Genomic Evolution of Domesticated Microorganisms

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Abstract

Strains of lactic acid bacteria, yeasts, and molds have been selected over thousands of years based on the unique sensory attributes they provide to food fermentations. Over the centuries they have evolved to their domesticated roles, leading to genome decay, loss of pathways, acquisition of genomic elements, and beneficial mutations that provide an advantage in their nutrient-rich food environments. This review highlights the evolutionary traits influenced by the domestication process as these microbes adapted to nutrient-rich foods developed by humans.

INTRODUCTION

Originally, fermentation of food substrates occurred spontaneously from naturally contaminating microorganisms. Humans repeated and controlled these processes in order to preserve and increase the sensory aspects of foods, resulting in established fermentation practices (Prajapati & Nair 2008). Over thousands of years, these practices essentially promoted the adaptation and domestication of microorganisms to various food systems. Continued passage of dominating cultures via backslopping in vegetable, cereal, dairy, and meat environments fits the definition of domestication, which is a "species bred in captivity and thereby modified from its wild ancestors in ways making it more useful to humans who control its reproduction and its food supply" (Diamond 2002, Legras et al. 2007, Lewis 1987, Makarova et al. 2006a).

After Louis Pasteur implicated microorganisms as the cause of fermentations in the mid-nineteenth century (Kosikowski & Mistry 1997), individual species that had been passed down over the centuries began to be identified with developing microbiological methods and linked to various fermentation traits. Historically, fermentations were natural and contained a variety of yeasts, molds, and bacteria (Rasic & Kurmann 1978), which likely played a role in the evolution of domesticated microorganisms. The domesticated strains, critical to safety, texture, flavor, and aroma of an end product, include eukaryotic molds and yeasts important in ethanol production, prokaryotic homofermentative lactic acid bacteria (LAB) important in lactic acid fermentations, and heterofermentative LAB, able to produce CO_2, acetic acid, lactic acid, and ethanol (Kandler 1983, Makarova et al. 2006a, Prajapati & Nair 2008). The widespread use and safe consumption of these microorganisms through human history has earned them a Generally Recognized as Safe, or GRAS, status from the FDA for consumption.

Some bacterial strains, including LAB and bifidobacteria are found naturally in the gastrointestinal (GI) microbiota of healthy humans. Although these particular strains have not been domesticated through centuries of selection for a particular fermentation trait, some are added to foods as probiotics, defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (**http://www.mesanders.com/docs/probio_report.pdf**). The association of live microorganisms with health benefits was realized more than 100 years ago. One notable example is Metchnikov's correlation of the long lives of the Bulgarian people and their consumption of a Bulgarian bacillus cultured milk (Metchnikoff 1907), which contained *Lactobacillus delbreuckii* subsp. *bulgaricus*. Compared with *L. bulgaricus*, probiotic strains isolated from oral, GI, and vaginal cavities have demonstrated significant potential for even greater health benefits. These mucosa-associated species provide an important contrast with which to compare the related, but genomically distinct, microorganisms associated with food fermentations.

Recently, genome sequencing of many microbes used as probiotics or involved in food fermentations has identified genes that were maintained, lost, or gained by each species as they evolved to a specific domesticated niche. Sequencing information has revealed high levels of horizontal gene transfer (HGT) in some LAB for specific fermentation and survival traits (Makarova et al. 2006a). With the advent of genomics and molecular techniques, a new chapter has begun in our understanding of microbe evolution and domestication, enabling genetic intervention and manipulation to enhance the beneficial roles of these species.

FERMENTED FOODS AND ASSOCIATED STRAINS

LAB, belonging to the Firmicutes phylum, *Bacilli* class, and *Lactobacillales* order, are Gram-positive, nonsporulating, microaerophilic bacteria that have been involved in fermentation of dairy foods, plants, and meats for thousands of years (Makarova et al. 2006a). They are associated with flavor

and aroma development because of unique amino acid and carbohydrate metabolic pathways that have been positively selected throughout their history with food fermentation and preservation (Hugenholtz 1993, Kandler 1983, Liu et al. 2008). Consequently, their evolution was influenced by domestication over multiple generations as humans intentionally selected the attributes that promoted desirable flavor, texture, quality, and safety.

LAB genomes range in size, protein-coding capacity, and pseudogene content in a manner reflecting the continuous evolution of this phylogenetic group as they become more competitive in their respective domesticated niches. The genomes are marked by species-specific gene loss in various metabolic pathways, sporulation, and oxidative stress responses (Makarova et al. 2006a). However, HGT between the LAB and species from other orders such as Actinobacteria have enabled gene acquisitions that have benefited the adaptation of the LAB to their respective environments (Makarova et al. 2006a, Makarova & Koonin 2007).

Although LAB species comprise a large portion of fermentation microorganisms, eukaryotes such as *Saccharomyces cerevisiae* and *Aspergillus oryzae* have also been domesticated through selection of desirable attributes over the centuries (Legras et al. 2007, Machida et al. 2005). The eukaryotes are marked by genome expansion and increases in a range of metabolic capabilities that underlie their beneficial use in several fermentation products (Forster et al. 2003, Machida et al. 2005).

The following sections review the genomic capabilities of fermentation microorganisms that have been domesticated for use in food substrates and products developed by humans. Specific gene losses and gains will be reviewed, along with the functional traits that have enabled these organisms to thrive in the stressful conditions often associated with domestic food processing.

Yogurt Cultures

The cultures, *Streptococcus thermophilus* strains CNR1066 and LMG13811, isolated from yogurt in France and the United Kingdom, and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842^T, isolated from Bulgarian yogurt, all showed significant genome decay and loss-of-function in pathways that are nonessential in a nutrient-rich dairy environment (Bolotin et al. 2004, van de Guchte et al. 2006). *S. thermophilus* is a homofermentative dairy LAB that has diverged from the pathogenic *Streptococcus* species by the degradation and loss of virulence-associated traits. The genome is almost 1.8 Mb, with a 39% guanine-cytosine (GC) content (Bolotin et al. 2004). *L. bulgaricus* is an obligatory homofermentative LAB that also has a 1.8 Mb genome with a GC content of 49.7%, which is significantly higher than most other LAB. The GC content is particularly high at codon position three, at 65% compared with the expected value of 54%, which suggests a more active and recent evolutionary process based on the faster rate of evolution generally associated with this codon position (van de Guchte et al. 2006).

S. thermophilus and *L. bulgaricus* developed a protocooperative state, where both species benefit from their interaction, which results from their history of cofermentation. To the mutual benefit of both species, these yogurt cultures were domestically enriched together for traits of rapid acidification and coagulation of milk (Bolotin et al. 2004, van de Guchte et al. 2006). *S. thermophilus* encodes enzymes for production of p-aminobenzoic acid (PABA), which *L. bulgaricus* is unable to produce. PABA serves as a key precursor for biosynthesis of the metabolic cofactor, folate, which is essential in human cellular functions as well as in both of these LAB (van de Guchte et al. 2006). Additionally, *S. thermophilus* encodes enzymes that are not present in *L. bulgaricus* but that are required for the production of the polyamine, putrescine. Certain pathways in *L. bulgaricus* suggest polyamines may be involved in protecting the strain from oxygen toxicity (Chattopadhyay et al. 2003, van de Guchte et al. 2006).

L. bulgaricus also encodes proteins that benefit *S. thermophilus*. PrtB is a protease encoded by *L. bulgaricus* that hydrolyzes casein to provide free amino acids and small peptides essential for growth in milk. Although *L. bulgaricus* is deficient in amino acid biosynthetic pathways (Beshkova et al. 1998, Gilbert et al. 1996, van de Guchte et al. 2006), *S. thermophilus* has retained most of them. Nevertheless, the growth of both species in coculture is stimulated by the amino acids and peptides made available by the *L. bulgaricus* protease (Beshkova et al. 1998, Hols et al. 2005). The mix of exopolysaccharides produced by both species in coculture can also contribute to yogurt texture (see below) (Bolotin et al. 2004, van de Guchte et al. 2006).

Compounds produced by *S. thermophilus* and *L. bulgaricus* that contribute to yogurt flavor include lactic acid, acetaldehyde, and the diketones, 2,3-butanedione and 2,3-pentanedione (Ott et al. 1999). Acetaldehyde, which contributes significantly to flavor, can be produced by several pathways, including pyruvate decarboxylation during lactose metabolism, degradation of threonine by deoxyribose aldolase, or threonine transformation by threonine aldolase (Chaves et al. 2002).

Cheese Cultures

The streptococci are closely related to the genus *Lactococcus*, which is commonly used in starter cultures for cheese and cultured dairy products. Although lactococci are widely found in nature on plants, these dairy-related bacteria have evolved to rapidly acidify and coagulate milk, largely via the acquisition of extrachromosomal elements. *L. lactis* strains can vary in their production of lactic acid, diacetyl, CO_2, and their ability to impart other flavor and texture characteristics. Two of the strains that have been sequenced are *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363. They are similar in genome size of approximately 2.5 Mb, with a GC content at 35%, typical of species comprising the LAB (Bolotin et al. 2001, Wegmann et al. 2007).

The fermentation products of *L. lactis* IL1403 depend on the available carbon source in its environment. *L. lactis* is able to undergo homolactic fermentation using carbon sources such as fructose and sucrose, which are imported with phosphoenolpyruvate-dependent transport systems (PEP-PTS). Most notable among these is the plasmid-encoded PEP-PTS system used to translocate and metabolize lactose (Bolotin et al. 2001, Cocaign-Bousquet et al. 1996, de Vos et al. 1990). Heterofermentative metabolism, requiring an active pentose-phosphate pathway, occurs in *L. lactis* for carbon sources without a PTS, such as maltose and galactose, resulting in a mixed-acid fermentation and ethanol products (Bolotin et al. 2001). *L. cremoris* MG1363 still retains metabolic pathways for a variety of carbon sources indicative of its plant-associated ancestry (Wegmann et al. 2007).

L. lactis IL1403 encodes numerous peptidases and aminotransferases that participate in degradation and uptake of amino acids from casein, which promote cell growth and lead to cheese flavor development (Bolotin et al. 2001). Branched-chain aminotransferase (BcAT), found in most lactococcal and streptococcal strains, transaminates isoleucine and valine, likely contributing to sweaty, sour, and sweet odors of some cheeses (Atiles et al. 2000, Liu et al. 2008, Yvon et al. 2000). Aromatic and aspartate aminotransferases (ArAT and AspAT) are also present in *L. lactis* species, where they contribute to growth in milk and to cheese flavor through the transamination of aromatic amino acids (Dudley & Steele 2001, Rijnen et al. 1999). Other LAB species contain putative ArAT and AspAT genes (Liu et al. 2008), but their contributions in most other species have not been characterized. The sulfur-containing amino acid methionine may also be processed in *L. lactis* via transamination by BcAT or ArAT, or by elimination through lyases, leading to aroma products such as dimethyldisulfide (DMDS) (Yvon & Rijnen 2001). A variety of enzymes associated with flavor formation from sulfur-containing amino acids were found in most sequenced LAB genomes, with the dairy fermentation species containing a greater number than the GI

tract–associated species (Liu et al. 2008), supporting the idea that these strains have evolved toward dairy fermentation roles.

EstA, an esterase involved in formation of short-chain fatty acid esters that can lead to fruity flavors in cheese, was encoded in *Lactococcus lactis* (Nardi et al. 2002) and *L. helveticus* cheese starter cultures (Fenster et al. 2000). A similar esterase, EstB, develops fruity flavor in cheese made with *L. casei* (Fenster et al. 2003). This flavor producing esterase was not found in the strains *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, and *Lactobacillus salivarius* (Liu et al. 2008), which are naturally associated with the GI tract and are not considered as domesticated members of the LAB in this review because these species are not typically used as starter cultures.

The diacetyl flavor desired in dairy products such as cottage cheese, cream cheese, and cultured buttermilk is a product of citrate utilization by a *L. lactis* ssp. *lactis* biovar *diacetylactis*. This biovar encodes the pathway to transport and metabolize citrate to pyruvate, which then converts to diacetyl through the chemical decarboxylation of the intermediate, α-acetolactate, in the presence of oxygen (Goupil et al. 1996, Hugenholtz et al. 2000).

The cheese culture, *Lactobacillus helveticus* DPC 4571, isolated from swiss cheese, contains high peptidolytic activity, enabling rapid lysis in the cheese matrix and promoting flavor development in certain cheese varieties. *L. helveticus* has a 2.08 Mb genome with a 37.7% GC content, similar to other dairy bacteria, but contains a far greater number of IS elements (~200) than any other species sequenced thus far. The proteolytic system and 24 peptidases encoded in *L. helveticus* provide the ability to hydrolyze casein and decrease bitter peptides, making this culture ideal for cheese fermentation and as an adjunct for accelerated ripening (Callanan et al. 2008).

Vegetable Cultures

Lactobacillus plantarum WCFS1 is a facultative heterofermentative LAB with a 3.3 Mb genome that encodes pathways for the metabolism of a wide variety of carbon sources. *L. plantarum* likely retained this metabolic capacity owing to its natural presence in a range of environments, including the GI tract and oral mucosa of humans, and dairy, meat, and plant-fermented foods (Ahrne et al. 1998, Kleerebezem et al. 2003). Use of *L. plantarum* in a range of fermentations is enabled by the presence of both the Embden-Meyerhoff-Parnas pathway and the phosphoketolase pathway, yielding both homo- and heterofermentative products from hexoses or pentoses. Pyruvate can also act as a substrate for various other pathways, leading to formate, 2,3 butanediol, and acetoin production (Kleerebezem et al. 2003).

Although *L. plantarum* does not contain a gene encoding a cell wall–associated Prt proteinase, it does encode 19 genes for intracellular peptidases. In addition, an *opp*-encoded oligopeptide transport system is present, as well as pathways for amino acid biosynthesis (Kleerebezem et al. 2003, Wegmann et al. 2007). The branched chain amino acids valine, leucine, and isoleucine are not synthesized but are obtained through specific transporters (Kleerebezem et al. 2003).

Leuconostoc mesenteroides subsp. *mesenteroides* is a heterofermentative LAB that initiates sauerkraut fermentations by lowering the pH with lactic and acetic acid production, and reducing the oxygen level through production of CO_2. After three days of domination by *L. mesenteroides*, the pH decreases and bacteriophages appear and eliminate the *Leuconostoc* population, which is succeeded by *L. plantarum* and other LAB to complete the fermentation (Lu et al. 2003). The genome sequencing of ATCC 8293 (2.0 Mb) revealed a phosphoketolase pathway and the genetic pathway to produce diacetyl, which can contribute flavor in both vegetable and dairy fermentations (Makarova et al. 2006a). The related *L. mesenteroides* subsp. *cremoris* is found in mixed species dairy starter cultures used for cultured milk and cheese fermentations. These L and DL type cultures

have been transferred repeatedly in milk for hundreds of years and are used primarily for diacetyl and CO_2 production (Cogan 1996).

Meat Cultures

Lactobacillus sakei 23K, a meat-associated LAB, has evolved to include metabolic pathways and stress response mechanisms specific to the fresh and fermented meat environments. The 1.88 Mb genome with a 41.25% GC content encodes pathways to utilize several sugars, although glucose is preferred. However, unlike dairy-associated LAB, *L. sakei* cannot metabolize lactose (Chaillou et al. 2005). The ability of *L. sakei* strains to compete with other bacteria for nutrients, tolerate low temperatures and salt, and produce bacteriocins and other antimicrobial agents reflects the adaptation of this organism to fresh and refrigerated meats. These properties have generated interest in this species as a natural biocontrol agent for raw meat (Chaillou et al. 2005, Dortu et al. 2008). *L. sakei* does not possess the aminotransferases and sulfur-associated enzymes involved in flavor development by many LAB cultures associated with cheese fermentations (Liu et al. 2008).

Soy, Rice, and Wine Cultures

Besides prokaryotic LAB species, some molds and yeasts have also been domesticated to food fermentation environments, including *Aspergillus oryzae* and *Saccharomyces cerevisiae*. The genomes of these species have been sequenced, revealing features that reflect an active domestic evolution process.

Although many prokaryotic LAB strains demonstrated genome decay and size reduction during domestication to a specific fermentation niche, the eukaryotic mold *Aspergillus oryzae* demonstrated genome expansion by 7–9 Mb when compared with related *Aspergillus* strains (Machida et al. 2005). *A. oryzae* harbors a 37 Mb genome with eight chromosomes and shows gene expansion in metabolic pathways and transporter genes. *A. oryzae* is used to ferment soy or rice, which are then used in traditional Asian products such as sake and soy sauce. *A. oryzae* highly expresses alcohol dehydrogenase and pyruvate decarboxylase enzymes, contributing to the ethanol content of final products. Similarly to *S. thermophilus*, *A. oryzae* was suggested to have evolved to a fermentation environment after diverging from a pathogen, in this case an *Aspergillus* species that produces aflatoxin. *A. oryzae* lost functions within the aflatoxin synthetic pathway as it evolved to a food fermentation niche (Bolotin et al. 2004, Machida et al. 2005).

Saccharomyces cerevisiae, a yeast associated with the fermentation of some breads and alcoholic beverages, contains 16 chromosomes as a result of a whole genome duplication event that took place 150 million years ago (Langkjaer et al. 2003). The genome encodes more than 6000 ORFs, including genes for amino acid and protein synthesis, growth on various carbon sources, and survival in a variety of stressful growth conditions (Forster et al. 2003, Giaever et al. 2002), providing an advantage for use in several types of fermentations. Another advantage is the production of sulfite, a metabolite in the reductive sulfate assimilation pathway that may have been selected as a desired trait in fermentations owing to its preservation abilities (Legras et al. 2007, Park & Bakalinsky 2000).

S. cerevisiae's evolution to specific domestic niches occurred over hundreds of generations in varying environments (Ferea et al. 1999). The strains associated with bread, beer, wine, and sake demonstrate genomic traits based on human-driven migration of yeast in Europe, Asia, and the Americas, generating the diversity of strains represented in different regions today. It has been suggested that a beer and a wine strain underwent tetraploidization to produce the bread strains, which were then selected and domesticated based on their fermentation products (Legras et al.

2007). Tetraploidization may alter gene expression in these strains owing to epigenetic remodeling and provide advantages such as protection from deleterious mutations through gene redundancy (Comai 2005).

In wine fermentations, the growth of *S. cerevisiae* must balance with the growth of *Oenococcus oeni*, as the yeast produces metabolites, such as ethanol and yeast autolysates, that may respectively inhibit or stimulate growth of this LAB strain (Alexandre et al. 2004). *O. oeni*, which has a 1.8 Mb genome, is used for malolactic fermentation of wine, resulting in deacidification of the wine after alcohol fermentation (Alexandre et al. 2004, Makarova et al. 2006a). *O. oeni* is one of the most acid- and alcohol-tolerant LAB and is exclusively associated with wine and cider habitats (G-Alegria et al. 2004). *O. oeni* diverged from the *Leuconostoc* species and adapted to wine fermentations with the loss of mismatch repair (MMR) genes, *mutS* and *mutL*, resulting in an increased rate of mutation and evolutionary capacity (Marcobal et al. 2008). The flavor compound, diacetyl, is also produced by *O. oeni*, utilizing similar genetic pathways to those described for *L. lactis* ssp. *lactis* biovar *diacetylactis* (Bartowsky & Henschke 2004, Hugenholtz 1993).

GENOME DECAY AND EVOLUTION RELATED TO NICHE ADAPTATION

Although the evolution of many LAB species has been intimately affected by their associations with food, another group of LAB is routinely associated with oral, gastrointestinal, and vaginal cavities. Although properties such as genome size and GC content do not vary to a great extent from the fermentation bacteria (**Table 1**), some metabolic and surface-associated proteins demonstrate specialization to niche-related roles as these groups of LAB diverged (O'Sullivan et al. 2009). The following sections will point out some of the similarities and differences between the fermentation-associated and the mucosal surface–associated LAB by examining evolutionary traits that have been selected in fermentation environments. Similar traits selected for in *S. cerevisiae* and *A. oryzae* will also be distinguished.

Loss of Gene Functions Related to Strain Domestication

The number of inactive genes (pseudogenes) due to mutation in fermentation strains is typically higher than in GI tract–associated strains (O'Sullivan et al. 2009) and demonstrates loss of function in nonessential genes during adaptation to fermentation environments (Makarova et al. 2006a) (**Table 1**). *S. thermophilus* CNRZ1066 and LMG18311 demonstrated lost function in transport protein and metabolism genes, including four of seven sugar PTS transporters that are unnecessary for growth in milk. Several of these genes appear to have retained function in the related oral-associated nondomesticated strain, *Streptococcus salivarius* (Bolotin et al. 2004). The probiotic strains *L. johnsonii*, *L. gasseri*, and *L. acidophilus* retained 16, 21, and 20 PTS transporters, respectively, and 3–5 ABC transporters, enabling utilization of a variety of sugars in the GI tract, including oligosaccharides that are largely undigested by humans (Altermann et al. 2005, Azcarate-Peril et al. 2008, Pridmore et al. 2004). Therefore, the highly domesticated *S. thermophilus* species has clearly evolved to preferentially ferment lactose and has lost its capacity to ferment a wide range of other carbohydrates.

L. helveticus DPC 4571 is a dairy-associated LAB that contains a similar number of pseudogenes to *S. thermophilus* (~10%) and, similarly, the greatest number are related to transport proteins and energy metabolism (Bolotin et al. 2004, Callanan et al. 2008). *L. helveticus* is phylogenetically most closely related to *L. acidophilus* NCFM, and their genomes share ~75% identical open reading frames and show considerable synteny (**Figure 1**) (Callanan et al. 2008). Although

Table 1 Features of representative sequenced fermentation- and epithelial-associated strains

Fermentation-associated LAB	Common niche	Genome size (protein coding genes)	% GC content	Pseudogene content	Prophage (complete)	IS elements/ transposons	Reference
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842^T	Dairy, yogurt	1.8 Mb (1562)	49.7	227	0	43	(van de Guchte et al. 2006)
Streptococcus thermophilus CNRZ1066 and LMG13811	Dairy, yogurt	1.8 Mb (~1900)	39	~180	0–1	~55	(Bolotin et al. 2004)
Lactococcus lactis subsp. *lactis* IL1403	Dairy foods	2.4 Mb (2321)	35.4	30	6	43	(Bolotin et al. 2001)
Lactococcus lactis subsp. *cremoris* MG1363	Dairy foods	2.5 Mb (2434)	35.8	81	2	71	(Wegmann et al. 2007)
Lactobacillus helveticus DPC 4571	Dairy, cheese	2.1 Mb (1610)	37.7	217	0	213	(Callanan et al. 2008)
Lactobacillus sakei 23K	Meat	1.9 Mb (1879)	41.3	30	0	12	(Chaillou et al. 2005)
Lactobacillus plantarum WCFS1	Dairy, meat, vegetables	3.3 Mb (3007)	44.5	39	2	2	(Kleerebezem et al. 2003)
Oenococcus oeni PSU-1	Wine	1.8 Mb (1691)	37.9	120	0	NA	(Makarova et al. 2006a)
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC 8293	Dairy, vegetables	2.0 Mb (1970)	37.7	17	1	NA	(Makarova et al. 2006a)
Pediococcus pentosaceus ATCC 25745	Meat	1.8 Mb (1755)	37.4	19	Possibly 2	NA	(Makarova et al. 2006a)
Lactobacillus casei ATCC 334	Dairy, cheese	2.9 Mb (2751)	46.6	82	2	NA	(Makarova et al. 2006a)
Mucosal-associated LAB							
Lactobacillus acidophilus NCFM	GI tract	2.0 Mb 1862 ORFS	34.7	NA	0	17	(Altermann et al. 2005)
Lactobacillus salivarius subsp. *salivarius* UCC118	GI tract, oral	1.8 Mb (1717)	33.0	73	2	37	(Claesson et al. 2006)
Lactobacillus johnsonii NCC 533	GI tract	2.0 Mb (1821)	34.6	NA	2	14	(O'Sullivan et al. 2009; Pridmore et al. 2004)
Lactobacillus gasseri ATCC 33323	GI tract	1.9 Mb (1755)	35.3	43	1	13	(Azcarate-Peril et al. 2008; Makarova et al. 2006a)
Lactobacillus reuteri JCM 1112	GI tract	2.0 Mb (1820)	38.9	NA	NA	55	(Morita et al. 2008; O'Sullivan et al. 2009)

NA, not available.

L. helveticus shares nearly 99% 16S ribosomal RNA sequence identity with *L. acidophilus*, the probiotic culture contains only a few pseudogenes (O'Sullivan et al. 2009), indicating that the high number of pseudogenes in *L. helveticus* reflects gene losses and genome decay that accompanied its domestication to milk as its natural habitat (Callanan et al. 2008). Interestingly, *L. helveticus* has lost many properties important to *L. acidophilus* for survival and activity in the GI tract, such as mucus binding proteins, sugar metabolism genes, and bile salt hydrolases (Callanan et al. 2008, O'Sullivan et al. 2009). As many as 36% of the pseudogenes in *L. helveticus* were proposed to be inactivated by IS elements in this genome encoding ~200 IS elements (Callanan et al. 2008). The loss of many of these pathways in fermentation strains compared with GI tract–associated strains provides strong evidence for the evolutionary divergence of domesticated strains by genome reduction and degradation.

Despite evolutionary divergence between fermentation LAB and GI tract–associated LAB, they often share an inability to synthesize many amino acids, vitamins, and cofactors, albeit this inability to synthesize required metabolites varies. For example, *L. plantarum* is able to synthesize all but three amino acids, whereas *L. johnsonii* can synthesize none (Kleerebezem et al. 2003, Pridmore et al. 2004). Basic gene loss and mutation patterns have also been established for *Leuconostoc mesenteroides*, associated with vegetable fermentations and some dairy fermentations (Orillo et al. 1969, Server-Busson et al. 1999), *Oenococcus oeni*, associated with wine fermentations, and *Pediococcus pentosaceus*, associated with meat fermentations (Makarova et al. 2006a). In most of these species, this dramatic gene decay resulted in increased nutritional requirements during growth (Goh & Klaenhammer 2009). To compensate for the loss of biosynthetic ability, the LAB acquired multiple transporters in order to scavenge important growth factors from their nutrient-rich environments (Altermann et al. 2005, Callanan et al. 2008, Claesson et al. 2006, Lorca et al. 2007, Pridmore et al. 2004).

The citric acid cycle is often present but incomplete in LAB fermentation strains, including *L. cremoris* MG1363, *L. lactis* IL1403, and *L. plantarum* WCFS1 (Bolotin et al. 2001, Kleerebezem et al. 2003, Wegmann et al. 2007). Probiotic strains may also encode an incomplete citric acid cycle, including *L. gasseri* ATCC 33323 and *L. acidophilus* NCFM (Altermann et al. 2005, Azcarate-Peril et al. 2008). In these microorganisms, the partial citric acid cycle may generate key intermediates for amino acid biosynthesis (Azcarate-Peril et al. 2008). Alternatively, eukaryotic fermentation strains *A. oryzae* and *S. cerevisiae* encode complete and active citric acid cycles for aerobic respiration (Abe et al. 2006, Camarasa et al. 2003). The citric acid cycle is also active in a reductive role during anaerobic fermentation in *S. cerevisiae*, leading to the production of organic acids that contribute to the sensory properties of wine (Camarasa, Grivet & Dequin 2003).

As mentioned previously, virulence factors have been lost from the fermentation strains *S. thermophilus* and *A. oryzae*, which both diverged from pathogenic strains. While *A. oryzae* lost functions in the pathway that encoded aflatoxin production, *S. thermophilus* lost a variety of virulence-related genes (VRGs), including genes encoding pathways to metabolize a variety of carbon sources and to modify and provide resistance to antibiotics (Bolotin et al. 2004, Machida et al. 2005).

Gain of Gene Functions Related to Strain Domestication

Optimal growth in nutritionally rich fermentation environments promoted the selection of genes encoding transporters for amino acids and carbohydrates, which can constitute up to 13–18% of the genomes of LAB (Lorca et al. 2007). Many genes appear to have been acquired by HGT, resulting in regions with a different GC content than the rest of the genome (O'Sullivan et al. 2009). This was observed for *L. plantarum*, *S. thermophilus*, *L. bulgaricus*, and *L. helveticus* (Bolotin

et al. 2004, Callanan et al. 2008, Kleerebezem et al. 2003, van de Guchte et al. 2006). For example, *S. thermophilus*, which was domesticated in the dairy environment, gained a lactose symporter (Bolotin et al. 2004).

Genetic mutations can lead to functional optimization in pathways that provide the ability to thrive in an environment. *L. bulgaricus* ATCC 11842^{T} contains an inactive *lacR* repressor, which promotes constitutive lactose uptake and utilization. Combined with the presence of *prtB*, a protease gene required for casein metabolism, *L. bulgaricus* has been genetically adapted for fast fermentation in a milk environment (Germond et al. 2003). It is suggested that the *prtB* gene in *L. bulgaricus* was gained by HGT because of its lower GC content relative to the rest of the *L. bulgaricus* genome (Germond et al. 2003). These genetic adaptations in *L. bulgaricus* for fast fermentation of milk become more obvious when similar genes are considered in other *L. delbrueckii* strains. Lactose and protein metabolism (*prtB*) are both regulated in *L. delbrueckii* subsp. *lactis* (Germond et al. 2003, Gilbert et al. 1997). Additionally, Lac and Prt encoding genes are absent from *L. delbrueckii* subsp. *delbrueckii* ATCC 9649^{T} and NCFB, which were selected from vegetable rather than dairy habitats (Germond et al. 2003).

L. bulgaricus shows high sequence homology to two gut symbionts, *L. acidophilus* NCFM and *L. johnsonii* NCC533 (Altermann et al. 2005, van de Guchte et al. 2006). Although *L. acidophilus* and *L. johnsonii* are naturally found in the GI tract, they also encode a Prt protease associated with casein degradation, usually conserved in dairy-associated LAB (Altermann et al. 2005, Pridmore et al. 2004). Considering the importance of milk as a nutritional source in mammals, it is not unexpected that commensal lactobacilli in the small intestine would retain their ability to hydrolyze milk casein. The GI tract–associated *L. salivarius* UCC118 also contains a PrtP homolog encoded on its megaplasmid, but as a pseudogene inactivated by a frameshift mutation (Claesson et al. 2006). Possibly, the genetic integrity of *Prt*-encoding genes is highly variable, and they are retained or lost in the gut microbiota based on the level of milk consumption by the host. Additionally, *Prt*-encoding genes may function in gut microbiota to degrade polypeptides from host food substrates or to cleave mucus layer glycoproteins, which may influence host interactions (Pridmore et al. 2004).

Genome analysis of *L. sakei* 23K did not reveal proteolytic pathways, but rather an array of amino acid and peptide transporters that can scavenge free amino acids in meat. Metabolism of the meat abundant amino acid, arginine, via the arginine deiminase (ADI) pathway improves *L. sakei* survival in anaerobic conditions and during stationary phase. Additionally, *L. sakei* contains catabolic genes for metabolism of the nucleosides inosine and adenosine as an alternative energy source (Chaillou et al. 2005, Zuniga et al. 1998).

L. plantarum contains genomic regions that are conserved in other LAB present in mixed fermentations, suggesting acquisition via HGT. Genes associated with the citric acid cycle, sucrose metabolism, and the lactose operon showed sequence conservation with *L. mesenteroides*, *P. pentosaceus*, and *Leuconostoc lactis*, respectively (Kleerebezem et al. 2003, Vaughan et al. 1996). One region of several hundred kilobases in *L. plantarum* was likely acquired by HGT based on a lower GC content (41.5%) relative to the rest of the genome (44.5%). This region encodes a potentially highly expressed (PHX) locus that contains numerous genes necessary for use of multiple carbon sources and growth in diverse environments, which varies in different isolates (Kleerebezem et al. 2003, Molenaar et al. 2005).

A. oryzae, the mold used in fermentation of several Japanese foods, encodes a higher number of enzymes for hydrolysis and metabolism of animo acid and complex sugars compared with related *Aspergillus* strains. These enzymes can promote growth on the surface of foods that initially provide few free nutrients and generate nutrients for succeeding fermentation. The presence of these additional metabolic capabilities provides evidence for the adaptation of *A. oryzae* to a number of specific fermentation environments (Machida et al. 2005).

Proteins Associated with Biofilms and Exopolysaccharides (EPS)

Exopolysaccharides (EPS) produced by several dairy LAB have contributed to the textural aspects of fermented foods to which they have adapted. Two basic phenotypes, ropy and mucoid, have been described, each providing unique textural qualities to a number of fermented dairy foods (Ruas-Madiedo & de los Reyes-Gavilan 2005). Homologs of *cps* or *eps* genes, encoding proteins involved in EPS synthesis, have been found in most LAB including varying species of *Lactococcus* and *Lactobacillus*, and in *S. thermophilus* (Bolotin et al. 2004, Bolotin et al. 2001, van de Guchte et al. 2006, van Kranenburg et al. 1997).

Putative EPS genes have been found in the meat-associated *L. sakei* genome (Chaillou et al. 2005). These include unique genes predicted to enable aggregation and biofilm formation on the surface of meat. One putative gene involved in biofilm formation contains a Gram-positive cell wall anchoring motif (PF00746) conserved in cell surface binding proteins of several GI tract–associated LAB (Altermann et al. 2005, Chaillou et al. 2005, Pridmore et al. 2004).

Although EPS production has been selected for fermentation strains as a beneficial texture attribute, many GI tract–associated strains also encode EPS systems, which may be secreted or cell surface attached (Kankainen et al. 2009, Pridmore et al. 2004). Putative EPS encoding genes are relatively conserved among the GI tract–associated probiotics *L. acidophilus*, *L. gasseri*, and *L. johnsonii*, with an active system demonstrated only in *L. johnsonii* (Altermann et al. 2005, Azcarate-Peril et al. 2008, Klaenhammer et al. 2005, Pridmore et al. 2004). *L. salivarius* contains two clusters of EPS-encoded genes, one of which is conserved in *L. plantarum* (Claesson et al. 2006). Other than these textural and biofilm properties, it is not clear whether or not EPS production affords any advantage to domesticated LAB.

Prophages, Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR), and Bacteriophage Resistance

Bacteriophages are a harmful but ubiquitous presence in industrial fermentation operations (Pfeiler & Klaenhammer 2007). However, in the case of sauerkraut fermentations, phages have been implicated in proper succession of LAB species, leading to a desirable final product (Lu et al. 2003). Most LAB genomes harbor one or more complete prophages or prophage remnants (**Table 1**). Bacteriophages have provided important vehicles for HGT and acquisition via lysogenic conversion through prophages. Prophage decay leads to remnants that may occasionally encode the genes that contribute to host survival, such as superinfection exclusion genes that may prevent phage infection (Desiere et al. 2002).

Evidence now exists for a bacteriophage resistance mechanism in many bacteria and archaea, encoded in sequences of palindromic repeats with nonrepetitive spacer regions in between, called clustered regularly interspaced short palindromic repeats (CRISPR), and adjacent *cas* genes (Sorek et al. 2008). CRISPR sequences have been documented in many LAB, including the yogurt cultures *S. thermophilus* and *L. bulgaricus* (Bolotin et al. 2004, Horvath et al. 2009, van de Guchte et al. 2006). In *S. thermophilus* strains, which can harbor up to two CRISPR sequences (Bolotin et al. 2004), a protective response to phage infection was demonstrated in the CRISPR1 sequence. Typically, one to four additional spacers of phage-derived sequences were observed in strains that became phage resistant. The added spacers are proposed to dictate phage specificity, whereas associated *cas* genes are expected to be necessary for spacer insertion and resulting resistance (Barrangou et al. 2007). Specifically, the *cas* genes have been proposed to encode analogs to proteins required for processing of interference RNA (siRNAs). The phage-derived spacers exhibit sequence complementarity to invading phages and thereby target them for degradation by *cas*-encoded proteins (Makarova et al.

2006b). From a fermentation technology viewpoint, CRISPR sequences are unique and can be used as a strain-specific DNA signature.

Plasmids: Roles in Evolution and Adaptation to Domesticated Niches

Plasmids are considered to be genetic agents of rapid evolution and adaptation; they are found in many LAB fermentation strains. *L. lactis* typically harbors numerous plasmids, ranging from 2–11 per strain, with many encoding essential traits for milk fermentation and survival (McKay 1983). Plasmid linkages include lactose metabolism (PEP-PTS transport and phospho-β-galactosidase), proteolytic activity (proteinase activities and oligopeptide transporters), bacteriocin production, citrate permease for diacetyl production, and multiple phage defense mechanisms (McKay 1983, Yu et al. 1996). Phage defenses consist of reducing phage adsorption, restriction and modification systems, disrupting phage development after infection (abortive systems), or causing premature lysis of phage-infected cells (Allison & Klaenhammer 1998, Durmaz & Klaenhammer 2007, O'Driscoll et al. 2006). Additionally, *Lactococcus* is genetically promiscuous, harboring conjugal episomes that allow high frequency mating and plasmid transmissions. Moreover, the widespread distribution of prophages in lactococci provides avenues for plasmid gene transfer via transduction (McKay 1983).

One of the more thorough studies of *Lactococcus* plasmids was the sequencing of the plasmid complement of *Lactococcus lactis* subsp. *cremoris* SK11. Four plasmids were annotated in detail, confirming the presence of genetic determinants for lactose utilization and oligopeptide transport related to growth, activity, and survival of dairy cultures in the milk fermentation environment (Siezen et al. 2005).

In addition to basic fermentation traits, lactococcal plasmids have been linked to cold shock proteins (Csp), which can increase survival of starter cultures in cold storage (O'Driscoll et al. 2006, Siezen et al. 2005). Additionally, the PABA synthase complex proteins PabA and PabB, required for folate synthesis, are encoded on pSK11P in *L. cremoris*. Folate is a necessary cofactor for single-carbon metabolism (Siezen et al. 2005). The common presence of plasmids in lactococci and their contribution to key fermentation properties is strong evidence for evolutionary domestication of the LAB to the dairy environment (McKay 1983).

Genes Associated with Survival in Stressful Environments

Fermentation microorganisms often must tolerate a range of changing growth and environmental conditions during bioprocessing. Changes in pH are typical of fermentations and can result in changes to strain balance because of the acid tolerance of the cultures involved. *L. plantarum* is the dominant strain in the later stages of sauerkraut fermentation because of its inherent acid tolerance and active F_0F_1-ATPase that regulates internal pH (Kleerebezem et al. 2003). *A. oryzae* encodes acid-tolerant proteinases that are absent in nonfermentation-associated *Aspergillus* strains (Machida et al. 2005).

Strains associated with the GI tract also require mechanisms for acid tolerance in order to survive passage through the stomach, and some are conserved within fermentation organisms. As with *L. plantarum* NCFS1, *L. acidophilus* NCFM contains an F_0F_1-ATPase system (Kullen & Klaenhammer 1999). Both *L. bulgaricus* ATCC11842T and *L. acidophilus* contain ornithine decarboxylases and proton pumps associated with pH stabilization and acid tolerance (Altermann et al. 2005, Azcarate-Peril et al. 2004, van de Guchte et al. 2006). These acid tolerance mechanisms can benefit GI strains that are now intentionally added to low pH food products as probiotics.

Genes associated with survival in high osmolarity conditions are encoded in cultures associated with high salt fermentations, such as *L. sakei* and *L. plantarum* (Chaillou et al. 2005, Kleerebezem et al. 2003). Similarly, the mold *A. oryzae*, associated with high solute environments, exhibits a gene duplication of the *Nik-1* gene associated with tolerance of high osmotic pressure (Machida et al. 2005). *S. cerevisiae* also encodes genes that respond to osmotic stress (Giaever et al. 2002).

Besides its ability to tolerate pH changes and salt environments, *L. plantarum* possesses multiple heat shock response mechanisms (GroES, GroEL, and Hsp), enabling survival during cheese pasteurization for later ripening (De Angelis et al. 2004) and Csp that may be beneficial to the food industry during cold storage of starter cultures and final products (Kim et al. 1998, Mayo et al. 1997). Conserved Csp sequences were found in several LAB species, where they may also be beneficial during cold storage, including the GI tract–associated strain *L. acidophilus* (Altermann et al. 2005) and the fermentation strains *L. sakei* 23K (Chaillou et al. 2005) and *L. bulgaricus* (Serror et al. 2003). Heat shock proteins are present in other LAB and can be expressed in stress situations other than elevated temperature. This was observed with *L. cremoris* MG1363, which expressed the DnaK, GroEL, and GroES heat shock chaperones under high salt conditions that are used to harden cheese surfaces (Kilstrup et al. 1997).

Mechanisms to prevent oxygen damage are also found in both fermentation and GI tract–associated strains. *L. bulgaricus* and *L. acidophilus* both encode a *potABCD* operon, which is an ABC transporter for the polyamines spermidine and putrescine, suggested to protect against oxygen toxicity (Chattopadhyay et al. 2003, van de Guchte et al. 2006). *L. sakei* 23K contains several genes to respond to changes in oxygen conditions (Chaillou et al. 2005). Many lactobacilli do not encode a superoxide dismutase gene for oxidative stress protection. An example of an alternate oxidative stress protection strategy can be found in *L. plantarum*, which encodes peroxidases as well as transport mechanisms to accumulate the oxygen radical scavenger, Mn^{2+}, from the vegetable environments that are rich in these ions (Kleerebezem et al. 2003).

S. cerevisiae cultures used in winemaking encode Ssu1, a plasma membrane protein necessary for sulfite efflux. This mechanism allows *S. cerevisiae* to tolerate sulfite, used to control wild yeasts and preserve wine (Legras et al. 2007, Park & Bakalinsky 2000).

In addition to their tolerance to environmental conditions found in fermented foods, LAB are also widely noted for their ability to produce bacteriocins that inhibit the growth of closely related LAB and Gram-positive bacteria in general. These bacteriocins are generally considered to be agents that can promote competition of LAB in nutritionally rich environments and as quorum sensing signals that direct high cell density population behavior and activities (Diep et al. 2006, Eijsink et al. 2002, Pridmore et al. 2004, Kleerebezem et al. 1997).

Use of Molecular Biology to Expand Fermentation Capabilities and Metabolite Production in Domesticated Microorganisms

The advent of genomic information has enabled the discovery of the genes directing pathways responsible for many desirable activities of fermentation microbes. Moreover, the explosion of biotechnological tools now enables improved control of bioprocessing behavior and engineering of strains to enhance their beneficial attributes. For example, *L. lactis* has been modified for increased production of diacetyl by inactivating AldB and decarboxylating α-acetolactate in the presence of oxygen (Hugenholtz et al. 2000). *S. thermophilus*, which produces acetaldehyde mainly through the threonine aldolase activity of serine hydroxymethyltransferase, encoded by the *glyA* gene, has demonstrated increased acetaldehyde production when *glyA* was overexpressed (Chaves et al. 2002). Engineering of LAB to increase the production of specific metabolites beneficial to humans, such as folate, is also being investigated to enhance the nutraceutical benefit of fermented products

(Kleerebezem & Hugenholtz 2003). The multitude of opportunities for genetic engineering of LAB strains is exploding, with genetic improvements possible from flavor- and texture-producing genes to phage-related CRISPR sequences (Barrangou et al. 2007, Mollet 1999). As more genomes are sequenced, the possibilities will only increase.

SUMMARY

Domesticated microbes that have evolved to food environments created by humans show remarkable patterns of adaptation. Predominant among these are: (*a*) genome decay and loss of biosynthetic capacity consistent with their evolution to nutritionally complex food environments; (*b*) acquisition of new competitive traits, such as nutrient transporters, by HGT or through the acquisition of extrachromosomal elements; and (*c*) specialization to unique food environments through adaptive mutations that promote key fermentation activities such as inactivation of the LacR repressor in the milk-adapted *L. bulgaricus* (**Figure 2**). Overall, this genomic evolution has revealed the ability of fermentation microbes to evolve and specialize to food as their natural habitat. In this process, our food supply has benefited dramatically in the variety of safe fermented foods and the diversity of their textures, flavors, and nutritional values.

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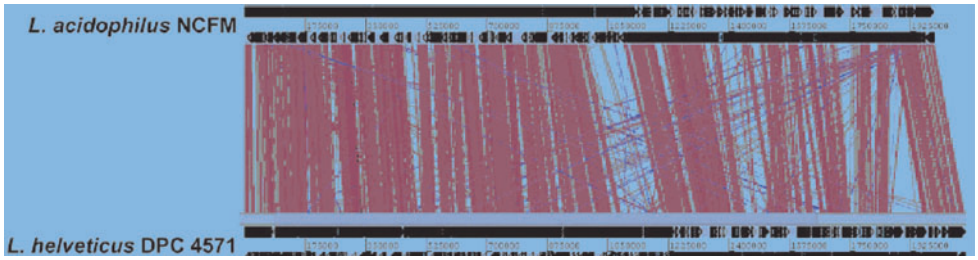

Figure 1

DNA similarities (BlastN matches) between *Lactobacillus acidophilus* NCFM and *Lactobacillus helveticus* DPC 4571, represented by the lines. Modified from Callanan et al. (2008).

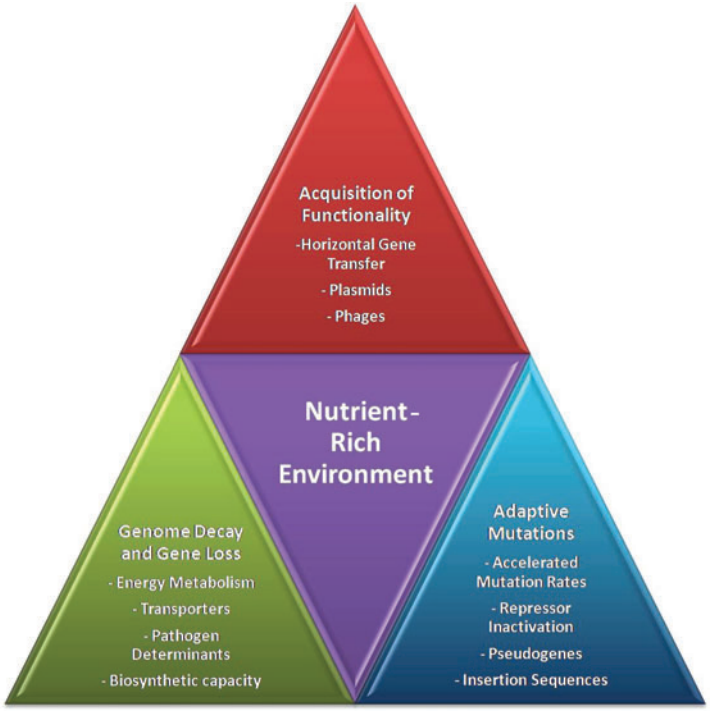

Figure 2

Genomic traits of domesticated microorganisms driven by evolution in nutrient-rich environments.

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Errata

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