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Introduction

Lactococci have been used for centuries in dairy fermentations. These Gram-positive, generally non-pathogenic, non-motile and non-sporulating bacteria are members of the Streptococcaccae family, which includes food, commensal and virulent species (**Fig. 1**). *L. lactis* is a relatively simple bacterium, with a 2.4 Mbp genome. Many of its functions of interest are non-redundant, which facilitates functional genetic studies of non-essential genes. Lactococci are also presumed to be devoid of virulence factors (although isolated cases did report *L. lactis* as the infectious agent in human and bovine infections; (1, 2)). The goal of this ~~updated~~ chapter is to confront previous and present information in different areas of lactococcal genetics, keeping in mind the relevance of findings to related bacteria, especially pathogens. Work on pathogens has long been focused on surface and secreted virulence factors, while work on lactococci has gone deeper in characterizing basic metabolic properties, nutrient uptake, and survival. Genes in basic metabolic pathways (e.g., respiration, metal homeostasis, amino acid metabolism) are now known to be essential not only for fitness, but also for virulence. Numerous lactococcus researchers who shifted their focus to pathogens have contributed to this understanding**.** The overall non-virulence of lactococci has also been useful in determining how metabolic and virulence factors participate in bacterial 'everyday life' outside the animal host. The deep knowledge of *L. lactis* physiology has led to new concepts and general findings, for example by, i~~)~~ establishing the bases for dialog between Firmicutes, ii~~)~~ giving concrete *in vivo* data on the biomedical or probiotic potential of recombinant and wild type lactic acid bacteria (LAB) (3, 4), and iii~~)~~ establishing the existence of an inverse correlation between bacterial mRNA concentration and stability (5).

This chapter, organized in five sections, highlights major work in lactococci, including metabolic capacities, physiology, stress response, interbacterial effects, and studies leading to novel uses of lactococci for protein delivery or as probiotics. Prophages have a primordial role in fermentation processes, genetic diversity, expression and cell lysis; this important area of

research, including abortive infection mechanisms is not a focus of this review (see (6-12)). Comparative *L. lactis* genome organization analyses are considered in the following publications and references therein (13, 14).

I. Basic features of lactococci: A primary description of *L. lactis*, and relationship to other Streptococcaccae are presented.

II. Metabolic options for lactococci: As lactococci are "industrial" bacteria, their studies focused on optimizing growth and flavor production during fermentation. Lactococci shift to a respiration metabolism when provided with an exogenous heme source. Metabolic flexibility provides a valuable prototype for lifestyle of other lactic acid bacteria and certain streptococcal pathogens, which respire when supplied with heme and also menaquinones. Nitrogen metabolism is extensively studied in *L. lactis*, as it determines the capacity to grow in dairy medium for food fermentation. Remarkably, nucleotide metabolism coordinate intracellulars pools with the envelope state.

III. Lactococcal stress responses: Lactococci, though largely consumed through fermented foods, have been considered as non-colonizers of the animal host. Lactococci express numerous and diverse factors that allow bacteria to respond and survive in a crowded or hostile environment, and were identified by simple selective systems. Unique structures discovered on the lactococcal envelope (pellicles and pili) may extend survival in harsh environments, notably the gut, and narrow the divide between food bacteria and pathogens.

IV. *L. lactis* and inter-bacterial dialogue: Lactococci impact expression and/or development of other bacteria in their biotopes, including pathogens. Several findings may prove valuable when considering the roles of cognate functions in pathogens.

V. Applications and genetic tools of lactococci: Lactococci are useful bacterial vectors for biomolecule delivery. Protein expression systems were developed in lactococci for applications in biotechnology, and have been used for expression in related low GC% Gram-positive bacteria

including pathogens; remarkably, some of them have stayed in use for over 25 years. As a simple non-pathogen, *L. lactis* is a useful host for separating potential virulence factors from pathogenic bacteria and analyzing their roles. While medical applications have not been marketed, numerous proof of concept studies show the feasibility of using *L. lactis*, or other lactic acid bacteria as biomolecule delivery vectors (see (15, 16) for reviews).

I. Basic features of lactococci

What is *L. lactis*?

Lactic acid bacteria (LAB) are named for their ability to produce lactic acid *via* a fermentation metabolism. *L. lactis* is a mesophilic LAB with an optimal growth temperature of ~30°C. It is the most extensively characterized LAB, which comprises a highly diverse group (**Table 1, Fig. 1**), including various cocci and bacilli. However, the term 'LAB' is misleading; although LAB generally refer to bacteria used in food fermentation, lactic acid producers also include opportunists and pathogens including Streptococci and Enterococci; of note, *Enterococcus faecalis*, despite its reported beneficial effects for fermentation, is now clearly emerged as an important clinical antibiotic-resistant opportunist pathogen that may lead to intestinal dysbiosis after antibiotic treatments (17-19). Among sequenced relatives, *L. lactis* is related to *Streptococcus mutans* (**Fig. 1**). The 2.4 Mb genome of *L. lactis* strain IL1403 ~~strain~~ is intermediate in size between Streptococcal pathogens like *Streptococcus pneumoniae* or *Streptococcus pyogenes* (reported as 2.1 and 1.9 Mb, respectively), and *E. faecalis* (3.4 Mb). Although described as "cocci", *L. lactis* subpopulations may differentiate to a rod shape, possibly reflecting a change in proportions of wall and division enzymes (20).

L. lactis is classified within the ~~Streptococcaceae~~ Streptococcaceae family, and it appears that *L. lactis* and the pathogenic Streptococci may have a common origin. In addition to high genetic relatedness, DNA motifs called Chi, which are required for chromosomal integrity and

are overrepresented in *L. lactis*, are identical to Chi sites present in both pathogenic and food-derived ~~Streptococcaceae~~Streptococcaceae, giving evidence that common core genome organization preceded divergence of streptococcal pathogens and food bacteria (21). Relatedness between *L. lactis* and streptococci surpasses by far that with many other LAB (including *Lactobacillus* species; **Fig. 1**). Streptococci also produce lactic acid, and so may be considered as a branch of lactic acid bacteria. The Streptococcaceae family diverged by unknown selective pressures to generate groups of pathogens, colonizers, and food bacteria, which seem to have diversified to adapt to their preferred biotopes.

Varied lifestyle of LAB

L. lactis and LAB in general seem to have a varied lifestyle. Lactococci are isolated from plants, and are likely to be ingested by grazing animals, together with milk, in the case of calves. Co-ingestion could explain how lactococci ended up in milk. The need for several plasmid- or transposon- encoded characteristics for growth in milk (e.g., enzymes for sugar and protein metabolism, DNA restriction and abortive phage infection functions, and bacteriocins) supports the hypothesis that milk is not the original habitat of LAB.

Genome plasticity

Studies of lactococci have focused on two organisms, IL1403, a subsp. *lactis* strain, and MG1363, a *L. lactis* subsp. *cremoris* strain. Despite ~80% sequence identity between these genomes, they differ by a large chromosomal inversion (22). Even closely related isolates of MG1363 show considerable polymorphism, corresponding to large rearrangements (23) that might be mediated by mobile elements. Studies of artificial chromosomal rearrangements suggest that lactococci tolerate certain large genomic inversions if the origin and terminus regions are not disturbed (24).

Genome transfer and rearrangements may occur in lactococci *via* conjugation, transposition or insertion sequences, and phage transduction, as suggested from identification of

the required elements in different species, or by experimental systems in which natural DNA transfer occurs (see (25-28) for examples). DNA transfer occurs in streptococci by natural competence, as first discovered and then characterized in *S. pneumoniae* (29). Since then, competence among LABs was shown for *Leuconostoc mesenteroides* and *Streptococcus thermophilus* (30-33). *L. lactis* strains encode homologs of all late competence genes that are regulated by ComX; *comX* overexpression led to induction of several genes linked to late competence (e.g., *comGA, GB, GC, GD, EA, EC, FA, FC, C, dprA, coiA*), but these bacteria seemed to be defective for competence (34). Nevertheless, overexpression of ComX did lead to competence in a subgroup of lactococci (35, 36), but not in *L. lactis* model strains IL1403 and MG1363. In *L. lactis* strains KW2 and KF147, comX overexpression led to a transformation rate of 10^{-5} - 10^{-7} transformants/total cell number/µg of plasmid. These rates are similar to those obtained by electroporation in glycine-treated cells (37).

L. lactis IL1403 contains 40 repertoried insertion elements, 14 of which correspond to an IS element similar to IS1070 from *Leuconostoc lactis*, as well as at least four prophage elements (38, 39). *L. lactis* and *S. thermophilus* share common integrative conjugative elements, as well as highly conserved regions coding, for example, exopolysaccharide synthesis enzymes (26). Existence of functional genes that have been transferred to *L. lactis* is suggested by the presence of atypical regions, i.e., containing DNA that differs structurally from its context, or unexpected Orfs, *e.g.*, a hemolysin-like protein. Similarly, an *S. thermophilus* isolate was shown to encode a pigment that was suspected to be transferred from pathogens, and usually lost in milk bacteria (40).

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Close interactions between micro-organisms, e.g., in the gastro-intestinal or vaginal mucosa of animals, or in industrial milk fermentation processes, could lead to horizontal genetic exchange. Contact between these bacteria is also suggested by the existence of nearly identical genes, e.g., in lactococci and *S. thermophilus* (26). Close physical contact, visualized

microscopically between *S. thermophilus* and *Lactobacillus bulgaricus*, may facilitate genetic exchange (41).

II. Metabolic options for Lactococci

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Lactococcal metabolism has been intensively studied for its industrial importance in fermentation processes, with a focus on metabolic pathways and their engineering (42). Importantly, basic metabolic functions may have far-reaching effects, and as will be described below, metabolic shifts can result in dramatic changes in *L. lactis* growth characteristics and survival.

A remarkable metabolic process in lactococci, respiration, was essentially overlooked with the focus on dairy fermentation (43). Researchers confirmed and developed a 1970 study, showing that Lactococci not only ferment sugars, but are also capable of forming an active electron transport chain to generate respiration metabolism (44-47). *L. lactis* respiration requires an external heme source, due to an incomplete biosynthetic pathway (48). The respiration process and relevance for certain pathogenic Streptococci and numerous lactobacilli is discussed.

The two energy metabolism 'options', fermentation and aerobic/anaerobic respiration, are presented. Pathways for nucleotide metabolism are also described, including a link established between nucleotide homeostasis and cell wall regulation.

Energy option 1: Fermentation in *L. lactis*

Simplistically, lactococci use sugars to provide energy, and amino acids to synthesize proteins (**Fig. 2**) Dairy lactococci have multiple nutritional requirements for amino acids and vitamins, probably resulting from their adaptation to a life in milk. Lactose is the major sugar source in milk, and through its uptake and degradation, Lactococci generate energy in glycolysis. Casein, the major protein component in milk, is degraded to provide the major carbon source for anabolism. The flow of carbon for energy production is therefore almost separable from the flow of carbon for anabolism in these bacteria, making them ideally suited for metabolic studies.

All species belonging to the genus *Lactococcus* produce acid from glucose, fructose, mannose and N-acetylglucosamine. *L. lactis* species used for dairy fermentation undergo mainly homolactic fermentation of lactose and other sugars. *L. lactis* subsp. *lactis* strains are more versatile than subsp. *cremoris* in their use of diverse sugar sources, including maltose, ribose and trehalose (reviewed in (49)). Sugars may be transported by plasmid-encoded phosphotransferase system (PTS) systems in dairy strains, or at a slower rate by a permease in non-dairy strains (50).

Carbohydrate fermentation may be shifted from homolactic (lactate production) to mixed acid fermentation (acetic acid, formic acid, CO_2, acetoin, and ethanol produced in addition to lactate; **Fig. 2**). Two sets of conditions accompany this change, namely, i)- altered redox state created by increased aeration during growth; or ii)- reduced entry of sugar that is used for energy production:

i)- Oxygen appears to be involved in maintaining the NADH/NAD ratio, which itself seems to regulate the switch between homolactic and mixed acid fermentation (50); aerobic conditions result in oxidation of NADH to NAD^+ (catalyzed by NADH oxidases), thereby reducing the NADH/NAD^+ ratio in the cell. Lactate dehydrogenase (Ldh) is active at high NADH/NAD^+ ratios (i.e., low oxygen), while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited. Increasing the amounts of water-forming NADH oxidase from a nisin-inducible promoter was shown to decrease the NADH/NAD ratio and to decrease *in vivo* activity of Ldh. The increased pyruvate pool was directed to acetoin and the flavor compound diacetyl. Other types of engineering (e.g., through mutations which block specific pathways) can also result in altered flavor properties in lactococcal fermentation (reviewed in (42), with descriptions of applications in (51-54)). Note that Ldh is essential in *Streptococcus mutans*, but not in *L. lactis*, possibly because *S. mutans* lacks alternative pathways for re-oxidation of NADH that are present in *L. lactis*. Among the three Ldh-like Orfs potentially present on the *L. lactis* IL1403 genome, studies suggest that at least two genes encoding functional Ldh are potentially expressed (55).

ii)- Sugar entry may be decreased if galactose is used as an energy source or when lactose is transported by a permease (56). All sugar-carbon utilization produces pyruvate regardless of the growth conditions and the pathway; activity of enzymes using pyruvate as a substrate determines if fermentation is homolactic or mixed acid. Decreased sugar flow thus favors activity of enzymes giving rise to mixed fermentation products. One hypothesis is that when carbon fluxes are high, GAPDH is a bottleneck in glycolysis, resulting in high pools of upstream intermediates. These pools would then inhibit pyruvate formate-lyase in one of the fermentation pathways from pyruvate, resulting in homolactic fermentation under anaerobic conditions. The extent to which GAPDH is a bottleneck in lactococci was examined by constructing strains with GAPDH activities ranging from 13% to 210% of the normal activity, and measuring metabolic fluxes (57, 58). Surprisingly, GAPDH was in large excess even when carbon fluxes are high; in terms of flux control, GAPDH would thus not be the controlling factor. Moreover, the fermentation pattern remains homolactic even after four-fold reduction in GAPDH activity, which shows that GAPDH has no control on mixed acid flux either. Similar studies were carried out to determine the importance of other glycolytic enzymes on the fermentation pattern. Phosphofructokinase had no control on glycolytic flux, nor on mixed acid flux (59), despite the presence of this enzyme in excess (60). Ldh had no control on glycolytic flux either but did exert a strong negative control on mixed acid flux (61). An intriguing possibility is that the excess glycolytic enzymes are somehow shuttled to the surface, where they could play other roles in bacterial dissemination; glycolytic enzymes, like GAPDH, have been reported to be present on the surface of numerous Streptococcaceae~~Streptococcaceae~~ (62-64).

The genetic organization of enzymes involved in sugar utilization may reveal regulation at the transcriptional level. *L. lactis* appears to coordinate the expression of 3 genes, each involved in key but distinct steps in fermentation, *pfk*, *pyk* (encoding phosphofructokinase and, pyruvate kinase, respectively) and *ldh* genes by having them present in one operon (called *las*).

The operon organization may prevent unwanted accumulation of glycolytic intermediates. In *S. pyogenes* and *S. pneumoniae*, the *ldh* gene is not within this operon, suggesting that a common regulation of the three genes may be unique to lactococci. The *las* operon is induced in the presence of glucose *via* the catabolite control protein CcpA (65). Expression of the entire *las* operon was modulated to determine its importance for metabolic flux; flux was highest when expression was at its normal level, and decreased rapidly when expression was reduced or increased (59).

In *Escherichia coli*, glycolytic metabolic flux is controlled almost exclusively by the demand for ATP. In *L. lactis*, the ATP demand also controls flux in slow or non-growing cells but not in fast-growing cells (59, 66-68).

The importance of individual enzymes for system properties such as fluxes, metabolite concentrations, frequencies of infection, survival rates, etc., can be accessed by modulating the activities of the respective components. Using synthetic promoters has proven useful for obtaining accurate tuning of gene expression and optimizing metabolic fluxes (57, 58). Moreover, this technique allows numerous genes to be modulated simultaneously and at differential levels in a cell; in one example an *L. lactis* strain was constructed in which all 10 genes encoding the enzymes of the glycolytic pathways were up-regulated using synthetic promoter libraries (69).

Energy option 2: Respiration in *L. lactis*.

Components of the electron transport chain: An active respiration chain comprises three elements: i) an electron donor, supplied by NADH dehydrogenases, ii) quinones (a non-protein component of the respiration chain), which deliver electrons to the terminal oxidoreductases, and iii) terminal oxidoreductases which contain heme as an essential cofactor and use oxygen as final electron acceptor (70, 71). Although *L. lactis* undergoes fermentation in a rich medium, addition

of a heme source to aerated medium activated a respiration metabolism in *L. lactis* (44, 46). Demonstration of the existence of an electron transport chain and heme-dependent membrane NADH oxidase activity, and identification of factors implicated in respiration, all point to the major impact of this metabolic mode on bacterial growth and survival (**Fig. 3**; (45-47, 72-75)). Heme--stimulated growth leads to an approximate doubling of cell biomass (in keeping with greater energy production by respiration activity), increased pH, and massive production of acetoin rather than lactic acid (46, 76). In combination with sugar--limiting conditions, as occurs during growth on maltose, the biomass yield increased further and indicated a reversal of the function of the H^+-ATPase towards the direction of ATP synthesis (77). An important feature of respiration metabolism is decreased oxidation in the cell, due to respiration-dependent oxygen elimination. This leads to a more reduced intracellular environment and consequently less oxygen-induced damage. This feature, together with increased pH, may explain the extraordinary increase in bacterial survival during respiration growth (46, 72).

Like other Gram-positive bacteria that grow *via* a respiratory metabolism, *L. lactis* utilizes only menaquinones as electron carrier to the terminal oxidoreductase. Inactivation of *menB*, which encodes a menaquinone biosynthesis enzyme (dihydroxynaphthonic acid synthase), totally abolished both quinone production and respiration in *L. lactis* (78). However, while other respiring bacteria have several oxidoreductases that assure respiration under different conditions, *L. lactis* uses a single enzyme, the cytochrome *bd* quinol oxidase (encoded by *cydAB*) (45, 46). This kind of oxidase has a high affinity for oxygen (79). Moreover, as shown for *S. agalactiae*, cytochrome bd quinol oxidase contributes to both virulence and colonization ((80, 81); YY, AG, and PG unpublished data).

Respiration in *L. lactis* requires uptake of heme (iron is insufficient), possibly suggesting the presence of a heme transporter. Genetic studies suggested that the *fhu* operon mediates heme uptake (75). However, more recent studies suggest that heme might diffuse into *L. lactis*

membranes and then be recovered by cytoplasmic heme-binding factors. Heme reduction by menaquinones appears to favor membrane incorporation (82). Other factors that facilitate heme entry *via* diffusion are being investigated.

Based on the dramatic differences in growth, survival, and metabolite production, we had anticipated that respiration growth would induce massive changes in gene expression when compared to fermentation. Surprisingly, expression of very few genes was specifically altered by respiration, as evaluated by proteomic (21 proteins) and transcriptomic (11 transcripts) analyses of late exponential phase cells (73, 83). These studies led to the hypothesis that NADH/NAD balance, which is shifted towards NAD by respiration chain activity, is responsible for modulating enzyme activities that accompany respiration growth (**Fig. 3**). Significant changes in expression were observed for a single operon, as discussed below (see section on heme homeostasis) (73).

Role of CcpA in respiration: The regulator CcpA, controlling catabolite carbon repression, imposes a hierarchical use of carbohydrates (84). CcpA controls carbohydrate catabolism (glycolysis) and positively controls production of Ldh (65), which converts pyruvate to lactate. CcpA seems to also coordinate respiration metabolism at different levels: The first control concerns sugar uptake, as *L. lactis* does not use amino acids or glycerol as carbon source (85). CcpA also controls the transporter *fhu*, which appears to contribute to heme uptake (75). Third, CcpA regulates expression of genes encoding protein components of the respiratory chain.

In *L. lactis*, *ndh* (encoding NADH dehydrogenase; Ndh) and *cydAB* are present on distinct operons (85, 86), while in other LAB they appear to be organized in a single operon (80, 87). The latter organization suggests coordinate expression of *ndh* and *cydAB*, and may indicate that the respiratory chain is fueled by just one NADH dehydrogenase. In *L. lactis*, two putative NADH dehydrogenase genes (*noxA llmg_1735*; *noxB*, *llmg_1734*) are present in the genome and both enzymes could drive electrons to menaquinones. Though *noxA* is adjacent to *noxB*, RNAseq

analyses indicated that they are not in an operon (86). In the case of *noxB*, direct control by CcpA is suggested by the presence of a CcpA binding motif cre (catabolite response element) in the promoter region. Altogether, *noxB* seems to be repressed by CcpA while *noxA* and *cydAB* are induced (88). Deletion of *noxA* abolished respiration activity on glucose growth, while the role of *noxB* is unknown (89). As *noxB* is likely induced late in growth (when CcpA is less active) it may drive electrons to menaquinone as does NoxA, but in late growth.

Interestingly, CcpA does not control respiration-induced acetoin production, as gene expression of this pathway was not affected by *ccpA* deletion (88). A challenging question concerns regulation of the pyruvate-acetoin pathway, which is functional late in respiration growth, as evidenced by acetoin accumulation in stationary phase cells; ~70% of the pyruvate pool is converted to acetoin (46, 73, 76) (**Fig. 3**). Interestingly, this pathway produces not only acetoin, but also carbon dioxide, which may contribute to lowering medium acidification in respiration growth (pH 6, compared to pH 4.5 in fermentation; both media contain 1% glucose as energy source) (PG and colleagues, submitted). The search for regulators and/or cofactors of the pyruvate-acetoin pathway, and for regulatory checkpoints of *L. lactis* respiration is ongoing.

Although *L. lactis* is only equipped for aerobic respiration, other LAB can also perform anaerobic respiration. In *Lactobacillus plantarum*, nitrate can be used as electron acceptor instead of oxygen. Reduction of nitrate into nitrite is performed by a quinone-nitrate reductase (NarG). Nitrate respiration is repressed by glucose suggesting potential participation of CcpA in anaerobic metabolism in this organism (89).

Heme homeostasis is a key to L. lactis respiration: Transcriptome studies of *L. lactis* in respiration *versus* fermentation conditions during exponential growth revealed strong up-regulation of components of a single operon *ygfCBA* encoding a putative transcriptional regulator (YgfC; also detected by proteomics (83)), a predicted permease (YgfB) and an ATPase (YgfA) (73). YgfB and YgfA are HrtB and HrtA orthologs, *hrtBA* for “heme-regulated

transporter", encoding HrtBA, an ABC transporter that was found in parallel in *Staphylococcus aureus* (90). Its function was subsequently clarified as a dedicated heme-efflux pump, which was necessary and sufficient to maintain intracellular heme concentrations at subtoxic levels while allowing sufficient intracellular amounts for functional respiration (**Fig. 4**). The predicted TetR family repressor YgfC, renamed HrtR, regulates expression of *hrtBA* in *L. lactis*. HrtR shows high-affinity for heme through a non-covalent hexacoordinated interaction with heme iron (74). HrtR binds a 15-nt palindromic sequence in the *hrtRBA* promoter region, which is needed for repression (74). Heme binding to HrtR modifies its conformation, releasing it from its target promoter and alleviating transcriptional repression of *hrtRBA* (74, 91).

The use of HrtR as an intracellular heme sensor and *hrtBA* regulator appears to be conserved among commensal bacteria. In contrast, numerous Gram-positive pathogens use an extracellular two-component system, *hssRS*, to regulate hrtBA (92). These findings point to an essential role of efflux for heme homeostasis in *L. lactis*. In contrast, heme import mechanisms remain elusive, despite a partial role for *fhu* (75). Membrane-associated menaquinones (MK) were shown to favor the accumulation of reduced heme in membranes (82). An oxidative environment, provided by oxygen, prevents and reverses hemin reduction by MK and thus limits heme accumulation in membranes (82). HrtBA counteracts MK-dependent membrane retention of excess heme in membrane, suggesting direct efflux from this compartment. Moreover, both HrtBA and MK-mediated reduction have a strong impact on heme intracellular pools, detected as induction of a HrtR heme sensor. This indicates that intracellular heme acquisition is controlled at the membrane level without the need for dedicated import systems, at least at high heme concentrations (82). While pathogens may have access to blood heme during infection, the existence of heme-responsive genes in lactococci and other commensal bacteria raises questions concerning the nature of heme sources in their natural ecosystems (48).

Roles of respiratory chain components in non-respiration processes: Milk, a main industrial medium for food fermentation, lacks heme and thus cannot support *L. lactis* respiration

metabolism. However, respiration chain components do affect fermentation by decreasing milk redox potential of. *L. lactis* is one of most reducing LAB, which provides a very negative redox potential value in milk. To establish low redox potential, oxygen must be removed and oxidized compounds should be reduced. During fermentation, water-forming NADH oxidase (NoxE) eliminates oxygen, and respiration chain components (NoxAB and MenC) provide lower redox potential by reducing oxidants in milk. Redox potential of milk is known to affect the microbiota and sensorial quality of fermented dairy products. Thus, although the *L. lactis* respiration chain is incomplete in milk due to the absence of a heme source, its reducing ability plays a significant role during fermentation (93).

L. lactis is a respiration prototype: Lactococci, unlike *E. coli* or *Bacillus subtilis*, respire using a restricted electron transport chain, and only if heme is provided. Comparative studies reveal that certain, but not all StreptococcaceaeStreptococcaceae, and certain other Firmicutes have the capacity to respire when provided with heme and a menaquinone source (45) (**Fig. 1**). They include *S. agalactiae* (80, 94), *E. faecalis* (95, 96), and *L. plantarum* (89). Since the first characterization in *L. lactis*, a far wider group of LAB are revealed to adopt respiration metabolism when heme, or heme and menaquinones, are added (see (48) for review).

Nitrogen metabolism

In a milk medium, lactococci derive amino acids from casein, via hydrolysis by the extracellular protease PrtP, transport of the generated peptides, and further degradation by a multitude of intracellular and envelope proteases and peptidases (97, 98). Amino acids readily available in milk are used both directly as amino acid building blocks, and also as a general carbon supply for other forms of anabolism in lactococci. Extracellular proteases, plus at least 14 intracellular peptidases are of key importance for amino acid utilization. Lactococci grow poorly or die in milk fermentation conditions in mutants that are devoid of different combinations of these peptidases (99).

Dairy lactococci differ from plant lactococci in that they require several amino acids for growth. Surprisingly, strains of both origins appear to have the necessary genes for biosynthesis. Nevertheless, dairy lactococci require Ile, Leu, Val and His, and sometimes Arg, Met, Pro and /or Glu (100). These amino acid requirements in dairy strains appear to result from multiple mutations rather than deletions in the structural genes (39). This may suggest that mutations accumulated as an economic measure in strains maintained in a dairy environment. Similar results are reported for *S. thermophilus*, a dairy bacterium related to *S. pyogenes*.

Nucleotide metabolism

Nucleotides are not only substrates for DNA and RNA polymerases, but are also substrates or allosteric effectors for many enzymes, and furthermore constitute parts of different coenzymes. Thus, mutants in nucleotide metabolism may display numerous phenotypes. Cell nucleotide pools are also influenced by the presence of exogenous nucleobases or nucleosides in the medium. Pathways of uptake and utilization of these compounds (the so-called salvage pathways, which vary in different organisms) are key contributors to bacterial responses to changes in the medium and to increased intracellular degradation of nucleic acids as illustrated below.

As seen above for operons involved in carbon metabolism, gene organization has unique characteristics in lactococci, which may impact regulation. For example, genes encoding pyrimidine (thymine, cytosine, and uracil) biosynthesis pathway leading to the formation of UMP are organized in 5 different operons in *L. lactis*, and in a single operon in most investigated Gram-positive bacteria. Similarly, purine (adenosine, guanine, and xanthine) biosynthesis genes involved in the first ten steps leading to IMP production are located in five separate operons in *L. lactis*, and in a single operon in *B. subtilis.*

Pyrimidine biosynthesis is regulated by attenuation and antitermination by PyrR. The PyrR protein binds to PyrR 'boxes' (5'-UCCAGAGAGGCUNGCAAG-3') present on the 5´ends of the untranslated mRNA´s on four of the identified pyrimidine biosynthetic operons (101, 102).

Mutation of the *pyrR* gene results in constitutively increased levels of the pyrimidine biosynthetic enzymes (101). The active RNA binding form of PyrR in *Bacillus* is a dimer which is formed when UMP, UTP or PRPP (5-phosphoribosyl-1-pyrophosphate) is bound to PyrR; GTP counteracts UTP binding and thus promotes transcriptional read-through (103). The regulatory mechanisms seem to be the same in *L. lactis* (104). Interestingly several *pyr* operons in *Mycobacterium smegmatis* have been found to be regulated by PyrR by translational repression (105).

Purine biosynthesis in *L. lactis* is positively controlled by PurR, and *purR* mutants are purine auxotrophs. PurR binds to a Pur box consensus sequence (5' ANNNCCGAACAAT 3') (106-108) to activate transcription of the *purC* and *purD* operons. Data show that the PurR activating effector is PRPP. As PRPP synthetase is inhibited by ADP and purine addition leads to increased purine pools (104), purine addition may indirectly decrease gene expression (108, 109). Interestingly, riboswitch control of the PurR-regulated *xpt* operon is modulated by purine pools (104). In operons activated by PurR, the distance between the pur box and the –10 region was 57-58 bp. Mutational studies of pur boxes supported the importance of the central CCGAAC sequence. Interestingly, while *L. lactis* PurR often acts an activator, the homologous PurR in *B. subtilis* usually works as a repressor. In addition to the importance of PurR binding sites, the two PurR proteins also differ in their PRPP binding properties. The two types of PurR proteins are related, and the *Bacillus* type seems to have evolved early on from the activator type of PurR (109).

The purR regulon includes purine biosynthetic genes, but also genes involved in purine uptake and conversion into purine monophosphates. Genes involved in C1 carbon metabolism, ppGpp metabolism, phosphonate transport and pyrophosphatase activity were also identified. Interestingly, a Pur box was also identified upstream of two ribosomal RNA operons, which

could imply an interconnection between the purine pathway and translation, but this link needs to be confirmed (104, 106).

L. lactis can utilize exogenous nucleobases or nucleosides present in the medium or formed from intracellular degradation of nucleic acids *via* the salvage pathways. Nucleobases uracil, guanine and adenine, as well as hypoxanthine and xanthine, are taken up and converted to nucleotide monophosphates, while cytosine is not utilized by lactococci (110). Orotate may be incorporated in plasmid-bearing *L. lactis* strains that express an orotate transporter, and may be used for UMP biosynthesis, since OMP is a normal intermediate (111, 112)**.** Thymine may be incorporated via pyrimidine phosphorylase to thymidine and further phosphorylated by thymidine kinase (*tdk*) to the corresponding monophosphate (110). Thus all common nucleobases except cytosine can be transported and incorporated into nucleotides. The ability of *L. lactis* to scavenge nucleobases or nucleosides can confer a survival advantage in stress conditions.

In *L. lactis*, all nucleosides except cytidine can be degraded to the corresponding nucleobase. However cytidine may first be deaminated to uridine and further to uracil. Pyrimidine nucleosides may also be directly converted to nucleotides since the corresponding nucleoside kinases (*udk* and *tdk*) are functional in lactococci. Two high affinity nucleoside transport systems were identified: UriP is specific for uridine and deoxyuridine, while BmpA-NupABC takes up cytidine and purine nucleosides, and the corresponding deoxyribonucleosides (113).

III. Lactococcal stress responses

Stress response is the adaptation to homeostatic changes due to the environment, and is generally modulated by sensors and regulators that sense the state and respond to signals. Major signals for *L. lactis* include metabolites like nucleotides, acid (self-induced), oxygen, salt, and metals such as heme (natural or industrially provoked), and toxic products (*e.g.*, disinfectants). We refer

readers to recent reviews on LAB stress responses (114) and on regulatory signals in *L. lactis* (115). Here we focus on specific effectors and responses that were not previously considered, *i.e.*, nucleotides, Spx, cyclic-di-AMP, and heme and respiration stress responses.

Regulation by nucleotide pools

Low nucleotide pool sizes may serve as internal stress signals that provoke expression of stress response genes in *L. lactis* (116, 117). An important link between nucleotide pools and cell wall synthesis was uncovered: As part of the uridine synthesis pathway, PyrB converts l-aspartate (l-Asp) to N-carbamoyl-l-aspartate. PyrB competes with cell wall enzymes for l-Asp; thus, l-Asp consumption during rapid growth favors wall flexibility, while its accumulation in stationary phase may lead to a more rigid cell envelope (118). Additionally, high UTP pools in the human pathogen *S. pneumoniae* correlated with more capsule formation and lower biomass yield (119). These examples suggest that nucleotide pools connect nucleotide metabolism to cell envelope structure.

The Spx family of regulators

A hot spot for *L. lactis* mutants that compensated stress sensitivity of a *recA* mutant, thermosensitivity of a *clpP* mutant, metal toxicity, and oxidative stress, all mapped to the *spx* gene (117, 120, 121). Spx is conserved in numerous Firmicutes and was shown in *B. subtilis* to be involved in oxidative stress regulation *via* thioredoxin gene expression (122). Seven *spx* homologues exist in *L. lactis* MG1363, five of which contain redox-reactive C-X-X-C regions (121). Exploration of other Spx proteins revealed that SpxB, a non-redox sensitive Spx, as a key regulator of cell wall resistance to lysozyme by activating O-acetylation of peptidoglycan (123). The Spx family is intrinsic to cell responses to oxidative stress and cell wall damage conditions.

Cyclic-di-AMP as second messenger in bacterial adaptation and potential role in heme sensitivity

Nucleotide pools are required for DNA synthesis, but are also involved in numerous stress responses, including amino acid starvation and acid stress (e.g., *via* ppGpp) (124). More recently, cyclic di-AMP was identified as a second messenger in stress adaptation (125). In *L. lactis*, cyclic-di-AMP homeostasis is a balance between synthesis via adenylate cyclase enzyme CdaA (Llmg_0448) and degradation by cyclic-di-AMP phosphodiesterase enzyme (GdpP, Llmg_1816). Increasing the cyclic di-AMP pool by deletion of *gdpP* increased heat resistance and salt hypersensibility in *L. lactis* (126). These phenotypes might be due to a change in peptidoglycan (PG) synthesis (127), as i)- salt sensibility in the *gdpP* mutant is suppressed by deletion of *cdaA* or *glmM* encoding a phosphoglutaminase enzyme involved in PG integrity; ii)- *cdaA* is in an operon with *glmM*; iii)- GlmM interacts with CdaA to modulate its activity; iv)- *L. lactis* cells spontaneously lyse when cyclic-di-AMP pools are decreased. The exact role of cyclic di-AMP in PG synthesis in *L. lactis*, though unknown, may be to modulate enzymatic activity to control cell wall plasticity. In contrast to CdaA, small molecules might interact with GdpP to modulate its activity. In *B. subtilis*, the GdpP homolog (YybT) harbors a PAS domain, which contains a heme binding site (128). Interestingly, *L. lactis* GdpP also harbors this domain and deletion of *L. lactis gdpP* gene increased sensitivity of cells to heme (128). These observations suggest a dialogue/link between heme homeostasis and cyclic-di-AMP pool enabling bacteria to adapt their physiology in response to exogenous heme.

In addition to its involvement in PG synthesis, cyclic di-AMP changed pyruvate carboxylase (*pycA*) activity in *L. lactis* (129). PycA is required for aspartate synthesis. Milk acidification is delayed in a *pycA* mutant compared to the wild type strain indicating that cyclic-di-AMP might modulate the fermentation process in some conditions.

Strategies for survival in stress conditions

L. lactis is equipped to deal with several stress conditions confronted in nature or in industrial settings, as summarized below. Salt and acid induce expression of GadB and GadC, which are

putatively involved in glutamate transport by an antiporter; glutamate transport presumably involves efflux of H^+, thereby maintaining intracellular pH (130, 131).

Toxic products such as bile, quaternary compounds, and antibiotics may be actively pumped out of the cell by specialized transport functions. Among the numerous transport systems that shuttle metabolites in and out of the cell, some mediate drug expulsion, and consequentially, can confer drug tolerance (132). In *L. lactis*, one multi-drug pump having specificity for a wide range of amphiphilic, cationic drugs (including antibiotics, quaternary ammonium compounds, aromatic dyes, and phosphonium ions) is LmrA. The *lmrA* gene encodes an efflux pump which is responsible for export of toxic molecules such as ethidium, etc in exchange for H^+ influx. LmrA (590 amino acids) is similar to the human multidrug resistance P-glycoprotein, thus raising questions about the origins of the pump. Judging from sequence analyses, an LmrA dimer would be the functional equivalent of the p-glycoprotein (LmrA is 32% identical to half of the P-glycoprotein particularly within known functional domains). Remarkably, LmrA is functional in eukaryotic cells, and is able to replace p-glycoprotein defects, thus making *L. lactis* an excellent model to study drug extrusion (132). Note that sequence comparisons predict an LmrA homologue in *S. pneumoniae* (an Orf with ~30% identity over 539 amino acids is present). The CmbT transporter (Llmg_1104), described initially as a cysteine and methionine biosynthesis transporter, was subsequently identified as a multi-drug efflux pump that effluxes a wide range of antibiotics and toxic drugs (133).

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Other efflux systems protect *L. lactis* from toxic metabolites: The dedicated heme-specific HrtBA efflux pump (see above) protects cells from heme toxicity. *L. lactis* homologs of heme and protoporphyrin IX efflux pumps identified in *S. agalactiae* may suggest that backup systems are available (134).

A series of exciting reports reveal that there is more to the envelope than what was previously thought: First, pili discovered in *L. lactis* led to the idea that these bacteria might

persist in the gut upon ingestion of fermented food products (135, 136). Second, a pellicle-like carbohydrate structure was visualized on the *L. lactis* MG1363 surface and characterized biochemically (137). A third structure, described as trapped within the peptidoglycan, corresponds to another pellicle-like carbohydrate thin outer layer surrounding *L. lactis* strain MG1363 (138).

Finally, basic cell metabolism can determine how well the bacterium copes with oxidative conditions. Respiration metabolism presents a clear advantage to lactococci in an aerobic environment for both growth and long term survival (Fig. 7; (46, 72)).

Selections leading to improved adaptation to environmental stress situations

Oxygen is a ubiquitous stress. If not eliminated, reactive oxygen derivatives provoke cell damage that can be lethal. One means of reducing oxygen-related damage is by removing oxygen. In fermenting cells, H_2O-forming NADH oxidases do eliminate oxygen during growth (139), although cells are sensitive to oxygen-related damage in stationary phase (140). Overproduction of H_2O-forming NADH oxidase could not only change metabolic end products (139), but also might improve survival in an oxidizing environment, due to oxygen removal. An alternative means of creating a more reducing environment is by adding glutathione, a redox peptide (141), or dithiothreitol, a reducing agent (75), to the medium. Lactococci lack catalase, which eliminates hydrogen peroxide in many aerobic bacteria. Hydrogen peroxide has been effectively removed by cloning catalase in *L. lactis* (142). Respiration metabolism in lactococci is a "natural" and efficient means of eliminating oxygen, compared to fermentation, leading to good survival in stationary phase (46, 72).

While acidification is ~~a~~ generated by the fermentation growth process itself, and as such is self-inflicted, cell survival is handicapped by acid accumulation (143). Significantly, acidification may be more severe if cells are immobilized, as acid diffusion may be slower. This situation may provide a natural selection for strains to escape from a constrained environment,

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and was shown experimentally to generate multi-stress-resistant mutants (116). Interestingly, acid resistant mutants evoked changes linking nucleotide pools (ppGpp) and cell wall alterations (116, 118). Studies using a semi-liquid medium have been used to impose a natural selection for mutants that can more readily escape a constraining environment (144). In one case, bacteria that make chains diffuse more slowly than single cells (**Fig. 8**). 'De-chaining' mutants, affected in penicillin binding protein and cell wall-synthesis enzyme PBP1A were isolated; the mutants no longer formed chains, and were able to disperse more readily in the semi-liquid medium; these mutants display greater permeability, probably due to increased cell wall breaks (144). In *S. agalactiae*, interruption of *ponA* results in reduced virulence that was linked to host antimicrobial peptide susceptibility (145, 146). In view of the identified role of PBP1A in lactococci, it is tempting to speculate that the *ponA S. agalactiae* mutant may also be defective in cell wall permeability and its chain-forming ability, thus impacting its *in vivo* localization. Use of semi-liquid medium for selections might prove effective in examining factors that are at work when bacteria are immobilized in their host, and has also proven useful in other types of selections in which surface properties are altered (137).

Transposon insertional mutagenesis was also used to select for stress-resistant strains of lactococci. Three examples are given: i)- A combination of stress conditions is lethal for lactococci (and possibly for other organisms), although each condition alone may be non-lethal. Simultaneous high temperature (37°C), oxygen, and either low pH (i.e., similar to conditions in the stomach) or a *recA* background, give rise to mutants of which many seem to affect intracellular metabolic pools of guanosine-phosphate and phosphate in stress response (116, 117). Low intracellular levels of these metabolite pools in the mutants may constitute a starvation signal to induce a stress response. These mutant strains show better long term survival than their non-mutated parents. It thus appears that a general stress response is induced in *L. lactis* when intracellular guanosine-phosphate and phosphate pools are low. In accordance with this, *L. lactis*

showed resistance to acid and heat stress when grown in milk or a synthetic medium without purines (147). ~~i~~Ii)~~-~~ Hydrogen peroxide is toxic to lactococci. An H_2O_2 -resistant mutant was isolated at high temperature (37°C), and although its resistance was 1000-fold greater than the parental strain, it displayed no other stress resistance phenotypes (148). One interest of this type of strain is its capacity to live in co-culture with strains producing mM amounts of H_2O_2, e.g., some Lactobacilli (149), and Streptococci (150). In the former case, use of H_2O_2-resistant lactococci could lead to development of new fermented products; in the latter, more efficient growth of lactococci could improve the hygiene of food products. ~~i~~Iii)~~-~~ Mutants were selected for increased resistance to dithiothreitol (DTT), a reducing agent, at elevated temperature. By preventing the formation of disulfide bonds, DTT disables a part of the oxidative stress response pathway (numerous lactococcal stress-response proteins contain one or more CXXC motifs). All 18 DTT-resistant mutants mapped within a single operon, *pst*, involved in phosphate transport. Greater oxidative stress tolerance of *pst* was linked to its effects on copper and zinc homeostasis (151).

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Stress resistant lactococci have several potential uses. First, such strains are potentially valuable in dairy fermentation. Their greater resistance to stress may overcome survival variability as seen in conventional strains. The specifically acid-resistant strains may provide resistance to extreme acid pH conditions, or may be better at maintaining a neutral internal pH. Stress resistant strains may survive longer in fermentation, and may also be more resistant to harsh storage conditions (like freezing and lyophilization). Second, as such strains may survive better to the harsh environments in the gut, they may be attractive for probiotic uses. In a proof of concept mouse inflammatory gut model, evidence was given that antioxidant superoxide dismutase produced by wild type *L. lactis* may improve gut integrity (4, 152, 153). Third, lactic acid bacteria are potentially valuable candidates for production of molecules with medical or biotechnological uses; Lactococci are non-toxic and have documented potential for expressing

and exporting proteins or other molecules of interest, either for industrial production, or in the gut.

IV. *L. lactis* and inter-bacterial dialogue

Several *L. lactis* properties were shown to either stimulate or inhibit bacterial growth, in some cases of opportunist pathogens, in unique and interesting ways. Three examples are described:

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Interbacterial cross-feeding

Via menaquinones: Other than *Lactococcus*, *Leuconostoc*, and *Enterococcus* species, most known LAB (pathogen or not) lack a complete menaquinone biosynthesis pathway, and require exogenous menaquinone or precursor, in addition to heme, for respiration growth (48, 89, 154, 155). Unexpectedly, menaquinone–deficient strains can overcome this deficiency *via* contacts with menaquinone producer species. For example, respiration of *S. agalactiae*, an opportunistic pathogen, is activated in the presence of *L. lactis via* menaquinone donation, and a heme source (78) (**Fig. 5**). Remarkably, *E. coli* expulses dihydroxynapthoate acid (DHNA), which is used by *S. agalactiae* to synthesize its own menaquinone (155). DHNA is coupled to an isoprenyl chain by menaquinone prenyltransferase, encoded by *menA*. In *Lb plantarum*, a *menA* ortholog is adjacent to the *cydAB* locus, suggesting that this organism can also use DHNA to synthesize menaquinones. Free menaquinones and DHNA are likely present in host ecosystems and can be used to activate metabolic pathways in commensal and pathogen bacteria as well. Thus, environmental non-pathogens might contribute to fitness and potentially virulence, of neighboring pathogens.

Via respiration: Respiration growth leads to higher pH and efficient oxygen consumption. The presence of a respiration-positive *L. lactis* strain (grown aerobically with heme) rescued growth and survival of a respiration-negative mutant strain (tested with *cydA*) (**Fig. 6**).

Interbacterial cross-inhibition

Via secreted products: Lactococci, like other Streptococcaceae, produce toxic hydrogen peroxide under aerobic fermentation conditions, which may inhibit growth of peroxide-sensitive bacteria (72). Numerous LAB produce bacteriocins, which have widely different host spectra (see (156) for review); lysis of heterologous bacteria by bacteriocins may provide needed nutrients for the producer cells. Production of lysins may act to regulate bacterial cell wall properties *in trans* (157).

Via regulation of expression in trans: Studies in mixed cultures give evidence for inhibitory effects of *L. lactis* on *S. aureus* growth and/or virulence gene expression. Tests conducted in either food or mammary gland cell lines indicate that i) *L. lactis* reducing activity contributes to inhibition of a major virulence gene regulator (*agr*), and ii) *L. lactis* at high bacterial concentrations inhibits *S. aureus* internalization in a mammary epithelial cell line (158, 159). These findings open prospects for antibiotic alternatives for prevention of infection, although their applicability to food industry and *in vivo* situations remain to be confirmed.

V. Applications and tools of Lactococci

The use of lactococci in 'bioprotein' delivery, i.e., for antigen or enzyme delivery *in vivo*, is based on the idea that *L. lactis* can act as an effective non-pathogenic carrier, which can be administered orally or nasally without provoking a reaction other than that induced by the bioprotein of interest. Prior to this update, numerous reports gave encouraging results for using lactococci for: i0 prophylaxis to prevent bacterial infection (160), ii) treatment of inflammatory bowel disease (3), iii) prophylaxis and/or treatment of virally-induced tumors (161), and iv) allergy prevention (162, 163). Other properties of lactococci, including the expression of pili that might mediate bacterial adhesion *in situ* (135, 136), and novel applications were since reported (164), which support their uses in biomolecule delivery. Despite a strong start, pharmaceutical drug development using *L. lactis* has not been reported, possibly due to the cost of development

and the existence of industrially-backed alternatives. Reported success with bioactive molecules might change this picture in the future.

Numerous applications using lactococci result from the development of genetic tools. Interestingly, a thermosensitive plasmid developed in *L. lactis* over 20 years ago, pG+host, is still the gene replacement and transposition tool of choice in numerous Firmicutes including pathogenic bacteria (165, 166). pG$^+$host is a derivative of broad host-range plasmid pWV01, and is non-replicative in *L. lactis*, streptococci, staphylococci, and in some lactobacilli at temperatures of 37.5°C or above. The thermosensitive replication protein has been retooled to produce numerous derivatives. Furthermore, pG+host is replicative in *E. coli*. The existence of an *E. coli* strain bearing a chromosomal copy of the non-temperature-sensitive *rep* gene (167) facilitates gene cloning, as well as characterization of pG$^+$host insertions.

Another tool that has withstood time is the nisin-inducible expression system. Nisin is a bacteriocin encoded by a conjugative transposon (168-170). The promoter for the nisin biosynthesis gene, *nisA*, is regulated by *nisR* and *nisK* gene products. In the absence of nisin, or of *nisR* and *nisK* genes, promoter activity is very low. Addition of sublethal amounts of nisin results in strong induction of promoter activity, as demonstrated in innumerable applications of this system. This system has been shown to be functional in other Firmicutes (see (171) for review). Expression systems have also been developed for wider use among Firmicutes, and may be advantageous in some cases over the nisin system. Controlled expression systems have arisen from studies of pH, salt, metal, heme, chloride, or sugar- regulated promoters (see (115) for review); some systems may overcome two potentially important limitations of the nisin system: i)- The requirement for either a specific host strain or a second plasmid (to provide *nisR* and *nisK* genes), and ii)- effects of nisin on the membrane, which may in particular be undesirable in studies of membrane proteins.

Highly regulated promoters can also be turned around for use as specific sensors. An interesting example concerns the HrtR repressor, which regulates heme efflux (**Fig. 4**). This, and other heme-responsive promoters have been developed as sensitive heme sensors, with proof of activity *in vivo* ((81), DL unpublished data).

Constitutive expression of promoters at fixed levels can be valuable for quantitative physiological studies or for fine-tuning of gene expression in biotechnology. *L. lactis* promoters P45 and P32 are commonly used, and strong constitutive promoters have been described (172). A set of synthetic promoters that differ by the sequence and length of spacers between the consensus sequences allow a broad range of constitutive activities (57, 173). A high expression promoter based on the PTS system is further enhanced by cellobiose and shown to be active in *B. subtilis* (174).

Site specific single copy integration

Lactococcal bacteriophages were initially studied with the goal of controlling starter culture lysis during fermentations. These phage studies led to the characterization of numerous bacterial strategies to abort phage activity. Phage studies have also been exploited to develop a site specific integration system. Integration of temperate phages makes use of a phage-specified integrase that catalyzes insertion of the phage at a specific bacterial target, which is often localized at, or near a tRNA gene. Using elements of lactococcal bacteriophage TP901-1, a site specific integrative vector was designed to obtain chromosomal single-copy integration (175). This system should allow stable insertion and expression of foreign genes, and can also be used to study expression of genes in single copy under different growth conditions. The TP901-1 integration system has also been used in human cells (176). Another integration system based on a lactococcal intron led to efficient and stable insertion of genes without the need for selection (177).

Immune system against phages: CRISPR-Cas system

Chromosomal manipulation was upstaged by a mechanistic breakthrough made in *S. themophilus* on phage resistance *via* the CRISPR-Cas system (CRISPR~~:~~, clustered regulatory interspaced short palindromic repeat, Cas: CRISPR-associalted), which comprises a natural immune system against phage infections. (178). During phage DNA infection, a bacteriophage DNA fragment is integrated in a specific chromosomal CRISPR locus. During a second attack with the same bacteriophage, small RNA expressed from the CRISPR system directs adjacently expressed Cas nuclease to cleave phage DNA, which prevents new infection. CRISPR-Cas is present in various LAB including *S. thermophilus* and lactobacilli, but is uncommon in lactococci (179), although applications of the system are seen as ubiquitous (180).

Protein export and display systems

Protein export reporters were developed to probe membrane protein structure and identify export signals (181) (182, 183). One such reporter, the nuclease of *S. aureus*, is a stable, well characterized protein which is active when present as an amino- or carboxy- terminal fusion to other peptides, and is faithful in reporting export events and in determining membrane protein topology. It is used in *L. lactis* and other Firmicutes to follow expression of exported proteins under different environmental conditions. The major advantages of using the nuclease over previously described export reporters are that it rapidly assumes its conformation and so avoids degradation, and as few as ~300 nuclease molecules per cell can be detected in colony assays (182-185).

The use of *L. lactis* as a ~~cell~~ bacterial factory has been developed to deliver proteins of interest in the extracellular medium, or on the bacterial surface. The export signal of Usp45, a secreted *L. lactis* protein, or signals from identified secreted native or heterologous proteins are routinely used. Secretion efficiency can be improved by introducing (if necessary) an overall negative charge at the N-terminal end of the mature, translocated secreted protein (184). Advantages of using LAB other than *L. lactis* concern transit time in the intestinal tract; systems

are thus being transposed to lactobacilli, such as *Lactobacillus plantarum* to improve efficacy (see (164) for review).

Anchoring of exported proteins *via* C-terminal LPXTG motifs is widely documented, particularly with respect to virulence factors, such as *S. pyogenes* M-protein (186-188). *L. lactis* encodes several anchored proteins, and at least two sortase-like proteins (189). Expression of the anchoring motif of the *S. pyogenes* M6 protein has been adopted to express recombinant proteins on the lactococcal surface (190). The AcmA autolysin is also cell wall-associated, due to the presence of a three-times repeated LysM motif (191). Anchoring *via* the AcmA binding motif was found to effectively present antigens at the lactococcal surface; a spacer between the anchoring domain and the protein of interest (a fimbrial protein) facilitates its access to target epithelial cells (192).

Field Code Changed

Expression strains

Lactococci are remarkable for their simple genomes, compared to complex bacterial models, such as *B. subtilis* or *E. coli*. An interesting example is HtrA, which is the only surface protease in *L. lactis*. In contrast, *B. subtilis* and *E. coli* both encode numerous exported proteases (193). An *htrA* mutation leads to temperature sensitivity; at 30°C, exported proteins showed increased stability, although activity was not necessarily improved (193).

Cell lysis systems

Controlled cell lysis is a potentially powerful means of arresting cellular and metabolic activity; in fermentation, it may additionally result in a coordinated release of enzymes which could accelerate product maturation. The host autolysin, AcmA (194), or bacteriophage encoded lysins and holins (which allow lysin release) are good candidates for this purpose. This application is potentially useful in controlling cell growth in fermented dairy products, as well as for enzyme release (195, 196). Expression of lysin and holin by a nisin-induced promoter does indeed appear to accelerate cheese ripening (197). Lysins, derived from bacteriophage specific to several

pathogens, effectively prevented or treated infections by streptococci, bacilli, and staphylococci in mice (198-200).

The cell envelope is an important barrier protecting the cell from stress situations. Cell wall damage via autolytic enzymes can render cells more sensitive to environmental conditions. Bacteria that have undergone even partial cell wall damage are permeable to small labeled probes used in standard *in situ* hybridization methods, while undamaged cells are not (201). As mentioned above, the SpxB protein mediates regulation of cell wall integrity (123). Rather than affect autolysin activity, SpxB is induced by lysozyme, and or peptidoglycan (PG) digestion products leading to PG O-acetylation, which renders it lysozyme-resistant. Such changes could have profound effects on bacterial state, fitness, and resistance to autolysins, with applications for probiotic uses.

Containment and food-grade strains

Inactivation of the *L. lactis* thymidylate synthase *thyA* gene results in a requirement for thymine or thymidine (66, 202). In *L. lactis*, a *thyA* mutant was exploited as a means of strain containment, i.e., strains can grow in a thymine-containing environment, such as the gut, but not in more limiting environments (202).

Nonsense suppressor strains, used in *E. coli* genetics to analyze phenotypes of point mutations. Plasmids carrying the suppressor genes could suppress an otherwise lethal nonsense mutation in the cell. This property of suppressors was exploited in *L. lactis* to construct and establish a food-grade plasmid (i.e., no foreign DNA) containing the suppressor genes in a suppressible purine auxotroph; this plasmid is stable in a milk media which cannot sustain growth of a purine auxotroph (203).

Higher antigenicity

Studies have compared effectiveness of presentation of different antigens as bacterial cytoplasmic, surface-anchored, or secreted proteins. Cell wall associated antigens seem to induce

greater immune response than secreted or cytoplasmically expressed proteins (204, 205). Furthermore, immune response was reportedly enhanced in mutant strains with cell wall defects due to mutation of the alanine racemase (206).

Conclusions

L. lactis is likely the microorganism most eaten by man. It belongs to a family comprised of pathogens (e.g., *S. pneumonaie, S. pyogenes*), commensal microorganisms (e.g., *S. gordonii, S. mutans*), and food microorganisms (e.g., *S. thermophilus* and *L. lactis*). Studies of *L. lactis* reveal that differences between pathogens and non-pathogens are limited. As a bacterium that acidifies its own medium, *L. lactis* may have a high capacity for stress resistance when pre-adapted; stress-resistant mutants with constitutive stress-resistance can be selected. Its metabolic flexibility by shifting to respiration metabolism can dispense with most acid production. As a food micro-organism, *L. lactis* comes into close contact with, and may cross-feed other bacteria in both the food environment and in the gut; as such, it may impact behavior of other bacteria in complex ecosystems. As a non-toxic bacterium that secretes relatively few proteins in quantity, *L. lactis* may also be an organism of choice for oral vaccine or protein delivery design and biotechnological uses (see (164, 207, 208) for reviews). As a simple organism with diverse biotopes, including plants, milk products, and the gut, *L. lactis* may be a good choice for studies on the influence of environmental stress on evolution.

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Table 1. Characteristics of *L. lactis*

Classification	Gram-positive, 38% G+C genome, 2.4 Mb, non-pathogenic food microorganism (referred to as a Lactic acid bacterium).
Close neighbors	Streptococci (food, commensal, pathogen)
Optimal growth temperature	30°C
Growth medium	Plants, milk, food silage, farm animal and human gut constutuents(?)
Environmental contacts	Plants (environmental niche) Farm animals Milk and other foods (environmental niche may rely on plasmid-encoded factors) Gastroeintestinal tract.
Metabolism	Fermentation. Respiration in aerobic, heme-containing medium.
Survival	Usually poor after fermentation growth. Good long term survival after growth by respiration.

Figure Legends

Fig. 1. Phylogenetic tree reveals similarities between lactococci and streptococcal pathogens. A phylogenetic tree built on 16S sequences was constructed directly on the Ribosomal Database interface (209). Branches with a bootstrap value below 60% are indicated with an asterisk. Respiration capacity (see text) is indicated by a red "R". R^c indicates conditional respiration: for *L. lactis*, *E. faecalis*,and leuconostoc sp., aerobic respiration is activated by exogenous heme. For all lactobacillus species, and *S. agalactiae*, respiration is activated by exogenous heme and menaquinone. Opportunist pathogens are indicated in bold. This figure is based on (154)..

Fig. 2. Basics of *L. lactis* fermentation. The NADH/NAD+ ratio is placed as a central determinant of carbon metabolic choice in *L. lactis* (56). ❶, Sugar fermentation generates ATP, which is used for amino acid anabolism. In anaerobic conditions and rapid sugar flux essentially all sugar is converted to lactate (homolactic fermentation) from pyruvate (glycolysis). When sugar flux is slower, or in the presence of sugars other than glucose or lactose, or in aerobic growth, mixed acid fermentation may occur. The latter conditions are characterized by lower $NADH/NAD^+$ ratios than those found during homolactic fermentations. Beside NADH, Glycolysis generates ATP and pyruvate from sugar degradation. ❷, Pyruvate dehydrogenase (Pdh) provides extra NADH from pyruvate when oxygen is present. ❸, Lactate dehydrogenase (Ldh) oxidizes NADH into NAD^+ by conversion of pyruvate into lactate thus maintaining glycolytic activity during fermentation. ❹, When oxygen is present, NADH can be oxidized by the cytoplasmic H_2O-forming NADH oxidase (NoxE), generating NAD+. ❺, The ATPase expulses H^+ at the expense of ATP to avoid acidification due to glycolysis. Pyruvate build-up leads to synthesis of acetate or the neutral acetoin and diacetyl (also see Fig 4). This figure is modified from (154).

Fig. 3. Basics of *L. lactis* respiration. Refer to **Fig. 2** for reactions ❶, ❷, and ❺, which are common to fermentation and respiration (numbering is the same). ❻,The membrane respiration chain (RC) comprises an electron donor (putatively encoded by *noxAnoxB*; (45, 89)), menaquinones (MKs) (encoded by *men* operon genes, or provided exogenously; (80)), and a terminal electron acceptor (the cytochrome oxidase encoded by *cydAcydB*; (46, 210)). Heme (red star) must be added exogenously (red arrow) to activate cytochrome oxidase. *S. agalactiae* and lactobacilli with respiration capacity (see **Fig. 1** legend) require MKs (schematic molecule with green center) and heme to activate respiration. Respiration chain activity results in H^+ expulsion. ❺, The ATPase might import H^+, which generates ATP, but with low efficiency (47, 77). *L. lactis* lacks a complete Krebs cycle. Thus, NADH, which is needed for the respiratory chain, is produced by carbon catabolism. ❶ Once phosphorylated, sugar is catabolized to pyruvate *via* glycolysis with production of ATP and NADH. As the respiration chain consumes NADH, Ldh activity decreases and pyruvic acid accumulates. Pyruvic acid dissociates to pyruvate and a proton, decreasing the internal pH. ❼, To avoid acidification, pyruvate/pyruvic acid is converted to acetolactate *via* acetolactate synthase (Als), then to the neutral compound acetoin with production of CO_2. Diacetyl is produced by spontaneous oxidation of acetolactate. This pathway raises the pH and improves cell survival. Some LAB convert acetoin to 2,3 butanediol. ❷, Pyruvate may also be converted to acetyl-CoA via Pyruvate dehydrogenase (Pdh), providing extra NADH and CO_2. Acetyl-CoA is further converted to acetate with production of ATP, promoting higher cell density. Acetate, acetoin, diacetyl, and 2,3-butanediol diffuse or are secreted into the medium. This figure is modified from (154).

Fig. 4. Schematic representation of *L. lactis* heme-sensing and HrtBA-mediated efflux, which regulate heme homeostasis. In *L. lactis* and numerous commensal bacteria, heme is suggested to be taken up by *fhuDBA* gene products (green ovals) and/or by diffusion through membranes (75, 82). Internalized heme binds to athe HrtR repressor, which releases binding to the *hrtRBA* operon. Consequent activation of *hrtBA* results in heme efflux (74). Red squares, heme.

Fig. 5. *L. lactis* produces menaquinones that cross-feed the opportunist pathogen *S. agalactiae.* Heme is present in the solid medium. A broad horizontal streak of an *S. agalactiae* strain (NEM316) is shown. Spots of cultures of *L. lactis* wild type (left) or *menB* that is defective for menaquinone synthesis (right) are deposited directly over the streaks. A stimulated growth zone is observed directly surrounding the WT *L. lactis* strain but not the *menB* mutant. From Mol. Microbiology (78).

Fig. 6. Respiring *L. lactis* can improve survival of non-respiring bacteria in co-culture. Differentially marked WT and *cydA* (non-respiring mutant) *L. lactis* strains were grown separately or together in co-culture. Non-respiring *cydA* grew less well, and showed poor survival when maintained in aerobic medium with heme over a 3-day period. In contrast, the respiring WT strain thrived. In contrast, the *cydA* strain fared much better when grown in co-culture with the WT strain, as determined by cell count determinations. From Mol. Microbiology (72).

Fig. 7. Respiration metabolism increases survival capacity of lactococci. When supplemented with hemin, aerobically grown lactococci can undergo respiration metabolism. As a result, cells

stored at 4°C show a markedly better survival, as compared to cells grown aerobically in the absence of hemin, or in static conditions. Improved survival was also observed when cells are maintained at 30°C. Experiment shown was performed by Karin Vido in authors' laboratory.

Fig. 8. Bacterial root formation in semi-liquid medium. Bacterial chains (here, an *acmA* mutant of *L. lactis*; 'parental strain'), diffuse slowly in a semi-liquid (0.035% agar) medium. Bacterial 'de-chained' mutants diffuse more quickly to form 'roots'. In this experiment, all the roots corresponded to independent mutants in the same gene, *ponA*, encoding PBP1A (reproduced from Kulakauskas and coworkers, reference (144)). Note that a similar strategy of semi-liquid medium selection was used to uncover the existence of a cell-surface carbohydrate pellicle in *L. lactis*; the system is readily applied to other bacteria (137).

References

1. Antolin J, Ciguenza R, Saluena I, Vazquez E, Hernandez J, Espinos D. 2004. Liver abscess caused by Lactococcus lactis cremoris: a new pathogen. Scand J Infect Dis 36:490-1.
2. Werner B, Moroni P, Gioia G, Lavin-Alconero L, Yousaf A, Charter ME, Carter BM, Bennett J, Nydam DV, Welcome F, Schukken YH. 2014. Short communication: Genotypic and phenotypic identification of environmental streptococci and association of Lactococcus lactis ssp. lactis with intramammary infections among different dairy farms. J Dairy Sci 97:6964-9.
3. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. 2000. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 289:1352-5.
4. Ballal SA, Veiga P, Fenn K, Michaud M, Kim JH, Gallini CA, Glickman JN, Quere G, Garault P, Beal C, Derrien M, Courtin P, Kulakauskas S, Chapot-Chartier MP, van Hylckama Vlieg J, Garrett WS. 2015. Host lysozyme-mediated lysis of Lactococcus lactis facilitates delivery of colitis-attenuating superoxide dismutase to inflamed colons. Proc Natl Acad Sci U S A 112:7803-8.
5. Nouaille S, Mondeil S, Finoux AL, Moulis C, Girbal L, Cocaign-Bousquet M. 2017. The stability of an mRNA is influenced by its concentration: a potential physical mechanism to regulate gene expression. Nucleic Acids Res 45:11711-11724.
6. Chopin MC, Chopin A, Bidnenko E. 2005. Phage abortive infection in lactococci: variations on a theme. Curr Opin Microbiol 8:473-9.
7. Deveau H, Labrie SJ, Chopin MC, Moineau S. 2006. Biodiversity and classification of lactococcal phages. Appl Environ Microbiol 72:4338-46.
8. Labrie SJ, Moineau S. 2007. Abortive infection mechanisms and prophage sequences significantly influence the genetic makeup of emerging lytic lactococcal phages. J Bacteriol 189:1482-7.
9. Mahony J, Cambillau C, van Sinderen D. 2017. Host recognition by lactic acid bacterial phages. FEMS Microbiol Rev 41:S16-S26.
10. Samson JE, Moineau S. 2013. Bacteriophages in food fermentations: new frontiers in a continuous arms race. Annu Rev Food Sci Technol 4:347-68.
11. Spinelli S, Veesler D, Bebeacua C, Cambillau C. 2014. Structures and host-adhesion mechanisms of lactococcal siphophages. Front Microbiol 5:3.
12. Sturino JM, Klaenhammer TR. 2004. Bacteriophage defense systems and strategies for lactic acid bacteria. Adv Appl Microbiol 56:331-78.
13. Douglas G, Azcarate-Peril M, Klaenhammer T. 2015. Genomic evolution of lactic acid bacteria: From Single Gene Function to the Pan-genome. . Biotechnology of lactic acid bacteria: Novel applications doi:doi.org/10.1002/9781118868386.ch3:32-54.
14. Campo N, Dias MJ, Daveran-Mingot ML, Ritzenthaler P, Le Bourgeois P. 2002. Genome plasticity in Lactococcus lactis. Antonie Van Leeuwenhoek 82:123-32.
15. Song AA, In LLA, Lim SHE, Rahim RA. 2017. A review on Lactococcus lactis: from food to factory. Microb Cell Fact 16:55.
16. de Moreno de LeBlanc A, Del Carmen S, Chatel JM, Miyoshi A, Azevedo V, Langella P, Bermudez-Humaran LG, LeBlanc JG. 2015. Current Review of Genetically Modified Lactic Acid Bacteria for the Prevention and Treatment of Colitis Using Murine Models. Gastroenterol Res Pract 2015:146972.
17. Pillar CM, Gilmore MS. 2004. Enterococcal virulence--pathogenicity island of E. Faecalis. Front Biosci 9:2335-46.
18. Nunez N, Derre-Bobillot A, Gaubert S, Herry JM, Deschamps J, Wei Y, Baranek T, Si-Tahar M, Briandet R, Serror P, Archambaud C. 2018. Exploration of the role of the virulence factor ElrA during Enterococcus faecalis cell infection. Sci Rep 8:1749.
19. Kim S, Covington A, Pamer EG. 2017. The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. Immunol Rev 279:90-105.
20. Perez-Nunez D, Briandet R, David B, Gautier C, Renault P, Hallet B, Hols P, Carballido-Lopez R, Guedon E. 2011. A new morphogenesis pathway in bacteria: unbalanced activity of cell wall synthesis machineries leads to coccus-to-rod transition and filamentation in ovococci. Mol Microbiol 79:759-71.
21. Halpern D, Chiapello H, Schbath S, Robin S, Hennequet-Antier C, Gruss A, El Karoui M. 2007. Identification of DNA motifs implicated in maintenance of bacterial core genomes by predictive modeling. PLoS Genet 3:1614-21.
22. Le Bourgeois P, Lautier M, van den Berghe L, Gasson MJ, Ritzenthaler P. 1995. Physical and genetic map of the Lactococcus lactis subsp. cremoris MG1363 chromosome: comparison with that of Lactococcus lactis subsp. lactis IL 1403 reveals a large genome inversion. J Bacteriol 177:2840-50.

23. Le Bourgeois P, Daveran-Mingot ML, Ritzenthaler P. 2000. Genome plasticity among related ++Lactococcus strains: identification of genetic events associated with macrorestriction polymorphisms. J Bacteriol 182:2481-91.
24. Campo N, Dias MJ, Daveran-Mingot ML, Ritzenthaler P, Le Bourgeois P. 2004. Chromosomal constraints in Gram-positive bacteria revealed by artificial inversions. Mol Microbiol 51:511-22.
25. McKay LL, Baldwin KA, Efstathiou JD. 1976. Transductional evidence for plasmid linkage of lactose metabolism in streptococcus lactis C2. Appl Environ Microbiol 32:45-52.
26. Bourgoin F, Pluvinet A, Gintz B, Decaris B, Guedon G. 1999. Are horizontal transfers involved in the evolution of the Streptococcus thermophilus exopolysaccharide synthesis loci? Gene 233:151-61.
27. Belhocine K, Plante I, Cousineau B. 2004. Conjugation mediates transfer of the Ll.LtrB group II intron between different bacterial species. Mol Microbiol 51:1459-69.
28. Kelly WJ, Altermann E, Lambie SC, Leahy SC. 2013. Interaction between the genomes of Lactococcus lactis and phages of the P335 species. Front Microbiol 4:257.
29. Griffith F. 1928. The Significance of Pneumococcal Types. J Hyg (Lond) 27:113-59.
30. Helmark S, Hansen ME, Jelle B, Sorensen KI, Jensen PR. 2004. Transformation of Leuconostoc carnosum 4010 and evidence for natural competence of the organism. Appl Environ Microbiol 70:3695-9.
31. Gardan R, Besset C, Guillot A, Gitton C, Monnet V. 2009. The oligopeptide transport system is essential for the development of natural competence in Streptococcus thermophilus strain LMD-9. J Bacteriol 191:4647-55.
32. Gardan R, Besset C, Gitton C, Guillot A, Fontaine L, Hols P, Monnet V. 2013. Extracellular life cycle of ComS, the competence-stimulating peptide of Streptococcus thermophilus. J Bacteriol 195:1845-55.
33. Fontaine L, Goffin P, Dubout H, Delplace B, Baulard A, Lecat-Guillet N, Chambellon E, Gardan R, Hols P. 2013. Mechanism of competence activation by the ComRS signalling system in streptococci. Mol Microbiol 87:1113-32.
34. Wydau S, Dervyn R, Anba J, Dusko Ehrlich S, Maguin E. 2006. Conservation of key elements of natural competence in Lactococcus lactis ssp. FEMS Microbiol Lett 257:32-42.
35. Mulder J, Wels M, Kuipers OP, Kleerebezem M, Bron PA. 2017. Unleashing natural competence in Lactococcus lactis by induction of the competence regulator ComX. Appl Environ Microbiol doi:10.1128/AEM.01320-17.
36. David B, Radziejwoski A, Toussaint F, Fontaine L, Henry de Frahan M, Patout C, van Dillen S, Boyaval P, Horvath P, Fremaux C, Hols P. 2017. Natural DNA transformation is functional in Lactococcus lactis ssp. cremoris KW2. Appl Environ Microbiol doi:10.1128/AEM.01074-17.
37. Holo H, Nes IF. 1989. High-Frequency Transformation, by Electroporation, of Lactococcus lactis subsp. cremoris Grown with Glycine in Osmotically Stabilized Media. Appl Environ Microbiol 55:3119-23.
38. Vaughan EE, de Vos WM. 1995. Identification and characterization of the insertion element IS1070 from Leuconostoc lactis NZ6009. Gene 155:95-100.
39. Bolotin A, Mauger S, Malarme K, Ehrlich SD, Sorokin A. 1999. Low-redundancy sequencing of the entire Lactococcus lactis IL1403 genome. Antonie Van Leeuwenhoek 76:27-76.
40. Delorme C, Bartholini C, Luraschi M, Pons N, Loux V, Almeida M, Guedon E, Gibrat JF, Renault P. 2011. Complete genome sequence of the pigmented Streptococcus thermophilus strain JIM8232. J Bacteriol 193:5581-2.
41. Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, Kulakauskas S, Lapidus A, Goltsman E, Mazur M, Pusch GD, Fonstein M, Overbeek R, Kyprides N, Purnelle B, Prozzi D, Ngui K, Masuy D, Hancy F, Burteau S, Boutry M, Delcour J, Goffeau A, Hols P. 2004. Complete sequence and comparative genome analysis of the dairy bacterium Streptococcus thermophilus. Nat Biotechnol 22:1554-8.
42. de Vos WM, Hugenholtz J. 2004. Engineering metabolic highways in Lactococci and other lactic acid bacteria. Trends Biotechnol 22:72-9.
43. Garrigues C, Johansen E, Pedersen MB, Mollgaard H, Sorensen KI, Gaudu P, Gruss A, Lamberet G. 2006. Getting high (OD) on heme. Nat Rev Microbiol 4:c2; author reply c3.
44. Sijpesteijn AK. 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in Streptococcus lactis and Leuconostoc mesenteroides. Antonie Van Leeuwenhoek 36:335-48.
45. Gaudu P, Vido K, Cesselin B, Kulakauskas S, Tremblay J, Rezaiki L, Lamberret G, Sourice S, Duwat P, Gruss A. 2002. Respiration capacity and consequences in Lactococcus lactis. Antonie Van Leeuwenhoek 82:263-9.
46. Duwat P, Sourice S, Cesselin B, Lamberet G, Vido K, Gaudu P, Le Loir Y, Violet F, Loubiere P, Gruss A. 2001. Respiration capacity of the fermenting bacterium Lactococcus lactis and its positive effects on growth and survival. J Bacteriol 183:4509-16.
47. Blank LM, Koebmann BJ, Michelsen O, Nielsen LK, Jensen PR. 2001. Hemin reconstitutes proton extrusion in an H(+)-ATPase-negative mutant of Lactococcus lactis. J Bacteriol 183:6707-9.

48. Gruss A, Borezee-Durant E, Lechardeur D. 2012. Environmental heme utilization by heme-auxotrophic bacteria. Adv Microb Physiol 61:69-124.
49. Neves AR, Pool WA, Kok J, Kuipers OP, Santos H. 2005. Overview on sugar metabolism and its control in Lactococcus lactis - the input from in vivo NMR. FEMS Microbiol Rev 29:531-54.
50. Cocaign-Bousquet M, Garrigues C, Loubiere P, Lindley ND. 1996. Physiology of pyruvate metabolism in Lactococcus lactis. Antonie Van Leeuwenhoek 70:253-67.
51. Liu J, Wang Z, Kandasamy V, Lee SY, Solem C, Jensen PR. 2017. Harnessing the respiration machinery for high-yield production of chemicals in metabolically engineered Lactococcus lactis. Metab Eng 44:22-29.
52. Kandasamy V, Liu J, Dantoft SH, Solem C, Jensen PR. 2016. Synthesis of (3R)-acetoin and 2,3-butanediol isomers by metabolically engineered Lactococcus lactis. Sci Rep 6:36769.
53. Hugenholtz J, Kleerebezem M, Starrenburg M, Delcour J, de Vos W, Hols P. 2000. Lactococcus lactis as a cell factory for high-level diacetyl production. Appl Environ Microbiol 66:4112-4.
54. Smid EJ, Kleerebezem M. 2014. Production of aroma compounds in lactic fermentations. Annu Rev Food Sci Technol 5:313-26.
55. Bongers RS, Hoefnagel MH, Starrenburg MJ, Siemerink MA, Arends JG, Hugenholtz J, Kleerebezem M. 2003. IS981-mediated adaptive evolution recovers lactate production by ldhB transcription activation in a lactate dehydrogenase-deficient strain of Lactococcus lactis. J Bacteriol 185:4499-507.
56. Garrigues C, Loubiere P, Lindley ND, Cocaign-Bousquet M. 1997. Control of the shift from homolactic acid to mixed-acid fermentation in Lactococcus lactis: predominant role of the NADH/NAD+ ratio. J Bacteriol 179:5282-7.
57. Solem C, Jensen PR. 2002. Modulation of gene expression made easy. Appl Environ Microbiol 68:2397-403.
58. Jensen PR, Hammer K. 1998. Artificial promoters for metabolic optimization. Biotechnol Bioeng 58:191-5.
59. Koebmann BJ, Andersen HW, Solem C, Jensen PR. 2002. Experimental determination of control of glycolysis in Lactococcus lactis. Antonie Van Leeuwenhoek 82:237-48.
60. Andersen HW, Solem C, Hammer K, Jensen PR. 2001. Twofold reduction of phosphofructokinase activity in Lactococcus lactis results in strong decreases in growth rate and in glycolytic flux. J Bacteriol 183:3458-67.
61. Andersen HW, Pedersen MB, Hammer K, Jensen PR. 2001. Lactate dehydrogenase has no control on lactate production but has a strong negative control on formate production in Lactococcus lactis. Eur J Biochem 268:6379-89.
62. Pancholi V, Fischetti VA. 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J Exp Med 176:415-26.
63. Henderson B, Martin A. 2011. Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. Infect Immun 79:3476-91.
64. Oliveira L, Madureira P, Andrade EB, Bouaboud A, Morello E, Ferreira P, Poyart C, Trieu-Cuot P, Dramsi S. 2012. Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. PLoS One 7:e29963.
65. Luesink EJ, van Herpen RE, Grossiord BP, Kuipers OP, de Vos WM. 1998. Transcriptional activation of the glycolytic las operon and catabolite repression of the gal operon in Lactococcus lactis are mediated by the catabolite control protein CcpA. Mol Microbiol 30:789-98.
66. Pedersen MB, Koebmann BJ, Jensen PR, Nilsson D. 2002. Increasing acidification of nonreplicating Lactococcus lactis deltathyA mutants by incorporating ATPase activity. Appl Environ Microbiol 68:5249-57.
67. Oliver S. 2002. Metabolism: demand management in cells. Nature 418:33-4.
68. Koebmann BJ, Solem C, Pedersen MB, Nilsson D, Jensen PR. 2002. Expression of genes encoding F(1)-ATPase results in uncoupling of glycolysis from biomass production in Lactococcus lactis. Appl Environ Microbiol 68:4274-82.
69. Dehli T, Solem C, Jensen PR. 2012. Tunable promoters in synthetic and systems biology. Subcell Biochem 64:181-201.
70. Richardson DJ. 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology 146 (Pt 3):551-71.
71. Poole RK, Cook GM. 2000. Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation. Adv Microb Physiol 43:165-224.
72. Rezaiki L, Cesselin B, Yamamoto Y, Vido K, van West E, Gaudu P, Gruss A. 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of Lactococcus lactis. Mol Microbiol 53:1331-42.

73. Pedersen MB, Garrigues C, Tuphile K, Brun C, Vido K, Bennedsen M, Mollgaard H, Gaudu P, Gruss A. 2008. Impact of aeration and heme-activated respiration on Lactococcus lactis gene expression: identification of a heme-responsive operon. J Bacteriol 190:4903-11.
74. Lechardeur D, Cesselin B, Liebl U, Vos MH, Fernandez A, Brun C, Gruss A, Gaudu P. 2012. Discovery of intracellular heme-binding protein HrtR, which controls heme efflux by the conserved HrtB-HrtA transporter in Lactococcus lactis. J Biol Chem 287:4752-8.
75. Gaudu P, Lamberet G, Poncet S, Gruss A. 2003. CcpA regulation of aerobic and respiration growth in Lactococcus lactis. Mol Microbiol 50:183-92.
76. Kaneko T, Takahashi M, Suzuki H. 1990. Acetoin Fermentation by Citrate-Positive Lactococcus lactis subsp. lactis 3022 Grown Aerobically in the Presence of Hemin or Cu. Appl Environ Microbiol 56:2644-9.
77. Koebmann B, Blank LM, Solem C, Petranovic D, Nielsen LK, Jensen PR. 2008. Increased biomass yield of Lactococcus lactis during energetically limited growth and respiratory conditions. Biotechnol Appl Biochem 50:25-33.
78. Rezaiki L, Lamberet G, Derre A, Gruss A, Gaudu P. 2008. Lactococcus lactis produces short-chain quinones that cross-feed Group B Streptococcus to activate respiration growth. Mol Microbiol 67:947-57.
79. D'Mello R, Hill S, Poole RK. 1996. The cytochrome bd quinol oxidase in Escherichia coli has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity in vivo by oxygen inhibition. Microbiology 142 (Pt 4):755-63.
80. Yamamoto Y, Poyart C, Trieu-Cuot P, Lamberet G, Gruss A, Gaudu P. 2005. Respiration metabolism of Group B Streptococcus is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol 56:525-34.
81. Joubert L, Dagieu JB, Fernandez A, Derre-Bobillot A, Borezee-Durant E, Fleurot I, Gruss A, Lechardeur D. 2017. Visualization of the role of host heme on the virulence of the heme auxotroph Streptococcus agalactiae. Sci Rep 7:40435.
82. Joubert L, Derre-Bobillot A, Gaudu P, Gruss A, Lechardeur D. 2014. HrtBA and menaquinones control haem homeostasis in Lactococcus lactis. Mol Microbiol 93:823-33.
83. Vido K, Le Bars D, Mistou MY, Anglade P, Gruss A, Gaudu P. 2004. Proteome analyses of heme-dependent respiration in Lactococcus lactis: involvement of the proteolytic system. J Bacteriol 186:1648-57.
84. Galinier A, Deutscher J. 2017. Sophisticated Regulation of Transcriptional Factors by the Bacterial Phosphoenolpyruvate: Sugar Phosphotransferase System. J Mol Biol 429:773-789.
85. Wegmann U, O'Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C, Ventura M, Goesmann A, Gasson MJ, Kuipers OP, van Sinderen D, Kok J. 2007. Complete genome sequence of the prototype lactic acid bacterium Lactococcus lactis subsp. cremoris MG1363. J Bacteriol 189:3256-70.
86. van der Meulen SB, de Jong A, Kok J. 2016. Transcriptome landscape of Lactococcus lactis reveals many novel RNAs including a small regulatory RNA involved in carbon uptake and metabolism. RNA Biol 13:353-66.
87. Rosinski-Chupin I, Sauvage E, Sismeiro O, Villain A, Da Cunha V, Caliot ME, Dillies MA, Trieu-Cuot P, Bouloc P, Lartigue MF, Glaser P. 2015. Single nucleotide resolution RNA-seq uncovers new regulatory mechanisms in the opportunistic pathogen Streptococcus agalactiae. BMC Genomics 16:419.
88. Zomer AL, Buist G, Larsen R, Kok J, Kuipers OP. 2007. Time-resolved determination of the CcpA regulon of Lactococcus lactis subsp. cremoris MG1363. J Bacteriol 189:1366-81.
89. Brooijmans RJ, de Vos WM, Hugenholtz J. 2009. Lactobacillus plantarum WCFS1 electron transport chains. Appl Environ Microbiol 75:3580-5.
90. Stauff DL, Bagaley D, Torres VJ, Joyce R, Anderson KL, Kuechenmeister L, Dunman PM, Skaar EP. 2008. Staphylococcus aureus HrtA is an ATPase required for protection against heme toxicity and prevention of a transcriptional heme stress response. J Bacteriol 190:3588-96.
91. Sawai H, Yamanaka M, Sugimoto H, Shiro Y, Aono S. 2012. Structural basis for the transcriptional regulation of heme homeostasis in Lactococcus lactis. J Biol Chem 287:30755-68.
92. Stauff DL, Torres VJ, Skaar EP. 2007. Signaling and DNA-binding activities of the Staphylococcus aureus HssR-HssS two-component system required for heme sensing. J Biol Chem 282:26111-21.
93. Tachon S, Brandsma JB, Yvon M. 2010. NoxE NADH oxidase and the electron transport chain are responsible for the ability of Lactococcus lactis to decrease the redox potential of milk. Appl Environ Microbiol 76:1311-9.
94. Yamamoto Y, Poyart C, Trieu-Cuot P, Lamberet G, Gruss A, Gaudu P. 2006. Roles of environmental heme, and menaquinone, in streptococcus agalactiae. Biometals 19:205-10.
95. Winstedt L, Frankenberg L, Hederstedt L, von Wachenfeldt C. 2000. Enterococcus faecalis V583 contains a cytochrome bd-type respiratory oxidase. J Bacteriol 182:3863-6.

96. Huycke MM, Moore D, Joyce W, Wise P, Shepard L, Kotake Y, Gilmore MS. 2001. Extracellular superoxide production by Enterococcus faecalis requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. Mol Microbiol 42:729-40.
97. Kunji ER, Mierau I, Hagting A, Poolman B, Konings WN. 1996. The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek 70:187-221.
98. Guillot A, Boulay M, Chambellon E, Gitton C, Monnet V, Juillard V. 2016. Mass Spectrometry Analysis of the Extracellular Peptidome of Lactococcus lactis: Lines of Evidence for the Coexistence of Extracellular Protein Hydrolysis and Intracellular Peptide Excretion. J Proteome Res 15:3214-24.
99. Mierau I, Kunji ER, Leenhouts KJ, Hellendoorn MA, Haandrikman AJ, Poolman B, Konings WN, Venema G, Kok J. 1996. Multiple-peptidase mutants of Lactococcus lactis are severely impaired in their ability to grow in milk. J Bacteriol 178:2794-803.
100. Cocaign-Bousquet M, Garrigues C, Novak L, Lindley ND, Loubiere P. 1995. Rational development of a simple synthetic medium for the sustained growth of Lactococcus lactis. J Appl Microbiol 79:108-116.
101. Martinussen J, Schallert J, Andersen B, Hammer K. 2001. The pyrimidine operon pyrRPB-carA from Lactococcus lactis. J Bacteriol 183:2785-94.
102. Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD, Sorokin A. 2001. The complete genome sequence of the lactic acid bacterium Lactococcus lactis ssp. lactis IL1403. Genome Res 11:731-53.
103. Jorgensen CM, Fields CJ, Chander P, Watt D, Burgner JW, 2nd, Smith JL, Switzer RL. 2008. pyr RNA binding to the Bacillus caldolyticus PyrR attenuation protein - characterization and regulation by uridine and guanosine nucleotides. FEBS J 275:655-70.
104. Jendresen CB, Dimitrov P, Gautier L, Liu M, Martinussen J, Kilstrup M. 2014. Towards in vivo regulon kinetics: PurR activation by 5-phosphoribosyl-alpha-1-pyrophosphate during purine depletion in Lactococcus lactis. Microbiology 160:1321-31.
105. Fields CJ, Switzer RL. 2007. Regulation of pyr gene expression in Mycobacterium smegmatis by PyrR-dependent translational repression. J Bacteriol 189:6236-45.
106. Jendresen CB, Martinussen J, Kilstrup M. 2012. The PurR regulon in Lactococcus lactis - transcriptional regulation of the purine nucleotide metabolism and translational machinery. Microbiology 158:2026-38.
107. Kilstrup M, Martinussen J. 1998. A transcriptional activator, homologous to the Bacillus subtilis PurR repressor, is required for expression of purine biosynthetic genes in Lactococcus lactis. J Bacteriol 180:3907-16.
108. Kilstrup M, Jessing SG, Wichmand-Jorgensen SB, Madsen M, Nilsson D. 1998. Activation control of pur gene expression in Lactococcus lactis: proposal for a consensus activator binding sequence based on deletion analysis and site-directed mutagenesis of purC and purD promoter regions. J Bacteriol 180:3900-6.
109. Hove-Jensen B, Andersen KR, Kilstrup M, Martinussen J, Switzer RL, Willemoes M. 2017. Phosphoribosyl Diphosphate (PRPP): Biosynthesis, Enzymology, Utilization, and Metabolic Significance. Microbiol Mol Biol Rev 81.
110. Martinussen J, Hammer K. 1995. Powerful methods to establish chromosomal markers in Lactococcus lactis: an analysis of pyrimidine salvage pathway mutants obtained by positive selections. Microbiology 141 (Pt 8):1883-90.
111. Solem C, Defoor E, Jensen PR, Martinussen J. 2008. Plasmid pCS1966, a new selection/counterselection tool for lactic acid bacterium strain construction based on the oroP gene, encoding an orotate transporter from Lactococcus lactis. Appl Environ Microbiol 74:4772-5.
112. Defoor E, Kryger MB, Martinussen J. 2007. The orotate transporter encoded by oroP from Lactococcus lactis is required for orotate utilization and has utility as a food-grade selectable marker. Microbiology 153:3645-59.
113. Martinussen J, Sorensen C, Jendresen CB, Kilstrup M. 2010. Two nucleoside transporters in Lactococcus lactis with different substrate specificities. Microbiology 156:3148-57.
114. Papadimitriou K, Alegria A, Bron PA, de Angelis M, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, Turroni F, van Sinderen D, Varmanen P, Ventura M, Zuniga M, Tsakalidou E, Kok J. 2016. Stress Physiology of Lactic Acid Bacteria. Microbiol Mol Biol Rev 80:837-90.
115. Kok J, van Gijtenbeek LA, de Jong A, van der Meulen SB, Solopova A, Kuipers OP. 2017. The Evolution of gene regulation research in Lactococcus lactis. FEMS Microbiol Rev 41:S220-S243.
116. Rallu F, Gruss A, Ehrlich SD, Maguin E. 2000. Acid- and multistress-resistant mutants of Lactococcus lactis : identification of intracellular stress signals. Mol Microbiol 35:517-28.
117. Duwat P, Ehrlich SD, Gruss A. 1999. Effects of metabolic flux on stress response pathways in Lactococcus lactis. Mol Microbiol 31:845-58.

118. Solopova A, Formosa-Dague C, Courtin P, Furlan S, Veiga P, Pechoux C, Armalyte J, Sadauskas M, Kok J, Hols P, Dufrene YF, Kuipers OP, Chapot-Chartier MP, Kulakauskas S. 2016. Regulation of Cell Wall Plasticity by Nucleotide Metabolism in Lactococcus lactis. J Biol Chem 291:11323-36.
119. Carvalho SM, Kloosterman TG, Manzoor I, Caldas J, Vinga S, Martinussen J, Saraiva LM, Kuipers OP, Neves AR. 2018. Interplay Between Capsule Expression and Uracil Metabolism in Streptococcus pneumoniae D39. Front Microbiol 9:321.
120. Tan YP, Giffard PM, Barry DG, Huston WM, Turner MS. 2008. Random mutagenesis identifies novel genes involved in the secretion of antimicrobial, cell wall-lytic enzymes by Lactococcus lactis. Appl Environ Microbiol 74:7490-6.
121. Frees D, Varmanen P, Ingmer H. 2001. Inactivation of a gene that is highly conserved in Gram-positive bacteria stimulates degradation of non-native proteins and concomitantly increases stress tolerance in Lactococcus lactis. Mol Microbiol 41:93-103.
122. Nakano S, Kuster-Schock E, Grossman AD, Zuber P. 2003. Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in Bacillus subtilis. Proc Natl Acad Sci U S A 100:13603-8.
123. Veiga P, Bulbarela-Sampieri C, Furlan S, Maisons A, Chapot-Chartier MP, Erkelenz M, Mervelet P, Noirot P, Frees D, Kuipers OP, Kok J, Gruss A, Buist G, Kulakauskas S. 2007. SpxB regulates O-acetylation-dependent resistance of Lactococcus lactis peptidoglycan to hydrolysis. J Biol Chem 282:19342-54.
124. Potrykus K, Cashel M. 2008. (p)ppGpp: still magical? Annu Rev Microbiol 62:35-51.
125. Corrigan RM, Abbott JC, Burhenne H, Kaever V, Grundling A. 2011. c-di-AMP is a new second messenger in Staphylococcus aureus with a role in controlling cell size and envelope stress. PLoS Pathog 7:e1002217.
126. Smith WM, Pham TH, Lei L, Dou J, Soomro AH, Beatson SA, Dykes GA, Turner MS. 2012. Heat resistance and salt hypersensitivity in Lactococcus lactis due to spontaneous mutation of llmg_1816 (gdpP) induced by high-temperature growth. Appl Environ Microbiol 78:7753-9.
127. Zhu Y, Pham TH, Nhiep TH, Vu NM, Marcellin E, Chakrabortti A, Wang Y, Waanders J, Lo R, Huston WM, Bansal N, Nielsen LK, Liang ZX, Turner MS. 2016. Cyclic-di-AMP synthesis by the diadenylate cyclase CdaA is modulated by the peptidoglycan biosynthesis enzyme GlmM in Lactococcus lactis. Mol Microbiol 99:1015-27.
128. Tan E, Rao F, Pasunooti S, Pham TH, Soehano I, Turner MS, Liew CW, Lescar J, Pervushin K, Liang ZX. 2013. Solution structure of the PAS domain of a thermophilic YybT protein homolog reveals a potential ligand-binding site. J Biol Chem 288:11949-59.
129. Choi PH, Vu TMN, Pham HT, Woodward JJ, Turner MS, Tong L. 2017. Structural and functional studies of pyruvate carboxylase regulation by cyclic di-AMP in lactic acid bacteria. Proc Natl Acad Sci U S A 114:E7226-E7235.
130. Sanders JW, Venema G, Kok J, Leenhouts K. 1998. Identification of a sodium chloride-regulated promoter in Lactococcus lactis by single-copy chromosomal fusion with a reporter gene. Mol Gen Genet 257:681-5.
131. Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G, Kok J. 1998. A chloride-inducible acid resistance mechanism in Lactococcus lactis and its regulation. Mol Microbiol 27:299-310.
132. Poelarends GJ, Mazurkiewicz P, Konings WN. 2002. Multidrug transporters and antibiotic resistance in Lactococcus lactis. Biochim Biophys Acta 1555:1-7.
133. Filipic B, Golic N, Jovcic B, Tolinacki M, Bay DC, Turner RJ, Antic-Stankovic J, Kojic M, Topisirovic L. 2013. The cmbT gene encodes a novel major facilitator multidrug resistance transporter in Lactococcus lactis. Res Microbiol 164:46-54.
134. Fernandez A, Lechardeur D, Derre-Bobillot A, Couve E, Gaudu P, Gruss A. 2010. Two coregulated efflux transporters modulate intracellular heme and protoporphyrin IX availability in Streptococcus agalactiae. PLoS Pathog 6:e1000860.
135. Oxaran V, Ledue-Clier F, Dieye Y, Herry JM, Pechoux C, Meylheuc T, Briandet R, Juillard V, Piard JC. 2012. Pilus biogenesis in Lactococcus lactis: molecular characterization and role in aggregation and biofilm formation. PLoS One 7:e50989.
136. Meyrand M, Guillot A, Goin M, Furlan S, Armalyte J, Kulakauskas S, Cortes-Perez NG, Thomas G, Chat S, Pechoux C, Dupres V, Hols P, Dufrene YF, Trugnan G, Chapot-Chartier MP. 2013. Surface proteome analysis of a natural isolate of Lactococcus lactis reveals the presence of pili able to bind human intestinal epithelial cells. Mol Cell Proteomics 12:3935-47.
137. Chapot-Chartier MP, Vinogradov E, Sadovskaya I, Andre G, Mistou MY, Trieu-Cuot P, Furlan S, Bidnenko E, Courtin P, Pechoux C, Hols P, Dufrene YF, Kulakauskas S. 2010. Cell surface of Lactococcus lactis is covered by a protective polysaccharide pellicle. J Biol Chem 285:10464-71.
138. Sadovskaya I, Vinogradov E, Courtin P, Armalyte J, Meyrand M, Giaouris E, Palussiere S, Furlan S, Pechoux C, Ainsworth S, Mahony J, van Sinderen D, Kulakauskas S, Guerardel Y, Chapot-Chartier MP.

2017. Another Brick in the Wall: a Rhamnan Polysaccharide Trapped inside Peptidoglycan of Lactococcus lactis. MBio 8.
139. Lopez de Felipe F, Hugenholtz J. 1999. Pyruvate flux distribution in NADH-oxidase-overproducing Lactococcus lactis strain as a function of culture conditions. FEMS Microbiol Lett 179:461-6.
140. Duwat P, Sourice S, Ehrlich SD, Gruss A. 1995. recA gene involvement in oxidative and thermal stress in Lactococcus lactis. Dev Biol Stand 85:455-67.
141. Li Y, Hugenholtz J, Abee T, Molenaar D. 2003. Glutathione protects Lactococcus lactis against oxidative stress. Appl Environ Microbiol 69:5739-45.
142. Rochat T, Miyoshi A, Gratadoux JJ, Duwat P, Sourice S, Azevedo V, Langella P. 2005. High-level resistance to oxidative stress in Lactococcus lactis conferred by Bacillus subtilis catalase KatE. Microbiology 151:3011-8.
143. Rallu F, Gruss A, Maguin E. 1996. Lactococcus lactis and stress. Antonie Van Leeuwenhoek 70:243-51.
144. Mercier C, Durrieu C, Briandet R, Domakova E, Tremblay J, Buist G, Kulakauskas S. 2002. Positive role of peptidoglycan breaks in lactococcal biofilm formation. Mol Microbiol 46:235-43.
145. Jones AL, Needham RH, Clancy A, Knoll KM, Rubens CE. 2003. Penicillin-binding proteins in Streptococcus agalactiae: a novel mechanism for evasion of immune clearance. Mol Microbiol 47:247-56.
146. Jones AL, Mertz RH, Carl DJ, Rubens CE. 2007. A streptococcal penicillin-binding protein is critical for resisting innate airway defenses in the neonatal lung. J Immunol 179:3196-202.
147. Ryssel M, Hviid AM, Dawish MS, Haaber J, Hammer K, Martinussen J, Kilstrup M. 2014. Multi-stress resistance in Lactococcus lactis is actually escape from purine-induced stress sensitivity. Microbiology 160:2551-9.
148. Rochat T, Gratadoux JJ, Corthier G, Coqueran B, Nader-Macias ME, Gruss A, Langella P. 2005. Lactococcus lactis SpOx spontaneous mutants: a family of oxidative-stress-resistant dairy strains. Appl Environ Microbiol 71:2782-8.
149. Ocana VS, Pesce de Ruiz Holgado AA, Nader-Macias ME. 1999. Selection of vaginal H2O2-generating Lactobacillus species for probiotic use. Curr Microbiol 38:279-84.
150. Seki M, Iida K, Saito M, Nakayama H, Yoshida S. 2004. Hydrogen peroxide production in Streptococcus pyogenes: involvement of lactate oxidase and coupling with aerobic utilization of lactate. J Bacteriol 186:2046-51.
151. Cesselin B, Ali D, Gratadoux JJ, Gaudu P, Duwat P, Gruss A, El Karoui M. 2009. Inactivation of the Lactococcus lactis high-affinity phosphate transporter confers oxygen and thiol resistance and alters metal homeostasis. Microbiology 155:2274-81.
152. Del Carmen S, de Moreno de LeBlanc A, Levit R, Azevedo V, Langella P, Bermudez-Humaran LG, LeBlanc JG. 2017. Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer mouse model. Int Immunopharmacol 42:122-129.
153. Watterlot L, Rochat T, Sokol H, Cherbuy C, Bouloufa I, Lefevre F, Gratadoux JJ, Honvo-Hueto E, Chilmonczyk S, Blugeon S, Corthier G, Langella P, Bermudez-Humaran LG. 2010. Intragastric administration of a superoxide dismutase-producing recombinant Lactobacillus casei BL23 strain attenuates DSS colitis in mice. Int J Food Microbiol 144:35-41.
154. Pedersen MB, Gaudu P, Lechardeur D, Petit MA, Gruss A. 2012. Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annu Rev Food Sci Technol 3:37-58.
155. Franza T, Delavenne E, Derre-Bobillot A, Juillard V, Boulay M, Demey E, Vinh J, Lamberet G, Gaudu P. 2016. A partial metabolic pathway enables group b streptococcus to overcome quinone deficiency in a host bacterial community. Mol Microbiol 102:81-91.
156. Diep DB, Nes IF. 2002. Ribosomally synthesized antibacterial peptides in Gram positive bacteria. Curr Drug Targets 3:107-22.
157. Mercier C, Domakova E, Tremblay J, Kulakauskas S. 2000. Effects of a muramidase on a mixed bacterial community. FEMS Microbiol Lett 187:47-52.
158. Nouaille S, Rault L, Jeanson S, Loubiere P, Le Loir Y, Even S. 2014. Contribution of Lactococcus lactis reducing properties to the downregulation of a major virulence regulator in Staphylococcus aureus, the agr system. Appl Environ Microbiol 80:7028-35.
159. Assis BS, Germon P, Silva AM, Even S, Nicoli JR, Le Loir Y. 2015. Lactococcus lactis V7 inhibits the cell invasion of bovine mammary epithelial cells by Escherichia coli and Staphylococcus aureus. Benef Microbes 6:879-86.
160. Mannam P, Jones KF, Geller BL. 2004. Mucosal vaccine made from live, recombinant Lactococcus lactis protects mice against pharyngeal infection with Streptococcus pyogenes. Infect Immun 72:3444-50.
161. Bermudez-Humaran LG, Langella P, Cortes-Perez NG, Gruss A, Tamez-Guerra RS, Oliveira SC, Saucedo-Cardenas O, Montes de Oca-Luna R, Le Loir Y. 2003. Intranasal immunization with

recombinant Lactococcus lactis secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect Immun 71:1887-96.
162. Repa A, Grangette C, Daniel C, Hochreiter R, Hoffmann-Sommergruber K, Thalhamer J, Kraft D, Breiteneder H, Mercenier A, Wiedermann U. 2003. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. Vaccine 22:87-95.
163. Chatel JM, Nouaille S, Adel-Patient K, Le Loir Y, Boe H, Gruss A, Wal JM, Langella P. 2003. Characterization of a Lactococcus lactis strain that secretes a major epitope of bovine beta-lactoglobulin and evaluation of its immunogenicity in mice. Appl Environ Microbiol 69:6620-7.
164. Michon C, Langella P, Eijsink VG, Mathiesen G, Chatel JM. 2016. Display of recombinant proteins at the surface of lactic acid bacteria: strategies and applications. Microb Cell Fact 15:70.
165. Maguin E, Prevost H, Ehrlich SD, Gruss A. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J Bacteriol 178:931-5.
166. Biswas I, Gruss A, Ehrlich SD, Maguin E. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol 175:3628-35.
167. Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet 253:217-24.
168. Siegers K, Heinzmann S, Entian KD. 1996. Biosynthesis of lantibiotic nisin. Posttranslational modification of its prepeptide occurs at a multimeric membrane-associated lanthionine synthetase complex. J Biol Chem 271:12294-301.
169. Siegers K, Entian KD. 1995. Genes involved in immunity to the lantibiotic nisin produced by Lactococcus lactis 6F3. Appl Environ Microbiol 61:1082-9.
170. Horn N, Swindell S, Dodd H, Gasson M. 1991. Nisin biosynthesis genes are encoded by a novel conjugative transposon. Mol Gen Genet 228:129-35.
171. Mierau I, Kleerebezem M. 2005. 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Appl Microbiol Biotechnol 68:705-17.
172. Zhu D, Liu F, Xu H, Bai Y, Zhang X, Saris PE, Qiao M. 2015. Isolation of strong constitutive promoters from Lactococcus lactis subsp. lactis N8. FEMS Microbiol Lett 362.
173. Jensen PR, Hammer K. 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Appl Environ Microbiol 64:82-7.
174. Ogaugwu CE, Cheng Q, Fieck A, Hurwitz I, Durvasula R. 2018. Characterization of a Lactococcus lactis promoter for heterologous protein production. Biotechnol Rep (Amst) 17:86-92.
175. Brondsted L, Hammer K. 1999. Use of the integration elements encoded by the temperate lactococcal bacteriophage TP901-1 to obtain chromosomal single-copy transcriptional fusions in Lactococcus lactis. Appl Environ Microbiol 65:752-8.
176. Stoll SM, Ginsburg DS, Calos MP. 2002. Phage TP901-1 site-specific integrase functions in human cells. J Bacteriol 184:3657-63.
177. Frazier CL, San Filippo J, Lambowitz AM, Mills DA. 2003. Genetic manipulation of Lactococcus lactis by using targeted group II introns: generation of stable insertions without selection. Appl Environ Microbiol 69:1121-8.
178. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709-12.
179. Millen AM, Horvath P, Boyaval P, Romero DA. 2012. Mobile CRISPR/Cas-mediated bacteriophage resistance in Lactococcus lactis. PLoS One 7:e51663.
180. Hidalgo-Cantabrana C, O'Flaherty S, Barrangou R. 2017. CRISPR-based engineering of next-generation lactic acid bacteria. Curr Opin Microbiol 37:79-87.
181. Kunji ER, Slotboom DJ, Poolman B. 2003. Lactococcus lactis as host for overproduction of functional membrane proteins. Biochim Biophys Acta 1610:97-108.
182. Poquet I, Ehrlich SD, Gruss A. 1998. An export-specific reporter designed for gram-positive bacteria: application to Lactococcus lactis. J Bacteriol 180:1904-12.
183. Le Loir Y, Gruss A, Ehrlich SD, Langella P. 1994. Direct screening of recombinants in gram-positive bacteria using the secreted staphylococcal nuclease as a reporter. J Bacteriol 176:5135-9.
184. Le Loir Y, Nouaille S, Commissaire J, Bretigny L, Gruss A, Langella P. 2001. Signal peptide and propeptide optimization for heterologous protein secretion in Lactococcus lactis. Appl Environ Microbiol 67:4119-27.
185. Le Loir Y, Gruss A, Ehrlich SD, Langella P. 1998. A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in Lactococcus lactis. J Bacteriol 180:1895-903.
186. Schneewind O, Mihaylova-Petkov D, Model P. 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. EMBO J 12:4803-11.

187. Navarre WW, Schneewind O. 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 63:174-229.
188. Fischetti VA, Pancholi V, Schneewind O. 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci. Mol Microbiol 4:1603-5.
189. Dieye Y, Oxaran V, Ledue-Clier F, Alkhalaf W, Buist G, Juillard V, Lee CW, Piard JC. 2010. Functionality of sortase A in Lactococcus lactis. Appl Environ Microbiol 76:7332-7.
190. Piard JC, Hautefort I, Fischetti VA, Ehrlich SD, Fons M, Gruss A. 1997. Cell wall anchoring of the Streptococcus pyogenes M6 protein in various lactic acid bacteria. J Bacteriol 179:3068-72.
191. Steen A, Buist G, Leenhouts KJ, El Khattabi M, Grijpstra F, Zomer AL, Venema G, Kuipers OP, Kok J. 2003. Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J Biol Chem 278:23874-81.
192. Lindholm A, Smeds A, Palva A. 2004. Receptor binding domain of Escherichia coli F18 fimbrial adhesin FedF can be both efficiently secreted and surface displayed in a functional form in Lactococcus lactis. Appl Environ Microbiol 70:2061-71.
193. Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A. 2000. HtrA is the unique surface housekeeping protease in Lactococcus lactis and is required for natural protein processing. Mol Microbiol 35:1042-51.
194. Buist G, Karsens H, Nauta A, van Sinderen D, Venema G, Kok J. 1997. Autolysis of Lactococcus lactis caused by induced overproduction of its major autolysin, AcmA. Appl Environ Microbiol 63:2722-8.
195. Gasson MJ. 1996. Lytic systems in lactic acid bacteria and their bacteriophages. Antonie Van Leeuwenhoek 70:147-59.
196. Sanders JW, Venema G, Kok J. 1997. A chloride-inducible gene expression cassette and its use in induced lysis of Lactococcus lactis. Appl Environ Microbiol 63:4877-82.
197. de Ruyter PG, Kuipers OP, Meijer WC, de Vos WM. 1997. Food-grade controlled lysis of Lactococcus lactis for accelerated cheese ripening. Nat Biotechnol 15:976-9.
198. Schuch R, Nelson D, Fischetti VA. 2002. A bacteriolytic agent that detects and kills Bacillus anthracis. Nature 418:884-9.
199. Loeffler JM, Nelson D, Fischetti VA. 2001. Rapid killing of Streptococcus pneumoniae with a bacteriophage cell wall hydrolase. Science 294:2170-2.
200. Nelson D, Loomis L, Fischetti VA. 2001. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. Proc Natl Acad Sci U S A 98:4107-12.
201. Bidnenko E, Mercier C, Tremblay J, Tailliez P, Kulakauskas S. 1998. Estimation of the state of the bacterial cell wall by fluorescent In situ hybridization. Appl Environ Microbiol 64:3059-62.
202. Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B, Cox E, Remon JP, Remaut E. 2003. Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. Nat Biotechnol 21:785-9.
203. Dickely F, Nilsson D, Hansen EB, Johansen E. 1995. Isolation of Lactococcus lactis nonsense suppressors and construction of a food-grade cloning vector. Mol Microbiol 15:839-47.
204. Bermudez-Humaran LG, Cortes-Perez NG, Le Loir Y, Alcocer-Gonzalez JM, Tamez-Guerra RS, de Oca-Luna RM, Langella P. 2004. An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. J Med Microbiol 53:427-33.
205. Norton PM, Brown HW, Wells JM, Macpherson AM, Wilson PW, Le Page RW. 1996. Factors affecting the immunogenicity of tetanus toxin fragment C expressed in Lactococcus lactis. FEMS Immunol Med Microbiol 14:167-77.
206. Grangette C, Muller-Alouf H, Hols P, Goudercourt D, Delcour J, Turneer M, Mercenier A. 2004. Enhanced mucosal delivery of antigen with cell wall mutants of lactic acid bacteria. Infect Immun 72:2731-7.
207. Wyszynska A, Kobierecka P, Bardowski J, Jagusztyn-Krynicka EK. 2015. Lactic acid bacteria--20 years exploring their potential as live vectors for mucosal vaccination. Appl Microbiol Biotechnol 99:2967-77.
208. Rosales-Mendoza S, Angulo C, Meza B. 2016. Food-Grade Organisms as Vaccine Biofactories and Oral Delivery Vehicles. Trends Biotechnol 34:124-136.
209. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:D141-5.
210. Lechardeur D, Cesselin B, Fernandez A, Lamberet G, Garrigues C, Pedersen M, Gaudu P, Gruss A. 2011. Using heme as an energy boost for lactic acid bacteria. Curr Opin Biotechnol 22:143-9.