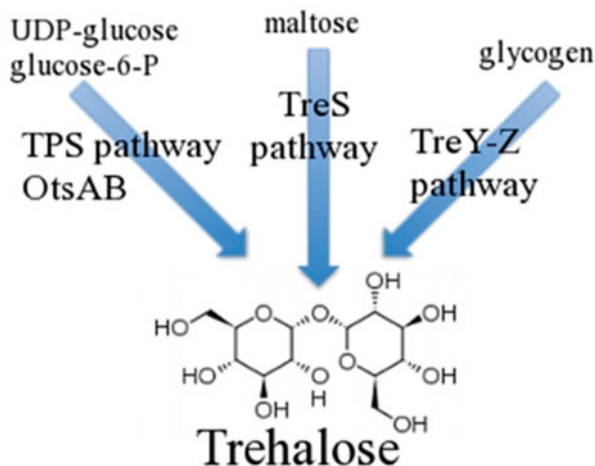


Fig. 2.2 Pathways for the synthesis of trehalose (From Avonce et al. 2006). *TPS* trehalose-6-phosphate synthase, *TPP* trehalose-6-phosphate phosphatase, *TreT* trehalose glycosyltransferase, *TreP* trehalose phosphorylase, *TreY* maltooligosyltrehalose synthase, *TreZ* maltooligosyltrehalose trehalohydrolase, *TreS* trehalose synthase

Fig. 2.3 Three pathways of trehalose biosynthesis in *C. glutamicum* (Wolf et al. 2003)



The lactobacilli whole genome sequences when compared by BLAST analysis for matches to trehalose biosynthetic genes (*L. johnsonii*, *L. plantarum*, *L. lactis*) in Avonce et al. (2006) were found to contain only *treP*. The role of trehalose as a stress protectant and compatible solute (desiccation-resistance and osmotolerance) has only been studied in a few organisms (e.g. *Escherichia coli*, *Saccharomyces cerevisiae*, cyanobacteria, streptomycete spores) including *C. glutamicum* (Wolf et al. 2003). This organism has three biosynthetic pathways for trehalose biosynthesis (Fig. 2.3) and Wolf et al. (2003) demonstrated that *C. glutamicum* synthesizes trehalose in response to osmotic stress using the *treY/treZ* pathway, from maltodextrins like glycogen, rather than the classical *otsA/B* (TPS/TPP) pathway studied in e.g. *E. coli* (Strom and Kaasen 1993; Welsh and Herbert 1999).

The widespread connections between glycogen and trehalose metabolism have been mapped onto sequenced genomes and reviewed by Chandra et al. (2011) but their analysis looks at the relationship more from the point of view of glycogen biosynthesis and only included the *treS* gene. Avonce et al. (2006) mapped the trehalose biosynthetic genes to whole genome sequences but only a few corynebacteria (*C. diphtheriae*, *C. efficiens* and *C. glutamicum*) sequences were available. *C. diphtheriae* only has the genes for the classic pathway (TPS/TPP) while *C. efficiens* and *C. glutamicum* have the three pathways (TPS/TPP, TS and *treYZ*).

In their study of osmoprotection in *C. glutamicum*, proline was the major compatible solute synthesized in response to the addition of 750 mM NaCl. But biosynthesis of trehalose by the *treYZ* pathway was critical to recovery from osmotic shock (Wolf et al. 2003). Since then at least 16 corynebacterial whole genomes have been submitted to the databases, including the cheese-related *C. casei* and *C. variable*. Some species, like *C. glutamicum*, have three pathways, some, including *C. casei* and *C. variable*, have two, lacking the *treYZ* pathway.

A similar picture emerges for *Arthrobacter*; the genes for all three pathways are present in the genome of *A. aurescens* TC1 and the formation of trehalose under

osmotic stress was observed by using NMR (Mongodin et al. 2006). There are eighteen genomes in projects listed in Genbank but only eight with data, including the cheese-specific *A. arilaitensis* (Monnet et al. 2010). Genome sequencing of another cheese isolate, *A. bergerei* Ca106, was registered at the end of 2012. The genes for the same three pathways identified in *A. aureescens* TC1 (Mongodin et al. 2006) are found by blastp in the genomes of the environmental *Arthrobacter* species sequenced, although except for good matches in *Arthrobacter* sp. Rue61a (Niewerth et al. 2012), the identities are in the 50–60 % range. Hits to the *A. arilaitensis* Re117 genome are lower 60 % (*otsA*) and 44 % (*otsB*) for the TPS/TPP pathway, a trehalose/maltose hydrolase (43 %), *treS* (33 %) and *treZ* (26 %) but no significant hit for *treY*.

Brevibacterium linens tolerates high salt concentrations (8–20 %), can grow from pH 5.5–9.5 (optimum pH 7.0) and survive carbohydrate starvation and drying for extended periods (Boyaval et al. 1985). Similar to the other cheese-related species, *B. linens* BL2 and the skin isolate *B. casei* lack the TreYZ pathway.

These cheese and skin isolates are probably subjected to higher desiccation and osmotic stresses on these surface biofilms than in soil but the lower capacity for trehalose biosynthesis may reflect the more consistent availability of compatible substrates for uptake from the cheese environment.

2.2.5 Competition -Bacteriocins

Some strains present in smear cheeses are able to produce bacteriocins (Ryser et al. 1994; Eppert et al. 1997; Carnio et al. 2000; Bleicher et al. 2010) which may provide them with a selective advantage. This property may also be used to prevent the growth of pathogens or spoilage bacteria (Motta and Brandelli 2008) during cheese ripening. The bacteriocins linecin A (Kato et al. 1991), linocin M18 (Valdés-Stauber and Scherer 1994) and linenscin OC2 (Maisnier-Patin and Richard 1995) are produced by some *Brevibacterium* isolates. The genome sequence of *B. aurantiacum* ATCC 9174 contains a gene (locus BlinB01003197) which shares homology (84 % at the amino acid level) with the gene encoding the linocin M18 in *B. linens* M18. Linocin M18 has antimicrobial activity against a wide range of Gram-positive bacteria, including *Listeria monocytogenes*, *Bacillus cereus* and *Staph. aureus*. No genes involved in the synthesis of bacteriocins have been identified in the genomes of *C. casei* UCMA 3821 and *C. variable* DSM 44702, *A. arilaitensis* Re117 and *Staph. equorum* Mu2.

2.2.6 Bacteriophage

Viruses that multiply by infecting bacteria are known as bacteriophage (or phage). An important problem in the dairy industry is the infection of lactic acid bacteria by bacteriophage, which causes delay or disruption of fermentation processes. To the authors' knowledge, no cases of growth arrest have been reported for the typical

bacteria involved in the ripening of smear cheeses. However, it is possible that the impact of bacteriophage on the ripening bacteria has been overlooked. Indeed, such infections would be more difficult to detect, in contrast to infections of lactic acid bacteria, which result in a reduced acidifying activity. It would be interesting to determine if bacteriophage could explain the difficulty of some strains that are deliberately inoculated into cheese milk to establish themselves on the surface of smear cheeses. The *C. variable* DSM 44702 genome contains a putative prophage (bacteriophage genome inserted in the bacterial chromosome) of about 48.3 kbp, which comprises 60 genes (Schröder et al. 2011). In the *C. casei* UCMA 3821 genome, 85 genes have been annotated as phage proteins. Furthermore, ten genes have been annotated as CRISPR (clustered regularly interspaced short palindromic repeats)-associated proteins and four CRISPR loci were identified using the CRISPR finder tool (Grissa et al. 2007). CRISPR loci provide acquired resistance to bacteriophage (Sorek et al. 2008). They typically consist of several non-contiguous direct repeats separated by variable sequences called spacers and often adjacent to CRISPR-associated genes. The presence of prophage and CRISPR loci in the genome of smear cheese bacteria indicates that bacteriophage may exert an influence on the development of these bacteria during the ripening of cheeses.

2.2.7 Production of Flavour Compounds

Volatile sulfur compounds are major flavour compounds in smear cheeses. They arise primarily from L-methionine degradation to methanethiol by the cheese-ripening microorganisms. This thiol is a common precursor of a variety of odorant sulfur-containing compounds (Amarita et al. 2004). The ability of some cheese ripening bacteria to produce high amounts of volatile sulfur compounds has been attributed to the presence of L-methionine-gamma-lyase (EC 4.4.1.11), cystathionine gamma-lyase (EC 4.4.1.1) or cystathionine beta-lyase (EC 4.4.1.8) activities (Ferchichi et al. 1985; Dias and Weimer 1998; Smit et al. 2005). However, the two latter enzymes are less effective than methionine-gamma-lyase for the production of methanethiol from methionine (Bruinenberg et al. 1997; Smacchi and Gobbetti 1998). No candidate genes for L-methionine-gamma-lyase, cystathionine gamma-lyase or cystathionine beta-lyase have been identified in *A. arilaitensis* Re117 (Monnet et al. 2010), which is consistent with the fact that *Arthrobacter* strains are generally considered as low producers of volatile sulfur compounds in cheeses (Bonnarme et al. 2000; Deetae et al. 2007). The *aecD* gene, which encodes a cystathionine beta-lyase, has been identified in *C. variable* DSM 44702 (CVAR_1048) and *C. casei* UCMA 3821 (CCAS_00895). In *Staph. equorum* Mu2, one gene encoding a cystathionine beta-lyase (SEQMU2_13200) and two genes encoding a cystathionine gamma-lyase (SEQMU2_13205 and SEQMU2_10740) are present. Study of the genome of *B. aurantiacum* ATCC 9174 revealed the presence of genes encoding a L-methionine-gamma-lyase, a cystathionine beta-lyase, and a gene encoding either a second cystathionine beta-lyase or a cystathionine gamma-lyase (Forquin et al. 2011).

2.2.8 Pigment Production

Cheese rind coloration is an important criterion of acceptance by cheese consumers and many smear cheeses are characterized by an orange-red or a yellowish colour. Cheese rind coloration is a complex process involving physical and chemical factors as well as biotic interactions (Bockelmann et al. 1997; Leclercq-Perlat et al. 2004; Galaup et al. 2007). The orange pigments produced by *Brevibacteriaceae* are aromatic carotenoids: isorenieratene, 3-hydroxy-isorenieratene and 3,3'-dihydroxy-isorenieratene (Dufossé et al. 2001). The genes corresponding to this pathway have been identified in *B. aurantiacum* ATCC 9175 (formerly *B. linens*), where they are located in the carotenogenic (*crt*) gene cluster (Krubasik and Sandmann 2000). This cluster is also present in *B. aurantiacum* ATCC 9174 (locus BlinB01002626 to BlinB01002637). For a long time, the coloration of smear cheeses was mainly imputed to the production of orange carotenoids by *Brevibacterium* strains. However, the predominant pigments present in the rind of the smear cheeses studied by Galaup et al. (2007) were produced by yellow bacteria such as *Microbacterium gubbeenense* and *A. arilaitensis*. Study of the cluster of carotenoid biosynthesis genes in *A. arilaitensis* Re 117 showed that the yellow pigment produced by this strain probably belongs to the C₅₀ carotenoid subfamily (Monnet et al. 2010).

2.2.9 Proteolysis

The major protein in milk is casein and the ability to break casein down to provide peptides and amino acids is critical for growth on cheese. This protein degradation contributes to changes in texture and flavour, as about 50 % of flavour compounds detected in cheese are derived from amino acid metabolism (Yvon and Rijnen 2001).

As has previously been discussed, milk evolved to transfer nutrients and health benefits from mother to newborn (Holt and Carver 2012; Oftedal 2012). All body fluids are super-saturated in calcium and phosphate relative to bone and contain casein-like precursor proteins to sequester Ca and P. But milk contains much higher concentrations (from 0.5 mM Ca in human milk to 100 mM in mouse milk) sequestered in casein micelles. Casein has evolved to deliver high concentration of calcium while preventing calcification in the mammary gland. Multiple caseins interact to form self-limiting micelles, β -casein sequesters Ca, as amorphous calcium phosphate nanoparticles, but forms continuously growing micelles. Caseins (α s1-, α s2-, β -, κ -), with an extended poly-proline backbone and multiple zipper amyloid fibril peptides, are present as unfolded proteins with phosphorylated peptides (present in α s1-, α s2-, β -caseins) binding the nanoparticles. The multiple caseins act as chaperone-like molecules, aggregating with one another rather than forming destructive amyloid fibrils, with κ -casein binding partly on the surface to limit micelle growth.

Protein degradation releases this Ca and phosphate, which should be bioavailable, a natural process along with coagulation in the stomach of young mammals to limit progress through the gut with the action of chymosin. This is a process that is at the

heart of cheese-making. Caseins are evolutionarily quite diverse but the ability to sequester calcium, across the wide range found in milk, is strictly correlated with the amount of casein in the milk. But caseins have evolved to generate bioactive peptides on digestion (Newburg 2005; Phadke et al. 2005) both antimicrobial e.g. isracidin in bovine milk (Hill et al. 1974) and immunomodulatory and opioid peptides such as β -casomorphins (Brantl et al. 1979).

Chymosin or rennin, an enzyme in rennet, cleaves the peptide bond between phenylalanine and methionine at positions 105 and 106 in κ -casein (Gilliland et al. 1991). Milk also contains several proteases, bovine milk contains plasmin (plasminogen and plasminogen activators), thrombin, cathepsin D, acid milk proteases, and aminopeptidase. Plasmin cleaves peptide bonds Lys-X, and Arg-X, and degrades caseins β - and α s2-casein > α s1-casein, but not κ -casein (Bastian and Brown 1996).

Casein is a major substrate in cheese and superimposed upon the changes in texture, flavour and organoleptic properties arising from the action of chymosin, is the extensive proteolytic activity from the cheese-making LAB, both secreted proteases and intracellular released proteases and peptidases, and the activity of proteolytic enzymes from the smear organisms.

Cheese-making LAB are auxotrophic for many amino acids and have complex proteolytic systems (Juillard et al. 1998; Upadhyay et al. 2004). Lactococci have a cell envelope-associated proteinase, lactocepin (PrtP) a subtilisin-like serine protease, loosely attached by Ca^{2+} to the cell surface, which degrades casein when lactococci grow in milk, but in cheese it degrades the casein peptides produced by chymosin and plasmin to shorter peptides (McSweeney 2004). LAB intracellular peptidases are essential for cheese ripening and the release of free amino acids.

On the smear cheese surface proteolytic enzymes from *B. linens* have been characterized most thoroughly (Ratray and Fox 1999). This organism produces extracellular proteinases and aminopeptidases in addition to a range of intracellular enzymes. These extracellular proteases are able to cleave both α s1- and β -casein to relatively high molecular weight peptides (Frings et al. 1993a; Fernandez et al. 2000).

Specific secreted proteases capable of cleaving casein, with a high content of proline and glutamine, have been characterised in *A. nicotianae* (Smacchi et al. 1999), found on smear cheeses such as Livarot, Limburger and Tilsit (Bora 2010) and related to *A. arilaitensis* and *A. bergerei* (Irlinger et al. 2005), and shown to be active on casein at the pH, temperature and NaCl concentrations found on cheese surfaces. The genome of *A. arilaitensis* (Monnet et al. 2010) contains at least 51 genes identified as protein degradation enzymes of which 9 are specific to *A. arilaitensis* compared to the environmental *Arthrobacter*, but which are active as casein-specific secreted proteases or adventitious lysis released proteases/peptidases is not clear. Similarly it is difficult to correlate the proteinases and aminopeptidases found in *B. linens* with the genome data for *B. linens* BL2.

An iminopeptidase has been purified and characterised from *C. variabilis* (Gobbetti et al. 2001) and analysis of the *C. variabile* genome (Schroder et al. 2011) identified the *pepI* gene as an iminopeptidase. A secretory serine peptidase, *sepP*, and aminopeptidase, *sepC*, were also highlighted as putative casein proteolytic enzymes.

2.2.10 Peptidomics

The peptide composition for each cheese depends upon the proteins and protein sequences, different for different milk caseins, and the specificities of proteases and peptidases, and stage in the ripening process. Typically on the order of 100 peptides are found in e.g. Emmental and Parmigiano-Reggiano (Gagnaire et al. 2001; Sforza et al. 2012). Like amino acids, short peptides can be bitter but di-, tri- and short peptides can be bioactive (Korhonen and Pihlanto 2003; Minkiewicz et al. 2008), with dairy products (Nagpal et al. 2011) and cheese (Lopez-Exposito et al. 2012) as potential sources.

Antibacterial peptides from milk proteins (Benkerroum 2010) have been known for some time (e.g. Hill et al. 1974) but their *in vitro* activity required high concentrations and have not been competitive compared to antibiotics. But the *in vivo* activity of isracidin is higher than its *in vitro* activity (Hayes et al. 2006). Conversely peptides have been identified that stimulate bifidobacteria (Liepke et al. 2002). Although the main focus of current active research is on the probiotic potential in functional foods, with cheese as a functional food, to establish the potential health benefits (Lopez-Exposito et al. 2012), the peptide profile, and the presence of antimicrobial and stimulatory peptides, may contribute to flavour and shape the microbial components of the smear, as well as influencing the microbial composition of the gut.

As well as exhibiting antimicrobial activity, peptides have been shown to be anti-hypertensive, anti-thrombotic, immunomodulatory, opiate and to influence mineral uptake (Korhonen 2009).

After antimicrobial peptides, angiotensin-converting enzyme (ACE) inhibiting peptides have been amongst the most studied and have been found in fermented milk products and in Manchego cheese (Gómez-Ruiz et al. 2002). There seems potential for them to be active against ACE in the brush border but as for all these bioactive peptides with peripheral targets in the body, uptake and transport to potential sites of action are problematic.

This can be seen with the milk-derived opioid peptides, the most studied opioid receptor ligands are those termed β -casomorphins (Brantl et al. 1979) derived from β -casein and these have been associated with the soporific effect of breast feeding on babies. In adult intestines the peptides would be broken down by enzymatic degradation during uptake but may penetrate the underdeveloped neonate intestinal wall. These opioid peptides are characterized by the presence of a tyrosine residue at the N-terminal and another aromatic amino acid at third or fourth position, a structural motif that fits the binding site of opioid receptors (Nagpal et al. 2011). These peptides may have local effects influencing gut transit.

Phosphopeptides from casein (caseinphosphopeptides CPPs) keep calcium and phosphate in solution at the pH in the intestine and keep these, and other, minerals bioavailable. These peptides are highly anionic and resistant to further digestion. Cheese is a rich source of these minerals and CPPs are found in cheeses such as emmental (Gagnaire et al. 2001). These specific peptide profiles are characteristic that aid in identification of cheeses and the proteolytic activities that generate them.

The evolution of milk to transfer health benefits to the young has generated a rich source of bioactivity, either present or potentially present in cheese, with demonstrated pharmacological activity but whose physiological significance has yet to be established.

2.3 Conclusion

The cheese microflora of smear cheeses adds another layer of activity to modify the organoleptic qualities of cheese. There is evidence of the evolution of cheese-specific strains both within LAB and some smear cheese species but many of the properties of these strains derive from evolutionary selection on the skin and in the soil. Both these habitats impose the same kind of osmotic and desiccation stresses as found on the cheese surface. The major selection pressure imposed by man is salt, the extent to which more subtle evolutionary selection pressure – choosing which cheeses to make by whom – consumer pressure, may have dictated which smear cheese flora have flourished and their properties will be difficult to establish. In fact specific microbiological selection pressure, in the generation and application of starter cultures, is a challenge.

Whether the dominance of actinomycetes in many smear cheeses, red smear cheese varieties, is derived from the early evolution of the actinobacteria during the invasion of the land is speculation but many of the properties of the species found on the cheese surface are shared with other environmental species in the same genera.

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Actinomycetes on European Smear Ripened Cheeses
Bora, N.; Dodd, C.; Desmasures, N. (Eds.)
2015, XII, 233 p. 33 illus., 22 illus. in color., Hardcover
ISBN: 978-3-319-10463-8