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Stress Physiology of Lactic Acid Bacteria

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SUMMARY

Lactic acid bacteria (LAB) are important starter, commensal, or pathogenic microorganisms. The stress physiology of LAB has been studied in depth for over 2 decades, fueled mostly by the technological implications of LAB robustness in the food industry. Survival of probiotic LAB in the host and the potential relatedness of LAB virulence to their stress resilience have intensified interest in the field. Thus, a wealth of information concerning stress responses exists today for strains as diverse as starter (e.g., *Lactococcus lactis*), probiotic (e.g., several *Lactobacillus* spp.), and pathogenic (e.g., *Enterococcus* and *Streptococcus* spp.) LAB. Here we present the state of the art for LAB stress behavior. We describe the multitude of stresses that LAB are confronted with, and we present the experimental context used to study the stress responses of LAB, focusing on adaptation, habituation, and cross-protection as well as on self-induced multistress resistance in stationary phase, biofilms, and dormancy. We also consider stress responses at the population and single-cell levels. Subsequently, we concentrate on the stress defense mechanisms that have been reported to date, grouping them according to their direct participation in preserving cell energy, defending macromolecules, and protecting the cell envelope. Stress-induced responses of probiotic LAB and commensal/pathogenic LAB are highlighted separately due to the complexity of the peculiar multistress conditions to which these bacteria are subjected in their hosts. Induction of prophages under environmental stresses is then discussed. Finally, we present systems-based strategies to characterize the "stressome" of LAB and to engineer new food-related and probiotic LAB with improved stress tolerance.

INTRODUCTION

Fermented foods are among the oldest forms of processed foods that have evidently survived into today's modern diet. They are produced during the biotransformation of raw materials into

the final product by the action of microorganisms. The vast majority of food biotransformations rely either on ethanol fermentation performed by the yeast *Saccharomyces cerevisiae* or on lactic acid fermentation performed by a relatively wide range of bacteria called lactic acid bacteria (LAB) (1). LAB were among the first bacteria to be studied because of their involvement in food fermentations and in human health.

In the early days, LAB taxonomy relied on morphological and physiological characteristics. The first technical definition, by Orla-Jensen, recognized LAB as Gram-positive cocci or bacilli that were nonsporulating and nonmotile and had the ability to catabolize sugars mainly into lactic acid (2). These classification criteria led to a broad definition of LAB comprising diverse bacteria. During the 1990s, advances in molecular techniques allowed a more elaborate description of LAB (3, 4). LAB generally have a low GC content (<50 mol%), while some lactobacilli have been reported to reach up to 57 mol% (5). They are Gram-positive, non-spore-forming, microaerophilic or anaerobic bacteria that produce lactic acid as the major end product of sugar fermentation. LAB are typically catalase and cytochrome negative, fastidious, aerotolerant, and acid tolerant. The most common genera of LAB considered to be food related are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Carnobacterium*, and *Weissella*. Even though it has been suggested that LAB are a heterogeneous group of bacteria and a universal technical definition may not exist, all the aforementioned genera have been shown to have diverged from a common ancestor. Both 16S rRNA gene and whole-genome phylogenies have revealed that the "core" LAB species form the distinct order *Lactobacillales* in the class *Bacilli* of the phylum *Firmicutes* (3, 4, 6). Based on this observation, a nonphylogenetic approach for defining LAB is rapidly becoming obsolete. For example, bifidobacteria or certain *Bacillus* species, which exhibit some characteristics in common with LAB, are no longer included in this group *sensu stricto*. According to the latest review about LAB taxonomy, the order *Lactobacillales* consists of six families with 38 genera and more than 400 species (7).

LAB starter cultures generate a bacteriostatic or even bactericidal environment for spoilage and pathogenic bacteria by lowering the pH of the food matrix during lactic acid fermentation. LAB fermentation combined with appropriate technological hurdles leads to safe food products with an extended shelf-life. LAB also play an important role in the development of the organoleptic properties of the product. Through their metabolic activities (e.g., lipolysis and proteolysis), LAB produce important aroma and flavor compounds, while they can also contribute to the texture (e.g., by the production of exopolysaccharides [EPS]). The involvement of LAB in food production is far from being unintentional. Food-related LAB are among the very few microorganisms that were domesticated by humans (1, 8). Domestication of LAB started several millennia ago. During this period, LAB genomes were streamlined by genome decay due to adaptation to food environments rich in nutrients (6). This process of reductive evolution resulted in metabolic simplification and in LAB strains with multiple auxotrophies due to the loss of several biosynthetic pathways. On the other hand, gene acquisition events allowed the gain of important technological properties (6). The continuous selective pressure for high-quality products exerted by humans led to the development of today's starters.

However, not all LAB are related to food fermentations. Even a superficial examination of LAB taxonomy reveals that several species are commensals and pathogens. This fact has not always been reflected in the literature. Frequently, food microbiologists and food technologists focused solely on benign LAB, leaving LAB pathogens to clinical microbiologists, and *vice versa*, irrespective of the underlying phylogenetic relationship. Recent metagenomic data support early observations of LAB being a part of the microbiomes of humans and other animals (9, 10). LAB are known to participate in the intricate balance of the microbiome ecosystem, which can be decisive for both health and disease. Interestingly, in some cases this is achieved by acidification of the environment by LAB in a manner similar to that in food fermentations (11). In addition to their antimicrobial properties, certain LAB can stimulate particular activities of the immune system of the host, prevent diarrheas following antibiotic treatment or viral infections, produce vitamins *in situ*, or lead to reduced cholesterol levels in blood (12). Thus, several LAB are used as probiotic bacteria, i.e., as "live microorganisms that confer a health benefit on the host when administered in adequate amounts" (13). Note that probiotic LAB strains may originate from food fermentations or might be commensals. Nonetheless, there are several formidable opportunistic LAB pathogens, i.e., commensals that can turn virulent given the right conditions. Such species are found mainly in the *Streptococcus* and *Enterococcus* genera (14). Group A streptococci (GAS), group B streptococci (GBS), and *Streptococcus pneumoniae* can cause invasive and life-threatening infections, while *Enterococcus faecalis* and *Enterococcus faecium* have emerged as major causative agents of nosocomial infections (14).

Given the involvement of LAB in food production and health, it is not surprising that they are among the best-studied microorganisms. Over the past 30 to 40 years, the physiology, biochemistry, genetics, and evolution of LAB have been the focus of research in many laboratories around the world. Our knowledge about LAB was revolutionized with the advent of genome sequencing, while recently developed meta-omics technologies allow monitoring of their behavior in complex food and microbiome ecosystems. A field of research that has received much attention early on is the stress physiology of LAB. Like all other microorganisms, LAB are exposed to stressful conditions, but studying the stress responses of the different categories of LAB is important for different reasons.

A key aspect of starter LAB is their robustness during the production and storage of fermented foods. Vulnerability of starters to technological hurdles may influence the fermentation process *per se*, which can have a serious impact on food quality and safety. Similarly, LAB need to be able to resist technological stresses during preparation of probiotic formulas to maintain high viable counts. After consumption, probiotic LAB must survive the harsh conditions in the gastrointestinal tract (GIT). Commensal LAB have to adapt to the various conditions prevailing in the different niches of the host, while pathogenic LAB need to counteract the host's innate immunity. LAB have also emerged as models for the study of bacterial stress physiology. Available genome sequences indicate that LAB are devoid of a dedicated stress sigma factor, such as σ^B, present in *Bacillus subtilis* and many other related Gram-positive bacteria (15). This is a major difference with important implications for gene regulation under stress conditions.

The latest review on the overall stress physiology of LAB was published by van de Guchte et al. in 2002 (16). Here we present a detailed overview of the latest developments in the study of the

stress behavior of LAB. After an initial presentation of central concepts of LAB stress physiology developed over the years, we concentrate on the stress defense mechanisms that have been reported to date. These responses are grouped according to their direct participation in preserving cell energy, defending macromolecules, and protecting the cell envelope. Important paradigms of stress-induced responses of probiotic and pathogenic LAB are described separately in order to highlight the relationship of stress to probiotic potential and virulence, respectively. The induction of prophages under a variety of environmental stresses is then discussed. The final part of the review is devoted to systems-based strategies used to characterize the "stressome" of LAB and to engineer LAB strains with improved stress tolerance. Considering the immensity of the relevant literature, only representative and/or key citations are included in this review.

STRESSES, EXPERIMENTAL CONTEXT, AND PHENOTYPES

Common Stresses Encountered by LAB in Their Ecological Niches

LAB are confronted with both abiotic and biotic stresses. Abiotic stresses are mainly those arising during food production and the manipulation of starter or probiotic cultures, while biotic stresses are encountered in the host or in complex ecosystems. In several instances, the type of elementary stress condition is the same regardless of the origin, be it technological or biological. The stresses that have been studied in LAB to date, along with some important characteristics, are presented below.

In the case of LAB, acid stress is a self-imposed stress. Lactic acid is the major end product of sugar fermentation and is virtually used as an antimicrobial agent against competing microorganisms. LAB are relatively acid tolerant, but the accumulation of lactic acid ultimately influences their physiology (17). It is not uncommon for LAB to cease to grow due to autoacidification rather than depletion of nutrients, while prolonged exposure to acidic conditions usually results in cell death. Acid stress is also relevant for probiotic LAB. In the GIT, the stomach retains a low pH between 2.0 and 4.0 via the production of HCl. The gastric juice also contains digestive enzymes (e.g., pepsin) that may additionally damage cells. Even though probiotics can be protected from the gastric environment by appropriate encapsulation, stomach transit with minimal loss of viability is still considered an important probiotic property (12). Likewise, pathogenic LAB are confronted with low pHs, e.g., in macrophages after phagocytosis. In brief, low pH damages both the cell wall and the cell membrane, thus influencing ΔpH and the membrane potential. Acidification of the cytosol is genotoxic and results in the denaturation of proteins. Overall metabolism is affected, which leads to energy depletion and cell death.

Research of bacterial responses to high temperatures led to the discovery of a set of heat-inducible proteins known as heat shock proteins (HSPs), which are employed to counteract the pleiotropic effects of heat stress (18). Major HSPs partake in the repair and turnover of damaged proteins. Heat stress is commonly encountered by many LAB. During food fermentation, high temperatures (>60°C) are used for pasteurization of raw materials. The indigenous LAB population has to cope with pasteurization, particularly in spontaneous fermentations. In fermentations where LAB are added as starters after the pasteurization step, they may be exposed to reheating steps that are necessary for the production of specific foods (e.g., in several types of cheese). In contrast to food-related LAB, commensals and pathogenic LAB encounter less drastic temperature fluctuations due to thermoregulation of the host. Still, fever may be considered a defense mechanism relying on heat stress to combat pathogens.

Increased osmolarity is an important hurdle used in the production of numerous fermented foods. As many food spoilage and pathogenic microorganisms are rather sensitive to high osmotic pressures compared to LAB, NaCl is usually added to aid the indigenous or starter LAB in initiating and taking over the fermentation process. Osmotic stress decreases the positive turgor of bacterial cells as a result of dehydration. Under such conditions, cells either produce or import small molecules, called osmolytes (e.g., glycine betaine, choline, or proline), to balance the difference between intracellular and extracellular osmolarities to allow rehydration through membrane-associated channels (19). Even though variations in osmolarity may exist among the different niches within a host, osmotic stress is generally not acknowledged as a major stress for commensal and pathogenic LAB.

Low temperatures are used for storing raw materials and foods to prevent spoilage. Also, LAB starter or probiotic cultures are most frequently stored in a frozen or freeze-dried form, with appropriate cryoprotectants included to increase viability (20). Cold stress leads to the induction of a set of proteins called cold shock proteins (CSPs) (21). All CSPs belong to one family of closely related low-molecular-weight proteins. They can bind to single-stranded nucleic acids and resolve secondary structures formed at low temperatures. CSPs are thus considered to support transcription and translation under cold stress. Cold temperatures above freezing may lead to growth arrest of LAB, but such conditions do not abruptly provoke cell death. In fact, many LAB can be stored at low temperatures (>0°C) for several days. In contrast, freezing of LAB cultures influences survival in a strain-dependent manner. As in the case of osmotic stress, cold stress is not commonly encountered by commensal and pathogenic LAB.

Although LAB are typically microaerophiles lacking a functional respiratory chain and catalases, several species are aerotolerant. Notwithstanding this, LAB are susceptible to aerobic conditions during food production and in the host. O_2 metabolism by LAB can also lead to the production of reactive oxygen species (ROS), and some strains can produce copious amounts of H_2O_2. They can also be exposed directly to ROS, e.g., during the oxidative burst in cells of the immune system. Oxidative stress influences the redox potential of the cell by affecting many enzymatic reactions. ROS are highly reactive moieties that can damage all major macromolecules of the cell, including proteins, DNA, and lipids. LAB possess a number of mechanisms for the detoxification of ROS. Nevertheless, several LAB have been shown to undergo respiration if heme and/or menaquinones are supplied exogenously (22). The implications of respiration in LAB are still a matter of investigation, but it is clearly an additional defense against oxidative stress.

Starvation, as a characteristic stress for free-living microorganisms, has been studied to some extent in LAB, although LAB reside in highly nutritious environments in which depletion of a nutrient rarely becomes the limiting factor for growth. It is generally acknowledged that starvation may be induced indirectly in LAB, as a side effect of another stress. A typical example is the starvation caused by lactic acid autoacidification through interference of the low pH with the action of transporters in the cytoplasmic mem-

brane and the consequent abolishment of nutrient uptake. Sugar starvation is important from a technological perspective because under these conditions, food-related LAB start to catabolize amino acids as an alternative carbon/energy source, resulting in the production of aroma compounds. Dairy LAB may convert branched-chain amino acids (BCAAs) to volatile branched-chain fatty acids (BCFAs) for ATP synthesis (23).

The cell envelope is the physical barrier separating the cell from its environment and, as such, the first line of defense against environmental perturbations. Changes in chemical composition of both the cell wall and the cell membrane triggered by stress have been shown to aid in cell survival. Maintaining the integrity of the cell wall under stress conditions is a matter of life or death for bacteria. The cell envelope is also a major cellular organelle with several physiological functions. It is thus not surprising that the cell envelope is the direct target of a multitude of antimicrobials (24). Over the past years, it has been demonstrated that LAB, like other bacteria, closely monitor the integrity of the cell envelope and that specialized repair mechanisms are induced in case of damage (25).

Apart from the stress conditions mentioned above that are relevant to the majority of LAB, there are stresses that may be faced exclusively by a limited number of species. Ethanol stress is of particular importance mainly for *Oenococcus oeni*, which performs malolactic fermentation (MLF) during wine making. There are also stresses that have gained momentum rather recently, such as metal stress (26). During acidification, LAB may cause the solubilization of metals, which may be toxic depending on their concentration. Moreover, there are stresses that have not been investigated in LAB as thoroughly as in other bacteria (e.g., DNA damage) and stresses that have not been considered physiologically relevant to the lifestyle of LAB (e.g., hypo-osmotic or alkaline stress).

In the majority of studies of the stress physiology of LAB, strains are exposed to only a single stressor to better dissect the physiological and molecular mechanisms underpinning the responses. In reality, LAB reside mostly in multistress environments that are fairly nutritious to compensate for their auxotrophies. The fastidious nature of LAB is often perceived as a vulnerability that may be associated with a diminished tolerance to environmental stresses. This assumption is far from the truth, and although no LAB has been characterized formally as an extremophile, several species/strains can tolerate or even grow in harsh environments. During transit through the GIT, several *Lactobacillus* spp. survive the low pH of the stomach (pH 2.0 to 4.0) and the subsequent exposure to bile salts and pancreatic juice in the duodenum (27). *Lactobacillus* spp. have also been found in the stomach microbiome (28). *Lactobacillus suebicus* isolated from fruit mash is able to grow at pH 3.0 and in the presence of 14% ethanol (29). *O. oeni* strains have been reported to proliferate in the presence of 13% ethanol at pH 3.2 and 18°C (30). *Tetragenococcus* spp. can survive and grow in salt at concentrations of up to 25% (wt/vol) (31), while *Leuconostoc gelidum* isolated from chilled products can grow at low temperatures, even at 1°C (32). In conclusion, there is ample evidence that LAB may be particularly robust bacteria.

Experimental Context for the Study of LAB Stress Physiology

A number of experimental approaches for the study of bacterial stress physiology have been adopted over the years. In practice, a stress is defined as a condition that results in reduced bacterial cell growth or survival. Growth and survival assays require an *a priori* determination of the optimal conditions to be used as controls for comparison. It has been argued that choosing the optimal conditions is arbitrary, since it is impossible to determine them experimentally without making at least some assumptions (e.g., suitability of the growth medium) (33). A more general definition of stress has been suggested to circumvent this discrepancy (33). In current terms, stress can be considered any transition of a bacterial cell from one condition to another that causes alterations to the cell's genome, transcriptome, proteome, and/or metabolome leading to reduced growth or survival potential. This definition of stress applies without the need to determine any "optimal" conditions.

Under stress, cells try to adapt by appropriate molecular responses in an attempt to ameliorate the negative effects and restore growth or survival potential. These adaptive or stress responses are the main focus of LAB stress physiology research. Bacteria continually monitor changes in their environment and respond whenever necessary (see below). Stress responses have been correlated with specific phenotypes so that they can be induced in a controllable and reproducible manner.

The phenotype appearing most frequently in the literature is that of the adapted cell. While adaptation is a general term describing the effort of an organism to resist and persist under stress, it has also been associated with a specific experimental setup in which cells are transiently exposed to mild nonlethal stress conditions that result in increased survival after a subsequent lethal challenge to the same stress. This type of adaptive response is mostly triggered rapidly, in the first minutes or hours of exposure to the mild stress. Two main variations of this basic experiment exist. First, cells may be left under suboptimal conditions much longer than needed to induce adaptation. This adaptive procedure is probably best described as habituation, a term used only scarcely for LAB (34). The molecular mechanisms underlying transient adaptation and habituation to a specific stress may overlap to a degree, but they are not completely identical (34–36). This may explain a number of contradictory results reported for some LAB species, since the two responses have sometimes been considered identical (37, 38). Alternatively, cells are transiently adapted to a stress and the lethal challenge is performed with a different stress. This treatment often results in increased survival, a phenomenon known as cross-protection. The exact combination of the two stresses that leads to cross-protection is species or even subspecies dependent. Cross-protection suggests an induction of molecular mechanisms during exposure to the first stressor that protects cells from the subsequent lethal challenge, and it may be of particular importance for LAB, as they are often exposed sequentially to a variety of stresses.

There are also phenotypes displaying a generalized resistance to many stresses at the same time. One such phenotype is observed when cells enter stationary phase. Cells enter stationary phase due to the exhaustion of nutrients and/or the accumulation of toxic metabolic products in their environment during growth. The environmental conditions of stationary phase become so stressful that the death rate of cells is accelerated (34, 39). Transition from the exponential to the stationary phase is accompanied by the induction of multiple regulons, resulting in the ability to cope with a number of different stresses. This phenotype increases the likelihood of survival until growth conditions are reestablished,

and it is especially important for LAB, which, unlike several other Gram-positive bacteria, are unable to form spores. Another multistress resistance phenotype has been shown for cells in biofilms. Such cells are more resilient than planktonic cells. Some bacterial species are naturally prone to forming biofilms, but biofilm formation may also be stress induced. In fact, early research with oral LAB, such as *Streptococcus mutans*, allowed the resistance phenotype of biofilms to be established (40). Biofilms are relevant for all types of LAB, including food-related ones (41), probiotic LAB (42), commensals (43), and pathogens (40). Interestingly, it has been demonstrated that during stationary-phase, biofilm formation, starvation, and other stressful conditions, some bacteria are viable but not culturable (VBNC). In this distinct physiological state, cells are metabolically active and multistress resistant but have lost the ability to proliferate. VBNC cells may resuscitate under specific conditions or in the presence of specific resuscitating molecules. Entry into the VBNC state is considered an adaptive strategy for long-term survival (44). A number of studies have addressed the VBNC state in LAB. However, since injured cells (see below) and VBNC cells may have similar phenotypes, it is not clear whether LAB truly exhibit the VBNC adaptive response. Finally, persister cells are subpopulations of multiple-antibiotic-resistant cells. In contrast to resistant cells that can grow in the presence of antibiotics, persister cells can resist lethal doses of antibiotics in the absence of growth. The persister cell phenotype has in a few cases been investigated in pathogenic LAB (for example, see references 45 and 46). Only recently was it suggested that the VBNC and persister physiological states may be closely related, with both employing dormancy as the main mode of stress resistance (47). The development and physiology of dormancy in LAB are poorly understood and surely deserve further investigation.

All adaptive responses described above rely basically on epigenetic mechanisms, since regrowth of adapted cells under optimal conditions abolishes any resistance phenotype. Even though it may take a number of generations before the resistance fades away, the fitness of the population ultimately returns to basal levels. Interestingly, stress-induced mutations can also occur; cells exposed to certain stresses can enter a hypermutable state, thereby increasing the diversity of a clonal population, which may lead to new genotypes allowing survival under conditions otherwise detrimental to the wild type (48). Increased mutation rates can be achieved by error-prone DNA polymerases, errors during transcription and/or translation, and activation of mobilizable elements. There is some evidence that adaptive mutations occur in LAB as a response to certain stresses (49, 50).

Adaptive responses, whether epigenetic or not, are the ultimate means to ensure bacterial survival under stress. Compelling evidence suggests that such responses are not inert but are characterized by some degree of plasticity. A number of studies show that diverse stress-resistant phenotypes may protect against the same stressor despite the fact that the molecular mechanisms involved may differ slightly or even in a major way (35, 36). Cells can apparently deploy multiple and overlapping resistance mechanisms to prevent or repair damage and achieve maximal survival.

Moving from the Population to the Single Cell

A fundamental problem inherently linked to the study of bacterial stress physiology is how to determine survival. The most practical methods rely on culturability, which imposes a binary logic to the assessment of survival (33). Cells are pronounced alive or dead depending on whether they are able to proliferate in a specific medium and under certain conditions. In reality, only culturable cells can be counted accurately on the basis of growth, while the number of dead cells is deduced indirectly from the untreated control population. However, there is at least one additional physiological state after exposure of a population of cells to stress, namely, that of the "injured" cell (51). Injured cells are metabolically active but have suffered damage to a degree that causes a transient or even permanent loss of proliferation capacity (34). They sometimes require extended recovery times or can recover only under special conditions. Injured cells may in a broad sense be considered VBNC, but they are not the result of an adaptive response. The assays to determine the presence or number of injured and VBNC cells are often the same. The simplest procedure is to use a fluorescent probe to reveal metabolic activity in the absence of culturability. A variety of probes are commonly used in *in situ* viability tests to measure, e.g., metabolic activity, membrane potential, replication, and membrane integrity (51, 52). Such probes have been coupled successfully with fluorescence microscopy or flow cytometry to assess the viability of stressed LAB. Novel quantitative PCR (qPCR)-based methods are also being developed. It is becoming increasingly evident that there is heterogeneity among live and injured cells. The more multiplex a viability assay is, for instance, by employing an increasing number of probes, the more subpopulations can be identified (51). Subpopulations determined by *in situ* assays can be characterized further by various culturability tests after cell sorting (34). *In situ* assays are appealing for industrial applications because they might offer very fast, nearly real-time monitoring of the physiological state of cells. Another important advantage of *in situ* assays is that they can be applied at the single-cell level. In the majority of studies, stress responses of LAB have been assessed only at the population level. Such strategies demonstrate the involvement of molecular mechanisms in an averaging manner, while the presence of any subpopulation(s) remains undetected. The coexistence of live, injured, and dead cells after lethal challenge is an indirect indication of an inherent heterogeneity in the original population. This population heterogeneity among bacterial clones has been attributed to asynchronous progression through the cell cycle, differences in cell age, mutations, variations in microenvironment conditions, and stochastic phenomena (52). Monitoring the kinetics of adaptation at the single-cell level has also revealed that the response is acquired on a cell-by-cell basis (34, 53), while not all cells are able to adapt within the same time frame or to the same extent (34). Current developments in "-omic" technologies are expected to allow for in-depth study of the stress physiology of single cells that is the basis of any stress response.

SENSING AND SIGNALING STRESSES IN LAB

Bacteria utilize a wide array of sensors to monitor the intracellular and extracellular environments and to regulate the cell physiology to cope with environmental changes (Fig. 1). Signal transduction systems can broadly be divided into two major categories: one-component systems (OCSs) and two-component systems (TCSs) (54). In OCSs, the sensory and output functions are located in the same polypeptide, while they are located on separate polypeptides in TCSs. In addition, other molecules, such as nucleic acids and lipids, can also act as sensors. Some of these, mostly RNA molecules, can elicit a response by themselves, while others transfer signals to protein partners that relay them or elicit the response.

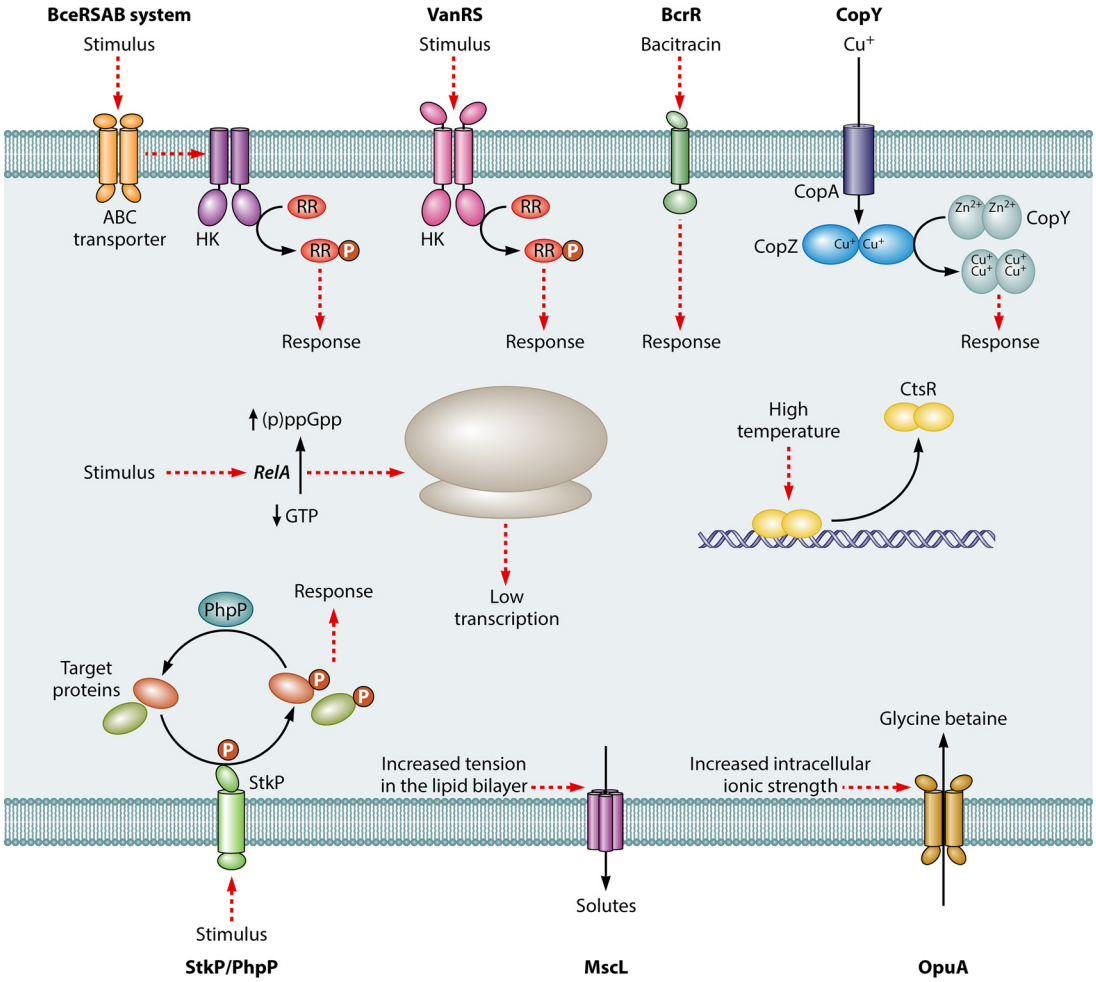

FIG 1 Schematic representation of selected LAB stress sensory systems. HK, histidine kinase; RR, response regulator.

Two-Component Systems

TCSs are signal transduction pathways typically consisting of a usually membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). HKs and RRs are modular proteins containing homologous domains, namely, a kinase domain and an H box in HKs and a receptor domain in RR, all of which are involved in the phosphotransfer reaction. They also contain heterologous sensory (HKs) and signaling (RRs) domains, which are involved in the reception of a specific stimulus and the delivery of the corresponding response, respectively. In general, detection of a specific stimulus triggers HK autophosphorylation on a conserved His residue and the subsequent transfer of the phosphate group to the receptor domain of its cognate RRs. Phosphorylation of the RR modulates its activity, which in most cases involves transcriptional regulation (55). Dephosphorylation of RRs is carried out by auxiliary phosphatases or, often, by the cognate HKs (56, 57). The final output response results from a balance between kinase and phosphatase activities.

TCS complements vary widely in LAB (58, 59). A clear correlation between genome size or lifestyle and the number of TCSs cannot be established, although, generally, species with the largest genomes encode the largest numbers of TCSs (58). Lactococci encode relatively few TCSs, ranging from 7 in *Lactococcus lactis* (*Lc. lactis*) strains IL1403 and MG1363 to 10 in *Lc. lactis* KF147 (59). The numbers of TCSs in streptococci vary from 8 in *Streptococcus thermophilus* LMD-9 to 31 in *Streptococcus pyogenes* MGAS8232 or *S. pyogenes* MGAS315 (59). In contrast to those in other LAB, orphan HKs and RRs are relatively frequent in streptococci.

The involvement of TCSs in stress responses in LAB has been evidenced mainly by phenotypic analyses of TCS-defective mutants (60–67). Inactivation of homologous TCSs often results in different phenotypes, suggesting that they have different physiological roles. For example, inactivation of the *rrp-31 hpk-31* (LSA0277-LSA0278) system of *Lactobacillus sakei* led to premature arrest of growth under reference conditions (MRS broth at 30°C), poor growth at high temperature (39°C), sensitivity to heat shock, aeration, and H_2O_2, and a higher resistance against vancomycin (60). In contrast, inactivation of the *Lactobacillus casei* (*Lb. casei*) BL23 homolog TCS01 resulted in normal growth in MRS broth at 37°C and sensitivity to acid and vancomycin (61). In many cases, it is not clear whether the observed effects correspond to a specific stress response or to physiological changes that result in an altered ability to respond to stress. The few examples in which control by TCSs of the response to specific stressors has been established mostly concern those involved in the cell enve-

lope stress response. These are dealt with further below, in Protecting the Cell Envelope.

One-Component Systems

Although the roles of TCSs in sensing and signaling have been studied extensively, OCSs are actually much more abundant in prokaryotes (54). OCSs are proteins containing sensory and signaling domains; they lack HK and receptor domains and can be identified by amino acid conservation in their DNA-binding domains and by different conserved motifs (54). Twenty families of major prokaryotic OCSs have been recognized so far (68). With the exception of the MetJ family, they are all represented in LAB. Knowledge about the involvement of OCSs in LAB stress responses is still scant, but a number of systems have been characterized, especially those involved in resistance against cationic antimicrobial peptides (CAMPs) (see Protecting the Cell Envelope), in oxidative stress, and in metal homeostasis and resistance.

Metal stress sensory and signaling mechanisms. Resistance mechanisms against metals are not well characterized for LAB (reviewed in reference 26), and most available information concerns pathogenic streptococci. In bacteria, three types of metal-sensing transcriptional regulators control gene expression in response to metal ion concentrations: derepressors (ArsR-SmtB, CopY, and CsoR-RcnR families), activators (MerR family), and corepressors (Fur, NikR, and DtxR families) (69).

(i) MarR family members CopY and AdcR/ZitR. Characterized LAB copper sensors include the CopZ-type copper chaperones and the CopY-type regulators (26). For *Enterococcus hirae*, it has been proposed that CopZ binds cytoplasmic Cu^{+} and transfers it to CopB for transporter-mediated export and to the CopY regulator for signaling (26) (Fig. 1). CopY is an OCS with an N-terminal DNA-binding domain and a C-terminal metal-binding domain (26). At a low Cu^{+} concentration, Zn^{2+} occupies the metal-binding site of CopY and the protein is bound to its target sequence, thereby inhibiting *cop* operon expression. When the Cu^{+} concentration increases, Cu^{+}-CopZ transfers its copper ion to CopY, displacing Zn^{2+} from the metal-binding site and resulting in CopY release from the DNA. This allows transcription of the *cop* operon (26). The *Lc. lactis* copper resistance sensing and signaling pathway is possibly similar to that described for *E. hirae* (26). *Lc. lactis* CopR, the CopY homolog, controls a regulon of 14 mostly uncharacterized genes (70). Recently, a different copper resistance mechanism was described for *S. pneumoniae* (71). This organism lacks a CopZ homolog. Instead, the resistance system consists of the membrane-bound Cu chaperone CupA, a copper exporter (CopA), and a CopY-type regulator (71). The *copY*, *cupA*, and *copA* genes are arranged in an operon whose expression was shown to be induced by Cu and repressed by CopY (72). The primary roles of CupA are proposed to be the sequestering of Cu^{+} and its transfer to CopA, activities that are essential for copper resistance. If the concentration of Cu^{+} exceeds the capacity of CupA, free cytoplasmic Cu^{+} may be bound by the repressor CopY, thus relieving repression of *copY*, *cupA*, and *copA* (71).

Bacteria require zinc, but an excess of zinc has toxic effects. In many bacteria, zinc homeostasis is maintained by the concerted action of pairs of sensors that regulate either the uptake or efflux of Zn^{2+} (73). Regulation in *Lc. lactis* of the Zn^{2+} uptake system ZitSQP is under the control of the repressor ZitR (74). Purified ZitR is a dimer with up to two zinc ligands per monomer. It specifically binds two intact palindromic operator sites overlapping the −35 and −10 boxes of the *zit* promoter (75). ZitR requires Zn^{2+} to bind DNA, and transcriptional analyses have shown that *zitSQP* expression is induced only at Zn^{2+} concentrations below 100 nM, indicating that ZitR acts as a sensor of Zn^{2+} scarcity (75). *S. pneumoniae* encodes a homologous Zn^{2+} uptake system (AdcABC) that is regulated by a ZitR homolog (AdcR) (76). AdcR also regulates the expression of the four pneumococcal histidine triad (Pht) proteins, PhtA, PhtB, PhtD, and PhtE (77), involved in surface adhesion, as well as the Zn^{2+}-specific AdcA homolog AdcAII and a Zn^{2+}-dependent alcohol dehydrogenase (78, 79). The *adc* operon is present in most streptococci and has also been studied in *Streptococcus gordonii*, where it is involved in Mn^{2+} homeostasis (80), and in *Streptococcus suis*, where it regulates the expression of a Zn^{2+}/Mn^{2+} uptake system, ribosomal genes, and *pht* genes (81).

(ii) TetR family sensors and zinc resistance. TetR proteins constitute a widespread family of OCSs regulating many aspects of bacterial physiology and interacting with a vast array of ligands (68). A small number of TetR family regulators have been characterized for LAB, among which only *S. pneumoniae* SczA has so far been implicated in a stress response. Resistance against zinc in *S. pneumoniae* depends mostly on the CzcD transporter, whose expression is under the control of SczA and whose activity is Zn^{2+} dependent (82). Despite extensive studies on other TetR regulators, metal-binding TetR regulators are quite uncommon in bacteria and await further characterization (68).

(iii) Regulators of the DtxR/MntR family. Regulators of the DtxR/MntR family are metal sensors that usually bind Fe^{2+} or Mn^{2+} (69). Cytoplasmic manganese plays a significant role in the protection against oxidative stress in LAB (83). In some streptococci, Mn^{2+} intake is mediated by the ScaCBA transporter, whose expression is under the control of ScaR, a regulator of the DtxR/MntR family (84). ScaR represses the expression of *scaCBA* in the presence of Mn^{2+}. Biochemical studies of the *S. gordonii* and *S. pneumoniae* ScaR proteins have shown that ScaR is a homodimer and contains two metal-binding sites per protomer (85, 86). In *S. pneumoniae* ScaR, Zn^{2+} occupies site 1, and although it is required for activation, it keeps ScaR in an inactive state. Activation of DNA-binding activity is accomplished only when Mn^{2+} occupies the lower-affinity site 2 (85). Zn^{2+} can also bind to site 2, resulting in ScaR having a low DNA-binding activity (85). This effect may partly explain the increase of expression of *scaCBA* in the presence of toxic levels of Zn^{2+} (85), although a recent study showed that Zn^{2+} competitively inhibits Mn^{2+} uptake and therefore leads to a depletion of intracellular Mn^{2+} content (87), thus relieving ScaR repression on *scaCBA*.

S. mutans SloR regulates the expression of the *sloABCR* operon, which encodes the Mn^{2+} and Fe^{2+} transporter SloABC (88). SloR represses *sloABCR* expression only in the presence of Mn^{2+} (88). Later studies showed that SloR controls a large regulon, acting both as a repressor and as an activator (69, 89). The homologous regulator MtsR of *S. pyogenes* also controls a large regulon that includes the genes encoding the Mn^{2+} and Fe^{2+} transporter MtsABC (90, 91). MtsR also represses the *dnaK* operon, suggesting that it mediates the response of *dnaK* to heat shock (90).

(iv) PerR, a regulator of the peroxide stress response. Although the oxidative stress response has been researched extensively in LAB, the regulatory mechanisms involved are still largely unknown (92). One of the best-characterized oxidative stress sensors in LAB is PerR, a regulator of the Fur family. Although most

members of this family are involved in metal homeostasis, PerR is a sensor of H_2O_2 (93). This has been documented for *E. faecalis* (94) and a number of streptococcal strains, showing that PerR links peroxide resistance and metal homeostasis in these organisms (95). *S. pyogenes* PerR regulates the expression of a ferritin-like protein of the MrgA/Dps family (Dpr) and PmtA, a CPx-type metal transporter (95, 96). Dpr binds Fe^{2+} as well as Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} (97), and it plays a key role in aerobic growth of *S. mutans* (98) and *S. suis* (99). PmtA is a putative Zn^{2+} efflux transporter, and its overexpression resulted in a strong induction of the AdcR-regulated genes (see the section on the MarR family, above) (95). Evidence suggests that PerR regulons may vary in different streptococcal species. For example, expression of the Mn^{2+} uptake system MntABC is under the control of PerR in *Streptococcus oligofermentans* (100).

Serine/threonine/tyrosine kinases. Regulation of protein activity by serine/threonine/tyrosine phosphorylation was first described for eukaryotes but was later also shown to play a central role in bacteria (101). Bacterial genomes contain eukaryotic-type kinases that are mainly responsible for serine and threonine phosphorylation. Phosphorylation at Ser, Thr, or Tyr residues is not as labile as phosphorylation at His or Asp. Therefore, Ser/Thr/Tyr kinases usually are associated with cognate phosphatases in order to quench signaling cascades (102).

Ser/Thr/Tyr kinases are scarce in LAB, although most of their genomes encode at least one kinase/phosphatase pair (103). A number of these have been characterized for some streptococci and *E. faecalis*. The *S. pneumoniae* StkP Ser/Thr kinase and PhpP phosphatase are involved in stress responses, among other physiological processes (104) (Fig. 1). StkP belongs to a conserved group of membrane-anchored Ser/Thr kinases that consist of a cytoplasmic kinase domain and an extracellular C-terminal region composed of several penicillin-binding and Ser/Thr kinase-associated (PASTA) domains. PASTA domains have been proposed to bind peptidoglycan (PG) fragments, which thereby act as signaling molecules (105). Indeed, *S. pneumoniae* StkP can bind PG subunits and β-lactam antibiotics (106). A recent study revealed that StkP requires the GspB protein for proper localization in the cell septum and for autophosphorylation and subsequent phosphorylation of its substrates (107). The StkP/PhpP pair regulates the activity of the phosphoglucosamine mutase GlmM (108), the cell division protein DivIVA (109), and the Mn^{2+}-dependent inorganic pyrophosphatase PpaC (109), among others. Interestingly, StkP phosphorylates the orphan RR RitR at the DNA-binding domain in an *in vitro* assay (110). RitR plays a key role in the response to iron and oxidative stresses by regulating the expression of the peroxide resistance protein Dpr and of iron uptake systems (111). The formation of a complex between DNA-RitR and the StkP phosphatase PhpP contributes to the regulation of RitR activity via a mechanism that remains unclear, to date (110).

The *E. faecalis* kinase/phosphatase pair IreK/IreP is involved in intrinsic resistance against cephalosporin, as evidenced by the fact that mutants lacking *ireK* exhibit cephalosporin susceptibility, whereas mutants lacking *ireP* are hyperresistant (112). A subsequent study suggested that IreK/IreP regulates the phosphorylation state of the IreB protein, which would control a cephalosporin resistance pathway through an undetermined mechanism (113).

Thermosensors in LAB

Bacteria possess two main routes to sense a sudden temperature change and to transmit the information. First is the evolutionarily conserved response to the heat-induced accumulation of denatured proteins, and the second is the direct sensing of temperature changes through primary thermosensory structures, such as DNA, RNA, proteins, or lipids, which either have a direct effect or lead to the activation of signal transduction pathways (114). The *Lc. lactis* CtsR regulator, a winged helix-turn-helix dimeric DNA-binding protein, has been shown to function as a thermosensor (115, 116) (Fig. 1). CtsR regulates the expression of *clp* and other HSP genes in LAB (117). It has been demonstrated for several CtsR proteins, including that of *Lc. lactis*, that their activity depends on the temperature, as they bind to DNA with a higher affinity at lower temperatures. The temperature sensitivity of CtsR is adapted to the specific living conditions of different low-GC Gram-positive bacteria (115). The thermosensor region is a highly conserved tetraglycine loop within the winged helix-turn-helix domain. This conserved region, which possesses high conformational entropy and therefore displays decreased thermostability, senses specific temperature shifts and regulates gene activity, whereas the more flexible regions of CtsR are responsible for adaptation to host-specific temperatures (115).

The Stringent Response: the Ribosome as a Sensor

Accumulation of the alarmone (p)ppGpp in the cell triggers the stringent response (SR), a highly conserved bacterial stress response originally defined as a response to amino acid starvation but nowadays recognized as being triggered by a wide range of environmental stress conditions (118) (Fig. 1). The SR induces large-scale transcriptional alterations that ultimately lead to a physiological shift to a nongrowth state. In most *Firmicutes*, up to three genes code for (p)ppGpp synthetases (Rels): the bifunctional RelA/SpoT homolog, also named RelA or Rel (RSH), and the small RelQ and RelP proteins, with only a (p)ppGpp synthetase domain (119). An evolutionary analysis of the RSH superfamily showed that members of the *Lactobacillales* encode anywhere from one (*O. oeni*) to four (one RSH and three small synthetases) RSH proteins in some streptococci (120).

The SR has been implicated in acid stress resistance in *Lc. lactis* (121) and *Lb. casei* (122) and in a number of stress conditions in *E. faecalis* (123, 124), *S. mutans* (125), and *S. pneumoniae* (126). A number of studies have shown that RSH is mainly involved in the classical SR, whereas the small synthetases may play different roles. In *E. faecalis*, RelQ apparently operates only in maintaining baseline levels of (p)ppGpp during homeostatic growth (124). There is also evidence indicating that RelP and RelQ may have distinct and specialized functions in *S. mutans* (127).

It remains to be established in full detail how (p)ppGpp modulates cell physiology in LAB. In *B. subtilis*, (p)ppGpp affects the transcription of rRNA genes by reducing the availability of the initiating nucleotide GTP (128), and a more recent study showed that GTP is a limiting factor for the growth rate (129): GTP levels become detrimental to growth when they reach a certain threshold. (p)ppGpp is crucial for maintaining GTP homeostasis (129). Interestingly, growth inhibition by GTP stress also occurs in *E. faecalis* cells unable to produce (p)ppGpp, suggesting that this phenomenon might also be conserved among LAB (130).

The mechanisms of the regulation of expression of (p)ppGpp-

producing enzymes in LAB are largely unknown. In *S. mutans*, *relP* is cotranscribed with the *relRS* TCS, whose inactivation results in a significant reduction in the basal level of (p)ppGpp. This indicates that RelRS is involved in the regulation of (p)ppGpp metabolism (127). The environmental signals to which RelRS responds have not been determined, although it has been suggested that RelRS may sense oxidative stressors or by-products of oxidative metabolism (131). Expression of *relPRS* is regulated by the MarR family transcriptional regulator RcrR, which is involved in stress tolerance and competence, although the signals to which RcrR responds remain unidentified (131, 132). There is some evidence suggesting that nucleotide pools may be involved in the modulation of (p)ppGpp production. Inactivation of the purine nucleoside phosphorylase DeoD in *S. thermophilus* resulted in a thermotolerant phenotype which correlated with an increased ppGpp content in this mutant (133).

The SOS Response in LAB: DNA as a Sensor

The SOS response is usually induced by damage to DNA or by a stop of replication resulting in exposure of single-stranded DNA (ssDNA). Triggering of the response is regulated by the concerted actions of the repressor LexA and the activator RecA, resulting in the induction of expression of proteins involved in DNA repair (134). The SOS response has received little attention in LAB, with the exception of *Streptococcaceae*. Some streptococci can elicit a DNA damage response mediated by the regulator HdiR, which represses its own gene and that of the DNA polymerase UmuC, a protein involved in the SOS response in other organisms (135). RecA catalyzes the self-cleavage of HdiR, although an additional cleavage of the N-terminal fragment of HdiR by ClpP was required for induction of HdiR-repressed genes (135). A homologous system was subsequently characterized in *Streptococcus uberis*, in which a gene cluster consisting of *hdiR*, *umuC*, and two uncharacterized genes was identified (49). Expression of this cluster is under the control of HdiR and is induced by DNA damage (49). A recent study showed that a LexA-like transcriptional regulator of *S. mutans* (SMU.2027) is induced in response to heat or DNA damage and through the CSP-ComDE quorum-sensing (QS) pathway (45). In contrast to the typical SOS response, activation of *S. mutans* LexA leads to the formation of persister cells (45).

In streptococci, such as *S. pneumoniae*, antibiotics causing DNA damage induce competence in a RecA-dependent way, but the mechanism remained unknown (136). It now appears that it is caused by the chromosomal location of early competence genes. Slager et al. (137) showed that antibiotics targeting DNA replication cause replication to stall while initiation of DNA replication continues. This results in a higher copy number of genes close to the replication origin, including the *comCDE* operon. The increase in gene dosage is sufficient to trigger the competent state (137). The origin-proximal location of early *com* genes thus constitutes a sensory mechanism that enables cells to activate competence in response to antibiotics interfering with DNA replication (137). In contrast, the SOS response mediated by HdiR and competence are antagonistic processes in *S. thermophilus* (138). Intriguingly, competence in this organism is regulated by the *comRS* genes, which are located far from the origin of replication (137).

Cyclic Nucleotides as Second Messengers in LAB

Several cyclic nucleotides (cyclic AMP [cAMP], cyclic GMP [cGMP], cyclic di-GMP [c-di-GMP], cyclic di-AMP [c-di-AMP], and cyclic AMP-GMP) play key roles in the regulation of bacterial cell physiology (139, 140). Very little is known about their role in LAB physiology. In recent years, c-di-AMP has been identified as a major second messenger (reviewed in reference 141). c-di-AMP is synthetized from two molecules of ATP by diadenylyl cyclases (DACs), and it is degraded to pApA or AMP by phosphodiesterases (PDEs) (142, 143).

Among LAB, the presence and synthesis of c-di-AMP were first described for *S. pyogenes* (144). Inactivation of a gene encoding a DAC homolog in *S. thermophilus*, *ossG*, resulted in a methyl viologen-sensitive phenotype, suggesting the involvement of this gene in the oxidative stress response of this organism (144, 145). On the other hand, inactivation of the *Lc. lactis* PDE-encoding gene *gdpP* resulted in increased heat resistance and salt hypersensitivity (146). Results obtained so far suggest that c-di-AMP is important in LAB stress responses, but much remains to be unraveled about its role in LAB.

Quorum Sensing

Bacterial quorum sensing regulates a number of cellular processes, including biofilm development, conjugation, competence, bacteriocin production, and pathogenesis. Furthermore, evidence suggests that it also plays a role in stress responses in oral streptococci, where production of competence-stimulating peptides is stimulated by stress conditions (147). Acidic conditions or exposure to spectinomycin increased the expression of the competence-stimulating peptide-encoding gene *comC* in *S. mutans* (148). Mutants of this organism impaired in the signaling pathway of the competence-stimulating peptides produced a significantly smaller number of persister cells following acid challenge, amino acid starvation, and/or oxidative stress (149).

Two kinds of signaling molecules have been identified in quorum-sensing systems in LAB: the autoinducer AI-2 and small pheromone peptides. AI-2 is a furanosyl borate diester produced and recognized by a wide variety of bacteria (for a review, see reference 150). AI-2 is produced from *S*-ribosylhomocysteine by the *S*-ribosylhomocysteine lyase LuxS, which is present in most *Lactobacillales* species, and a number of studies have shown that many LAB respond to AI-2 (see the references in references 150 and 151). Inactivation of *luxS* in *S. pyogenes* resulted in increased acid tolerance (152), while AI-2 has been shown to influence biofilm formation in other streptococci, such as *Streptococcus intermedius* (153), *S. gordonii*, and *Streptococcus oralis* (154). It still remains to be established clearly, however, whether AI-2 plays a relevant role in stress responses.

The role of small pheromone peptides or autoinducing peptides (AIs) in intercellular communication of LAB is better characterized. AIs can be detected at the cell surface by specific TCSs or recognized by intracellular receptors after internalization. An example of a pheromone peptide is the previously discussed CSP regulating competence in *S. pneumoniae*. *S. mutans* uses a different CSP and a paralogous TCS system to regulate both competence and production of bacteriocins (155). The second type of pheromone peptides is exemplified by the ComRS system of streptococci from the bovis, pyogenes, and salivarius groups (156, 157). The involvement of competence quorum-sensing systems in stress responses is discussed above.

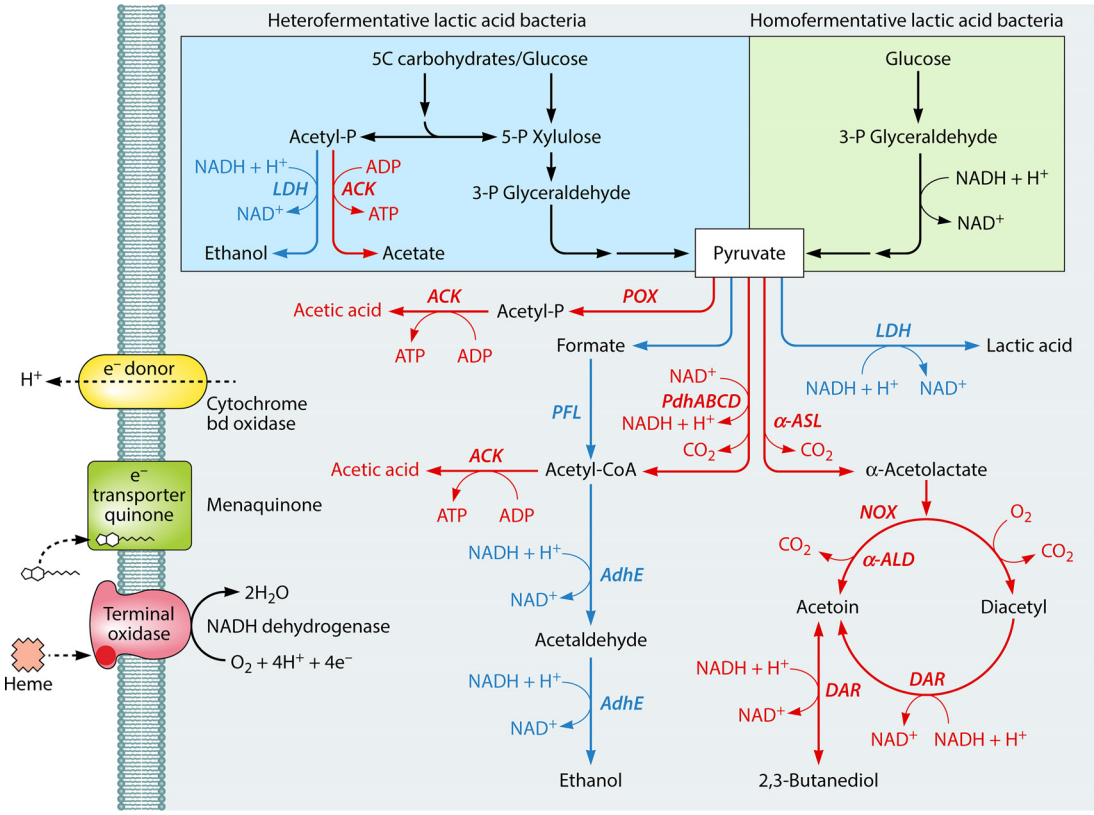

FIG 2 Schematic representation of changes of the carbohydrate metabolism, glycolysis, and fate of pyruvate in lactic acid bacteria. Colored arrows and enzymes indicate common reactions (black), those mainly induced during fermentation by unstressed cells (blue), and those induced in respiratory and/or environmentally stressed cells (red). LDH, lactate dehydrogenase; ACK, acetate kinase; POX, pyruvate oxidase; PFL, pyruvate formate lyase; PdhABCD, pyruvate dehydrogenase complex; α-ASL, α-acetolactate synthase; AdhE, alcohol dehydrogenase; NOX, NADH oxidase; α-ALD, α-acetolactate decarboxylase; DAR, diacetyl reductase.

PERTURBATIONS OF METABOLISM AND METABOLIC ADAPTATIONS OF LAB UNDER STRESS CONDITIONS

LAB are subjected to marked metabolic perturbations under environmental stress conditions (Fig. 2 and 3). As a consequence of stress, cells lower their metabolic activities, which decreases energy production and the generation of a proton motive force (PMF) and alters growth and viability (17, 158). Overall, the responses consist of the selection of alternative fates of pyruvate, the utilization of other carbon sources, the activation of the proteolytic system, and/or the catabolism of free amino acids (FAA) by cells. Metabolic adaptation is crucial for survival because it stimulates the production of additional energy and lowers the stress level, e.g., through alkalization of the cytosol under acidic conditions (159). The extent of metabolic perturbation and the main routes to reprogram pathways responsible for substrate catabolism in order to adapt to environmental stresses vary between LAB species (160).

Metabolism of Carbon Sources and Energy Production

Under environmental stress conditions, LAB change metabolic and energy fluxes, modify the rate of growth, and adapt the metabolism of carbon sources to the new environment by modifying the synthesis of enzymes and metabolites (161). Environmental stresses inhibit the glycolytic pathway of *Lc. lactis* and decrease the synthesis of biochemical energy (17). Similar metabolic perturbations are common in other LAB. Consequently, the ability of LAB to efficiently transport and metabolize carbohydrates and other carbon sources, such as malate and citrate, under environmental stress conditions is crucial for growth and persistence.

Transport and fermentation pathways of carbohydrates. LAB express numerous proteins responsible for carbohydrate transport and utilization. Proton-coupled active transport by proteins from the major facilitator superfamily (MFS), the glycoside-pentoside-hexuronide (GPH) superfamily, and the ATP-binding cassette (ABC) superfamily and by group translocators, such as the phosphotransferase system–glucose-fructose-lactose (PTS-GFL) superfamily, is used most frequently (162). LAB modulate the synthesis of specific transporters depending on the type of carbohydrate available (163, 164). Under acid stress conditions, *Lb. casei* and *S. mutans* markedly decreased the synthesis of the phosphoenolpyruvate phosphotransferase system (PEP-PTS) for glucose, which is the primary carbohydrate transport system belonging to the PTS-GFL superfamily (165–167). Under the same conditions, an acid-resistant mutant of *Lb. casei* showed the highest level of PEP-PTS and of the phosphocarrier protein (HPr). High levels of PEP-PTS may improve the acid resistance. The glucose PTS is upregulated at low pH in *Streptococcus sobrinus* (168) and *Streptococcus macedonicus* (34, 36). Under optimal conditions and when cells are growing in glucose-rich media, HPr inhibits PEP-PTSs for carbohydrates other than glucose, preventing their

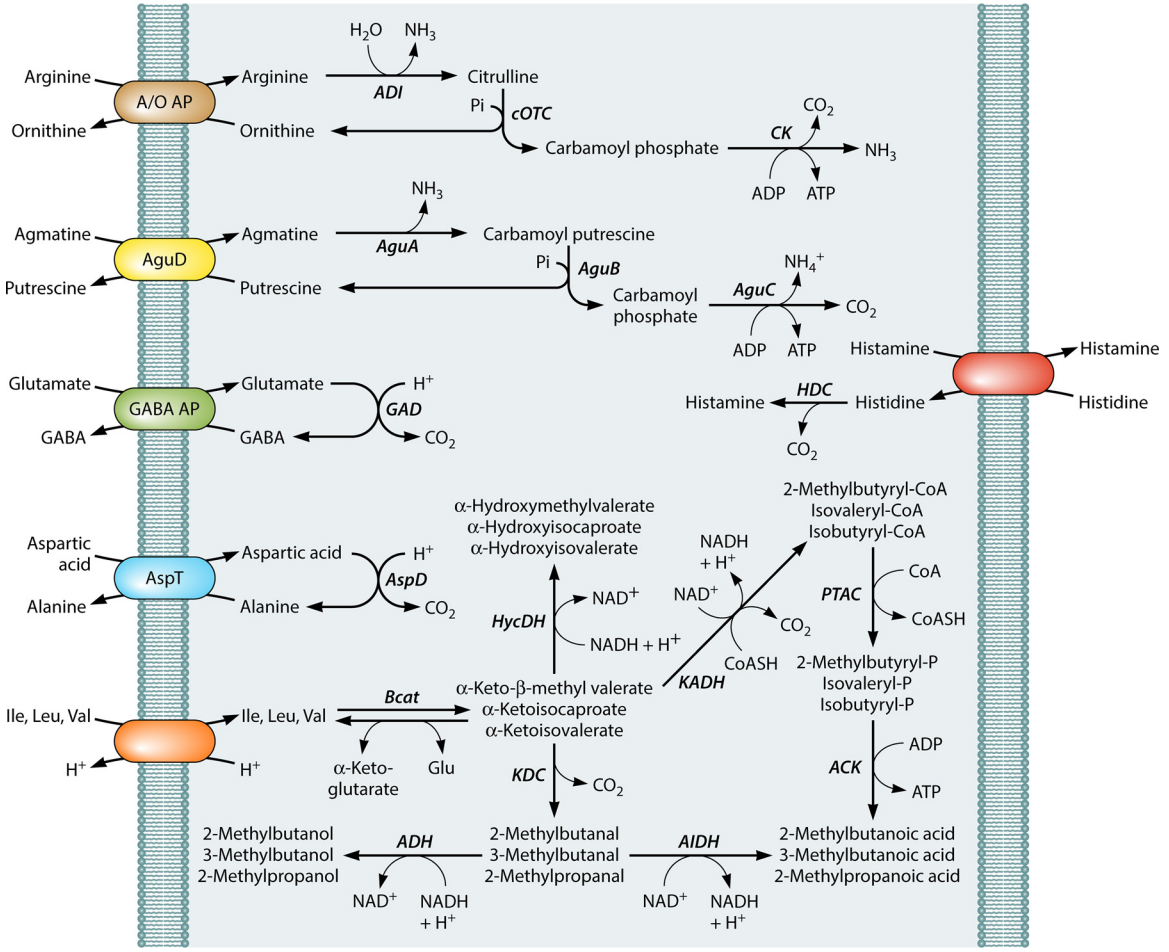

FIG 3 Schematic representation of the main free amino acid pathways of lactic acid bacteria induced under acid stress and/or starvation conditions. GABA, γ-aminobutyric acid; ADI, arginine deiminase; cOTC, catabolic ornithine transcarbamoylase; CK, carbamate kinase; AguA, agmatine deiminase; AguB, putrescine carbamoyl transferase; AguC, carbamate kinase; AguD, agmatine/putrescine antiporter; GAD, glutamate decarboxylase; HDC, histidine decarboxylase; AspD, aspartic acid decarboxylase; AspT, aspartate-alanine antiporter; Bcat, α-ketoglutarate-dependent branched-chain aminotransferase; HycDH, hydroxy-acid dehydrogenase; KaDH, keto acid dehydrogenase; KDC, 2-keto acid decarboxylase; ADH, alcohol dehydrogenase; AIDH, aldehyde dehydrogenase; PTAC, phosphotransacylase; ACK, acetate kinase.

transport into the cell. In addition, glycolytic intermediates (e.g., fructose-1,6-bisphosphate) activate the phosphorylation of HPr at the serine residue at position 46 (169). The resulting P-Ser-HPr interacts with the global transcriptional regulator CcpA, and the complex prevents the catabolism of carbon sources other than glucose, the preferred sugar in most bacteria, by binding to the catabolic repression element (*cre*) upstream of the responsible genes and shutting them down. Under low-pH conditions, some LAB increase pyruvate kinase activity, which may accelerate the depletion of fructose-1,6-bisphosphate, relieving CcpA repression and allowing the use of alternative carbon sources (170).

Acid stress causes intracellular acidification, which decreases the activity of cytoplasmic enzymes (17). Transcriptomic and proteomic studies have highlighted that many LAB enhance the levels of glycolytic enzymes under acid, thermal, and osmotic stresses, but without increasing the synthesis of lactic acid (171, 172). LAB such as *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, and *Lc. lactis* modify pyruvate metabolism at the expense of lactic acid, and they increase the synthesis of basic compounds (e.g., lysine and diacetyl/acetoin) (173, 174), energy-rich intermediates (such as ATP and NADH) (175), EPS, and/or glycogen (176). The level of lactate dehydrogenase (Ldh), which is responsible for the synthesis of lactic acid from pyruvate, markedly decreases. Pyruvate oxidase and phosphate acetyltransferase, used to synthesize acetyl-coenzyme A (acetyl-CoA), are induced in *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb. rhamnosus* under acid stress conditions (161, 170). Acetyl-CoA is rerouted toward the biosynthesis of fatty acids instead of butanoate (161, 170), which may enhance the rigidity and impermeability of the cytoplasmic membrane (177, 178). Changes in pyruvate metabolism were also observed in *S. mutans* under acid stress (166, 167).

Metabolic adaptations in the presence of oxygen and ROS. Despite having a fermentative metabolism, several LAB species possess genes that code for a respiratory electron transport chain (179). They harbor the *cydABCD* operon, encoding a heme-dependent cytochrome with the capacity to generate PMF. Compared to when they are fermenting, LAB display an increase of extracellular pH and biomass under energetically favorable respiration conditions, that is, in the presence of oxygen, heme, and menaquinone (179–181) (Fig. 2). *Lc. lactis* growing anaerobically

displays a fermentative metabolism in which carbohydrates are converted into (mainly) lactic acid (182). Two molecules of NADH, produced from the oxidation of glyceraldehyde-3-phosphate, are reoxidized through the action of Ldh. The NADH/NAD^+ ratio plays a key role in *Lc. lactis* in controlling the shift from homolactic to mixed-acid fermentation (183). Under oxygenic conditions, enhanced expression and activities of NADH oxidase and NADH peroxidase compete with Ldh for NADH molecules (184). Thus, the production of lactic acid is reduced and the glycolytic flux is redirected toward production of acetate, ethanol, acetoin, diacetyl, and CO_2. The consumption of oxygen reduces its cytoplasmic concentration and limits the oxidative stress. Hydrogen peroxide is produced under these circumstances, which may affect the growth rate of *Lc. lactis* and may ultimately result in cell death. The regulation of respiration in *Lc. lactis* is mediated via CcpA (185). In glucose-rich media, CcpA represses heme intake and cells grow through fermentation, synthesizing only lactic acid (Fig. 2). As glucose is consumed, heme intake occurs and *Lc. lactis* respires pyruvate and/or lactic acid. The shift toward the respiratory pathway produces metabolic changes, which mainly involve pyruvate oxidase (POX) activity for converting pyruvate into acetyl-P. The latter compound is used via acetate kinase (ACK), producing acetic acid and ATP. The respiratory metabolism of *Lc. lactis* results in the irreversible inactivation of pyruvate formate lyase (PFL), impeding the conversion of pyruvate into acetyl-CoA. The level of alcohol dehydrogenase (AdhE), which uses NADH to convert acetyl-CoA into acetaldehyde and ethanol, is reduced. Under such conditions, the pyruvate dehydrogenase complex (PdhABCD), which catalyzes the conversion of pyruvate into acetyl-CoA and NADH, is activated. The combined activity of POX and PdhABCD leads to a decrease of pyruvate available for Ldh. LAB decrease the synthesis of lactic acid and ethanol under oxidative stress. At low pH and a high concentration of carbohydrates, LAB decrease the synthesis of lactic acid, and the excess of pyruvate is metabolized via α-acetolactate synthase (α-ASL) to form α-acetolactate. Under aerobic conditions, α-acetolactate is nonenzymatically decarboxylated to diacetyl. Under limiting oxygen conditions, α-acetolactate is decarboxylated into acetoin via α-acetolactate decarboxylase (α-ALD). Diacetyl reductase (DAR) converts acetoin into diacetyl (186). Depending on the cellular redox state, DAR may also catalyze the reduction of acetoin into butanediol.

Carbohydrate starvation. Under carbohydrate starvation conditions, LAB such as *Lc. lactis* lose the ability to form colonies, and they enter into the VBNC state for at least 2 weeks (187). They shift the physiological state by lowering the synthesis of DNA and proteins and achieving the stationary phase of growth. At the beginning of starvation, cells accumulate glycolytic intermediates (PEP, 3-phosphoglycerate, and 2-phosphoglycerate). Further, glycolytic intermediates are metabolized to pyruvate and ATP, and the levels of PEP-PTSs markedly decrease. Under lactose starvation conditions, *Lb. casei* and *Lb. rhamnosus* also regulated their glycolytic enzymes differently (176, 188). *S. mutans* modulated its metabolic activities through transcriptional and enzyme allosteric regulation in order to optimize the flow of carbohydrates and to maintain an optimal energy state (189). An increase of the catabolism of carbon sources (e.g., inositol, glycerol, RNA, lipids, proteins, peptides, and especially FAA) was also seen when LAB were faced with carbohydrate starvation (188). This mechanism allows cells to survive without carbohydrates (187). Under long-term starvation conditions, repression of PEP-PTS transporters for carbohydrates and of glycolytic enzymes is mainly due to derepression of carbon catabolite repression (CCR) mediated by CcpA.

Malolactic fermentation pathway. Species belonging to the *Oenococcus*, *Pediococcus*, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* genera and oral *Streptococcus* species (190) use the MLF pathway to enhance survival under environmental stress conditions, such as low pH, a high concentration of carbohydrates, or starvation. This pathway involves the decarboxylation of L-malic acid to L-lactic acid and CO_2. The regulatory protein (MleR), malate permease (MleP), and malolactic enzyme (MleS) are the components of the route. The uptake of the dianionic malic acid is coupled to the exit of its decarboxylation monoionic product, lactic acid (malate/lactate antiporter system). Cytosolic H^+ ions are consumed during malic acid decarboxylation, which promotes $\Delta\psi$ and ΔpH gradients over the cell membrane. Therefore, MLF increases the cytoplasmic pH and PMF, which helps the cell to take up other nutrients (158). The PMF formed via MLF is sufficient to drive ATP synthesis through F-ATPase (F_1F_o-H^+ ATPase or ATP synthase). The CO_2 produced by MLF is partially used by carbonic anhydrase to form bicarbonate, further increasing the cytosolic pH. Under acidic conditions and/or osmotic stress conditions, *Lb. plantarum* decreases glucose intake and uses malic acid as a preferred energy source (164, 191, 192). MLF has also a protective role for *S. mutans* against oxidative stress and starvation (193). Malate and low pH represent inducing signals for the MLF pathway, except in *O. oeni*.

Metabolism of citrate. Under acid stress conditions, lactococci and thermophilic lactobacilli metabolize citrate, producing pyruvate. Citrate metabolism requires the following three steps: (i) citrate intake via permease (CitP), (ii) formation of oxaloacetate from pyruvate through the activity of citrate lyase (CL), and (iii) decarboxylation of oxaloacetate into pyruvate via oxaloacetate decarboxylase (OAD). The end products are acetic acid, CO_2, and pyruvate. As described above, the environmental conditions determine the fate of pyruvate. Because of the increased pH in the cytoplasm and extracellularly, LAB take advantage of the cofermentation of citrate with other carbohydrates, such as lactose or glucose. ^{13}C-assisted nuclear magnetic resonance (NMR) analysis showed that *Lc. lactis* possesses a citrate/lactate antiporter (194). As described for the MLF pathway, transport and metabolism of citrate increase the PMF due to the generation of $\Delta\psi$ and ΔpH gradients over the cytoplasmic membrane. The main protective effects of cofermentation differ among LAB. In the presence of glucose and citrate, homofermentative and facultatively heterofermentative species (e.g., *Lactococcus* sp., *Enterococcus* sp., and *Lb. plantarum*) use the glycolytic pathway to form 2 mol each of lactic acid and ATP for every 1 mol of glucose consumed. Each mole of citrate produces pyruvate and, subsequently, acetoin, butanediol, and diacetyl, which protect the cell against acid stress. Furthermore, *Lc. lactis* and *Lb. rhamnosus* metabolize citrate and synthesize acetic acid and ATP under carbohydrate starvation conditions (176, 195). The cofermentation of citrate and glucose in obligately heterofermentative LAB follows the pentose phosphate pathway, mainly producing lactic acid, ethanol/acetic acid, and ATP from the consumed glucose. One additional mole each of acetic acid and ATP is synthetized for each mole of citrate metabolized.

Metabolism of Nitrogen

The metabolism of nitrogen, which includes proteolytic and urease systems, plays a pivotal role in LAB growth and survival. The regulation of nitrogen metabolism occurs mainly via GlnR and CodY (196, 197). A gene for GlnR is present in all sequenced genomes of LAB, while CodY is present only in strains belonging to the *Lactococcus*, *Streptococcus*, and *Enterococcus* genera. Under conditions of high nitrogen availability, GlnR controls the import of nitrogen-containing compounds and the synthesis of intracellular NH_3. On the other hand, CodY interacts with its BCAA coeffector to control amino acid biosynthesis, transport systems, and peptidases.

The proteolytic system. Genomes of LAB encode extracellular or cell wall-associated proteinases that hydrolyze environmental proteins into oligopeptides, as well as specific transport systems to take up oligo-, di-, and tripeptides and FAA from the environment. Intracellular oligopeptidases and peptidases hydrolyze oligopeptides and peptides into smaller peptides and FAA. LAB modify proteolysis as a response to environmental stresses. Under acidic conditions, they reduce the abundance of amino acid transporters, and FAA become limiting. Consequently, many lactobacilli increase the activities of peptidases, such as PepN and PepO (161, 165, 170, 171). The proteolytic system of LAB is also influenced by carbon metabolism (196). *Lc. lactis* increased the levels of endo- and exopeptidases (PepO and PepC) during growth under respiratory compared to fermentative conditions (181). VBNC cells of *Lc. lactis* increased the expression of *pepXP*, *pepDA*, and *pepDB*, while *pepQ* and *pepV* were repressed in comparison to their expression in exponentially growing cells. Much more needs to be uncovered regarding the mechanisms of carbon and nitrogen coregulation. CcpA forms complexes with CodY in *B. subtilis*, and it would be very interesting to see whether such a regulatory mechanism also occurs in LAB (196). Recently, it was shown that the aminotransferase IlvE of *S. mutans* is regulated by both CcpA and CodY under acid stress conditions, supporting the hypothesis of carbon and nitrogen coregulation in this species (198).

Metabolism of FAA. The metabolism of FAA not only contributes to flavor development in fermented foods but also is crucial for LAB stress resistance. It allows the production of precursors for the biosynthesis of amino acids, fatty acids, nucleotides, and vitamins, generates energy under starvation conditions, and increases the intracellular pH (Fig. 3). Deamination and decarboxylation of FAA are the main routes that enhance LAB growth and survival under environmental stress conditions. Deamination leads to the synthesis of α-keto acids and NH_3, the latter of which increases the intra- and extracellular pH, protecting cells against acid stress. α-Keto acids are used to synthesize other amino acids for anabolic purposes or are catabolized into ketones, aldehydes, alcohols, or acids to improve the cellular redox potential and/or to synthesize ATP. Amino acid decarboxylation leads to the conversion of an amino acid into the corresponding amine and CO_2. This reaction is coupled to the exchange between substrate and product (e.g., glutamic acid/γ-aminobutyric acid [GABA] or aspartic acid/alanine), which is mediated by an antiporter. The translocation is driven by the opposite gradients of substrate and product over the membrane, which are maintained by decarboxylation. These combined activities generate a PMF because proton consumption during decarboxylation leads to an increase of the intracellular pH (199). Therefore, the antiporter/decarboxylase system is an indirect proton pump analogous to other mechanisms providing energy and protecting from acidic conditions (200). Several LAB genomes (e.g., that of *Lactobacillus acidophilus*) encode amino acid decarboxylases, cation transport ATPases, and chaperones involved in pH regulation at the cytoplasmic level (201). When they are in the VBNC and/or carbohydrate starvation state, LAB metabolize BCAAs to synthesize ATP and BCFAs (184). The main FAA pathways induced under environmental stress responses are discussed in the following paragraphs.

(i) ADI pathway. The arginine deiminase (ADI) pathway consists of three enzymes, namely, arginine deiminase (ADI; encoded by the *arcA* gene), catabolic ornithine transcarbamoylase (cOTC; encoded by *arcB*), and carbamate kinase (CK; encoded by *arcC*), and one membrane transport protein (encoded by *arcD*) (Fig. 3). All genetic components of this pathway are present in LAB genomes (202). Arginine is degraded through the ADI pathway into ornithine, NH_3, and CO_2. Arginine enters the cytoplasm via an arginine/ornithine antiporter system, without energetic cost. As shown for several LAB species isolated from sourdough, cheese, meat, and wine, this pathway is induced by arginine and/or environmental stresses, such as acidity and starvation (203). Nutrient sources (e.g., carbohydrates), growth rate, pH, and arginine all affect the ADI pathway of *S. gordonii* (204). The ADI pathway of *Streptococcus rattus* is subject to substrate induction and catabolite repression (205). The NH_3 produced alkalizes the cytoplasm, thus increasing acid resistance (203, 206). In addition, the extra ATP produced via the ADI pathway enhances cell survival after carbohydrate depletion (202), while the intermediate carbamoyl phosphate is used to synthesize pyrimidines. ADI pathway regulation is connected to carbon metabolism via CCR. The intergenic regions of the *arcABC* genes have several *cis*-regulatory element sites, which is suggestive of direct regulation of the ADI pathway through CcpA (202, 207). Members of the CRP/FNR family of transcriptional regulators also seem to exert a positive regulation. The *arcABC* genes of *Lc. lactis* are repressed under aerobic and respiratory conditions (180).

(ii) AgDI pathway. The agmatine deiminase (AgDI) pathway, analogous to the ADI system, consists of three enzymes, i.e., putrescine carbamoyl transferase (AguB), agmatine deiminase (AguA), and carbamate kinase (AguC), and one agmatine/putrescine antiporter (AguD) (Fig. 3). All components are encoded by the single operon *aguRBDAC*, which has been studied in *S. mutans*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *E. faecalis*, *Lc. lactis*, and *S. mutans* (208–210). This pathway allows the hydrolysis of agmatine into putrescine, NH_3, CO_2, and ATP and is a response mechanism to acid and starvation stresses. It represents a potential health risk for humans due to the synthesis of putrescine, a biogenic amine. Like the ADI pathway, the AgDI pathway is induced by the substrate (agmatine) and regulated via CCR through mechanisms that are dependent or independent of CcpA (208, 210). The AgDI pathway of *S. mutans* was also induced in response to heat stress (209).

(iii) GAD pathway. The glutamate decarboxylase (GAD) pathway consists of GAD, a pyridoxal 5′-phosphate (PLP)-dependent enzyme, and a glutamate/GABA antiporter (Fig. 3). The GAD pathway is encoded by *gadB* and *gadC* and is regulated by an activator, GadR (206). GAD catalyzes the decarboxylation of glutamate into GABA, a bioactive compound that acts as a neurotransmitter in humans. The uptake of glutamate and the expulsion of GABA are mediated by the electrogenic antiporter, which,

together with the decarboxylation of glutamate, produces PMF. Three decarboxylation-antiporter cycles are needed to synthesize 1 mol of ATP. The cytoplasmic pH increases due to the removal of H^+ ions. Concomitantly, the extracellular pH increases slightly due to the exchange between glutamate and the more alkaline compound GABA. Glutamate and/or environmental stresses (e.g., acidity, osmotic stress, or starvation) induce the GAD pathway in the dairy starters *Lc. lactis*, *S. thermophilus*, and *Lb. delbrueckii* subsp. *bulgaricus*, in nonstarter lactic acid bacteria (NSLAB), such as *Lb. brevis*, *Lactobacillus paracasei*, and *Lb. plantarum*, and in lactobacilli isolated from human and animal intestines (211, 212).

(iv) AspD pathway. The aspartic acid decarboxylase (AspD) pathway catalyzes the β-decarboxylation of aspartic acid with the synthesis of alanine and CO_2 (Fig. 3). The uptake of aspartic acid and the expulsion of alanine are mediated by an electrogenic antiporter, which, together with the decarboxylation reaction, produces PMF (212). This pathway has been described for *Lactobacillus* sp. M3 (199), *Lactobacillus buchneri*, and *Lb. acidophilus* (201). Other LAB, including *Lb. plantarum* WCFS1 and *E. faecalis* V583, encode putative aspartic acid decarboxylases (213, 214). The exact mechanism of regulation of the AspD pathway is presently unknown.

(v) HDC. Histidine decarboxylase (HDC) catalyzes the β-decarboxylation of histidine into histamine and CO_2 (Fig. 3). Genes encoding HDC (*hdcA*) have been characterized for *Lactobacillus* sp. 30a, *Lb. buchneri* B301, and *Lactobacillus hilgardii* (215–217). In these organisms, *hdcA* forms an operon with a downstream gene (*hdcB*) of unknown function. *Lb. buchneri* and *Lb. hilgardii* harbor genes encoding a histidine/histamine antiporter and a histidine aminoacyl-tRNA synthase (*hisS*), which are located up- and downstream of the *hdcAB* operon, respectively. HDC has a dual role in protecting cells against acid stress and generating a PMF. HDC activity is regulated via the cytosolic pH. The enzyme is active at low pH, but a neutral or alkaline pH disrupts its substrate-binding site. The survival of *Lc. lactis* and *S. thermophilus* is increased under acid stress conditions when the glycolytic pathway is coupled to HDC activity (218).

(vi) Catabolism of BCAAs in VBNC cells. During starvation, LAB increase the catabolism of the BCAAs isoleucine, leucine, and valine through the activity of α-ketoglutarate-dependent branched-chain aminotransferase (BcaT) (Fig. 3). The presence and activity of BcaT vary greatly among LAB. VBNC cells of *Lc. lactis* metabolize leucine into isobutyric, propionic, and acetic acids. BCAAs are imported via PMF-dependent mechanisms that generate ATP during short (several days)- and long (several years)-term starvation (184). BcaT and aromatic amino acid aminotransferases (AraT) play a pivotal role in the regulation of the proteolytic system of *Lc. lactis*, as they control the intracellular pool of BCAAs, which in turn affects CodY activity. BCAAs directly interact with CodY and enhance its affinity for target genes (219). BcaT is repressed by glucose, which confirms that a link exists between carbon and nitrogen regulation.

The urease system. The hydrolysis of urea enhances survival of LAB under acid stress conditions. Among LAB, the urease system has been studied mainly in *S. thermophilus*, *Streptococcus salivarius*, *Lactobacillus reuteri*, and *Lactobacillus fermentum* (220–222). The urease system is quite complex, as it is encoded by *ureI* followed by structural (*ureABC*) and accessory (*ureEFGD*) genes (223). The urease system produces NH_3 and CO_2 from urea, thus protecting cells against acid stress. CO_2 serves in several anaplerotic reactions that are responsible for the biosynthesis of amino acids and nucleic acids. The urease operon of the oral LAB *S. salivarius* is positively regulated by urea, low pH, and an excess of carbohydrates. The urease system is induced when *S. salivarius* is growing in a biofilm, which helps to modulate the pH of dental plaque. The expression of the urease operon in *S. salivarius* 57.I is mediated via CodY (224).

Accumulation of polyphosphate in LAB. Inorganic polyphosphate (poly-P) is a polymer of phosphoanhydride-linked phosphate residues that can be found as chains of a few to thousands of residues in living organisms (225). In bacteria, the accumulation of poly-P is dependent on the activity of polyphosphate kinase (Ppk; EC 2.7.4.1), which catalyzes the ATP-dependent formation of a phosphoanhydride bond between a poly-P chain and orthophosphate (226). The hydrolysis of poly-P is catalyzed by exopolyphosphatases (Ppx; EC 3.6.1.11) and the guanosine pentaphosphate phosphohydrolase (GppA; EC 3.6.1.40). *Lactobacillus* organisms, such as *Lb. plantarum* WCFS1, *Lb. rhamnosus* GG, *Lb. casei* BL23 and ATCC 334, and *Lb. reuteri* DSM20016, contain all three enzymes (Ppk, Ppx, and GppA). The ability of lactobacilli to accumulate poly-P is correlated with the presence of *ppk* genes in the strains and the availability of phosphate in the medium (227, 228). However, *Lb. plantarum* strains containing *ppk* genes also accumulate poly-P in media with low phosphate levels (228). Poly-P has a protective role against oxidative stress in bacteria, where it acts as a chaperone preventing the aggregation of damaged proteins (225). Poly-P protects some LAB, such as *Lb. plantarum* lacking superoxide dismutase, from toxic oxygen-derived compounds (O_2^-; free radicals from the Fenton reaction) and improves the expulsion of toxic reactive metals (Fe^{2+} and Cu^{2+}). The synthesis of poly-P in *Escherichia coli* is regulated by hypochlorous acid (229). Alcántara and coworkers (228) showed that disruption of the *Lb. casei ppk* gene resulted in a lack of synthesis and intracellular accumulation of poly-P. *Lb. casei* BL23 Δ*ppk* exhibited decreased growth under high-salt or low-pH conditions and increased sensitivity to oxidative stress compared to those of the wild type (228). Poly-P-accumulating LAB (*Lb. casei* and *Lb. fermentum*) have also been isolated from natural whey starters, and a positive role has been postulated for poly-P in acid stress resistance of LAB strains during mozzarella cheese production (230). Poly-P is also involved in the regulation of general stress resistance pathways in different bacteria (225), but more research is required to fully understand its role in LAB (228).

PROTECTION OF MACROMOLECULES IN LAB

Preventing Macromolecules from Being Damaged

Some stresses are imposed on cells through specific molecules, such as protons, ROS, metal ions, and salts (such as NaCl). LAB have developed various mechanisms to directly counteract the negative effects of these molecules (Fig. 4). Evidence suggests that other stress-causing events, such as irradiation, starvation, and highly/lowly osmotic environments, are indirectly manifested through some of the aforementioned molecules, especially ROS. Consequently, toxic molecules are not always associated with a single type of stress.

The F-ATPase proton pump. Among the many mechanisms that LAB species employ to counteract stress damage, the F-ATPase system deserves attention as a system that protects against protons. The F-ATPase acts in parallel to the above-described metabolic

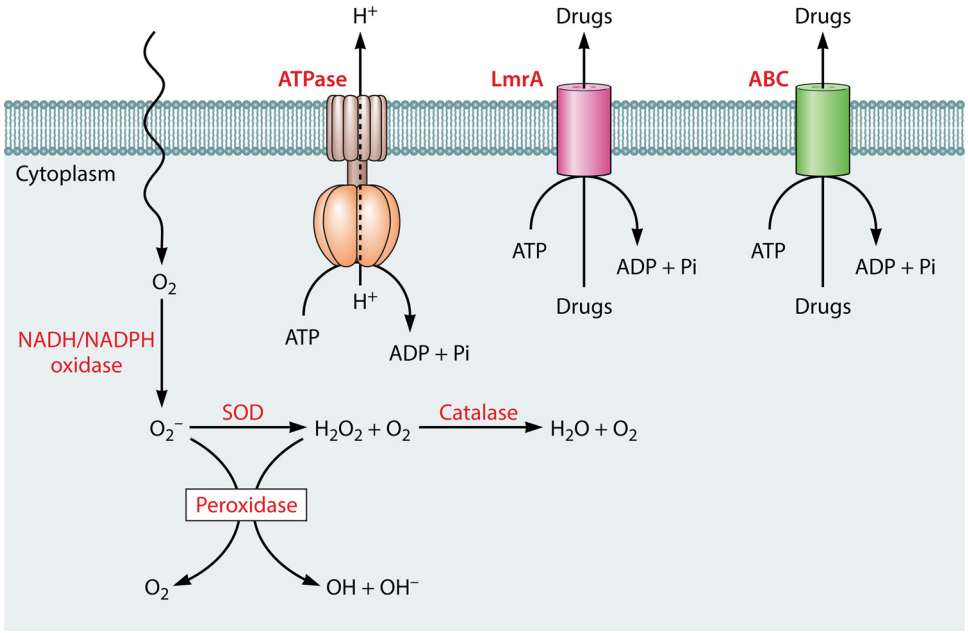

FIG 4 Schematic representation of the main molecular mechanisms preventing macromolecules from being damaged in LAB. SOD, superoxide dismutase.

adaptations that result in alkalization of the intracellular and extracellular environments. F-ATPase is a multimeric enzyme that in Gram-positive facultative anaerobes hydrolyzes ATP synthetized during carbohydrate fermentation or FAA catabolism, generating a PMF (Fig. 4). F-ATPase is a reversible enzyme that can also synthesize ATP by using protons that flow from the environment into the cell. Under acidic conditions, LAB use F-ATPase as a proton pump to maintain intracellular pH homeostasis. This reaction requires ATP because the expulsion of protons from the cytoplasm to the outside environment takes place against an increasing proton gradient that is a consequence of the synthesis of lactic acid during carbohydrate fermentation (231). The level of F-ATPase activity depends on proton transport demand, substrate catabolism, and availability of ATP. The F-ATPase systems of *Lb. casei* and *Lb. plantarum* have optimum activities at pH values (pH 5.0 to 5.5) lower than those (pH 7.0 to 7.5) of *S. thermophilus* and *Lc. lactis* (232). Distinctly aciduric oral streptococci, such as *S. mutans* GS-5 and *E. hirae* ATCC 9790, possess an F-ATPase with a lower optimal pH than that of the more acid-sensitive oral streptococci (233). Interestingly, LAB species or strains that possess an F-ATPase with an optimal activity at low pH are highly protected against acid stress. The capacity to pump protons out of the cell under acidic conditions protects important glycolytic enzymes and allows long-term survival (234). Lactobacilli increase the synthesis of one or more of their ATP synthase components (e.g., AtpB, AtpA, AtpG, and AtpD) under acidic conditions. The F-ATPase complex not only is involved in the maintenance of cytoplasmic pH homeostasis but also responds to bile salt exposure (235–237). It was noticed that in *Lb. casei* BL23 (235) as well as in *Lb. rhamnosus* GG (236), the exposure of cells to bile salt caused an enhanced abundance of F-ATPase, though this may have been a direct consequence of PMF dissipation, as reported for other LAB treated in the same manner (238).

Detoxification of ROS in LAB. Oxygen *per se* does not damage cells, but during the operation of metabolic pathways it is partially reduced to water, leading to the synthesis of ROS, including superoxide anion radicals, hydroxyl radicals, and H_2O_2. These display a considerable oxidizing potential and are highly toxic to cells (239, 240). ROS toxicity is also exerted through chemical modification and/or inactivation of most types of biomolecules (184). As detailed above, *Lc. lactis* can use oxygen under certain growth conditions (179). Also, H_2O_2 may be formed during mixed-acid fermentation as a consequence of the activity of NADH oxidases (241). Superoxide anion as well as hydroxyl radicals are produced during the Fenton reaction (242, 243). Metal ions can catalyze certain reactions leading to the formation of ROS, which is in fact considered one of the underlying mechanisms of metal-induced stress. Iron, copper, and other redox-active metal ions exert their effects by stimulating the Fenton reaction.

(i) Cellular damage produced by ROS. At the molecular level, ROS can react with macromolecules, such as proteins, lipids, and nucleic acids. Superoxide anions have a limited oxidizing potential and may attack compounds such as polyphenols, ascorbate, and catecholamines (239). H_2O_2 can inactivate proteins through the oxidation of cysteinyl residues (240) or can react with cations, such as Fe^{2+} and Cu^{2+}, producing hydroxyl radicals by the Fenton reaction (239, 244). The damage caused by the hydroxyl radical, which is the most destructive oxidative agent, includes strand breakage and chemical modification of DNA (239, 244).

(ii) ROS resistome. Bacteria such as *Lc. lactis*, *E. coli*, and *B. subtilis* are able to cope with oxidative stress through a number of enzymes that counteract the negative effects of oxidation (245). Notably, induction of genes encoding ROS-protective enzymes is growth phase dependent and confers multistress resistance (184, 246). Among the main players in LAB that help to counteract the deleterious effects of oxidative stress are (i) NADH oxidase/NADH peroxidase, (ii) superoxide dismutase (SOD), (iii) cytochrome *d* oxidase, (iv) catalase in the presence of necessary cofactors, and (v) nonenzymatic dismutation of H_2O_2 by Mn^{2+}.

The most conserved oxidative resistance mechanism in LAB

results from the coupling of NADH oxidase and NADH peroxidase (184). Oxygen is initially used for the oxidation of NADH to NAD^+ via the NADH oxidase, a reaction that produces H_2O_2. H_2O_2 is further reduced to water by the NADH peroxidase. NADH peroxidase activity is rather low in *Lc. lactis*, and the detoxification of H_2O_2 is incomplete. It was recently proposed that metabolically synthetized pyruvate reacts nonenzymatically with residual H_2O_2 to produce water (247). Multiple enzymes with peroxidase activity have been described for LAB, such as AhpCF, Npr, and Tpx of *E. faecalis* and AhpCF and Tpx of *S. mutans* (248).

Two distinct NADH oxidases have been identified in *S. mutans*. They catalyze either the two-electron reduction of oxygen to H_2O_2 (performed by Nox1) or the four-electron reduction of oxygen to water (AhpCF) and thereby provide a very efficient enzymatic defense against oxidative stress (249). *S. thermophilus*, on the other hand, does not appear to encode a Nox1 enzyme but uses a single NADH oxidase to reduce oxygen directly to water (250).

Another key player in resistance to oxidative stress is SOD, which eliminates superoxide anion radicals. Bacteria possess different types of SOD enzymes depending on the bivalent metal cofactor with which the enzyme is coupled, i.e., Fe^{2+}, Mn^{2+}, and/or Cu^{2+}-Zn^{2+}. LAB produce mainly Mn^{2+}-binding SODs (251). Analysis of an *Lc. lactis sod* mutant clearly established that SOD protects against superoxide anion radicals (251). An alternative strategy to counteract superoxide anion radicals is by the production of high levels of intracellular glutathione (252). Various enzymes that are known to counter oxidative stress, namely, MsrAB, AhpCF, Tpx, Npr, KatA, Sod, and HypR, have been characterized for *E. faecalis* (94, 248, 253–255).

E. faecalis possesses a respiratory chain that produces extracellular superoxide, whose production level depends on the availability of hematin as a cofactor for cytochrome *bd* or on fumarate as a terminal electron acceptor (256, 257). When this pathway is impaired by restricting access to these nutrients, extracellular superoxide is generated by partially reduced demethylmenaquinones upon exposure to oxygen (257). Thus, *E. faecalis* necessarily expresses multiple antioxidant defense mechanisms, such as NADH peroxidase and manganese-containing SOD (MnSOD).

Bacteria can also cope with oxidative stress through catalase, an enzyme catalyzing the detoxification of H_2O_2. The following three classes of bacterial catalases are currently recognized: monofunctional catalases, catalase-peroxidases, and manganese catalases (pseudocatalases) (258). The monofunctional catalases and the catalase-peroxidases contain heme as a prosthetic group. The monofunctional catalase specified by the genome of *E. faecalis* has been shown to remove H_2O_2 (259).

In order to retain the low intracellular redox potential required to keep proteins in their reduced form, the presence of glutathione as well as the thioredoxin systems is crucial (260). Members of the genus *Streptococcus* contain glutathione. They either synthesize glutathione or import it from the medium (261). In addition, streptococci encode a glutathione reductase, which catalyzes the reduction of glutathione disulfide that is produced upon oxidation of glutathione by NADPH (262). The glutathione reductase gene in *S. thermophilus* is induced in the presence of oxygen (262). The *S. thermophilus* genome also contains genes encoding two thioredoxins, a thioredoxin reductase, and a single glutaredoxin. This relatively large genetic repertoire emphasizes the importance of such enzymes in oxidative stress resistance (for a review, see reference 250). *E. faecalis* is able to synthesize glutathione and expresses glutathione reductase (261, 263).

Adaptation is also part of the survival strategy of LAB to cope with oxidative stress. Upon exposure to sublethal concentrations of H_2O_2, *Lc. lactis* can survive in the presence of otherwise lethal concentrations of this molecule (264). *Lb. plantarum* Lp80 *poxB*, encoding pyruvate oxidase, is linked to oxidative stress resistance (265–267). PoxB utilizes oxygen to convert pyruvate to acetylphosphate (Fig. 2), which results in the production of CO_2 and H_2O_2. Detoxification of the latter molecule is subsequently done by NADH peroxidase (268). Notably, PoxB activity is induced by oxygen or H_2O_2 and repressed by glucose (265). *In silico* analyses of *Lb. plantarum* Lp80 revealed a strongly conserved putative binding site in the promoter region of *poxB* for OhR, a regulator repressing the *ohrA* gene involved in peroxide detoxification. This is suggestive of regulation of *poxB* transcription in response to oxidative stress.

Other specific mechanisms involved in the adaptation of *S. thermophilus* to oxidative stress that support the aerotolerance of this bacterium imply the existence of a detoxifying mechanism against H_2O_2. Genome analysis of *S. thermophilus* revealed the existence of a thiol-peroxidase-encoding gene, which may explain this oxygen-tolerant phenotype (269).

(iii) Modulation of ROS-based stress. The mechanisms that evolved in LAB to overcome oxidative stress seem to overlap, and thus cells subjected to a specific stress condition can trigger different stress responses (for a review, see reference 184).

The cAMP receptor protein, the fumarate and nitrate reduction regulator (FNR), and FNR-like proteins (Flp) are part of a superfamily of proteins that encompasses structurally related transcription factors implicated in the control of stress responses (270). As mentioned above, such proteins display structural features allowing transmission of environmental and metabolic signals to the cell and which may trigger general and specific responses to various (stressful) physiological conditions (270). In *Lb. casei*, FNP and Flp exert a key role in the prevention of oxidative injuries by interacting with the 4Fe-4S oxygen-labile cluster (271). The *Lc. lactis* genome contains the two Flp-encoding genes *flpA* and *flpB*. They are located in two paralogous operons in which the proximal genes are predicted to encode metal ion transport systems (272). Targeted mutagenesis experiments showed that the FlpA/FlpB proteins control Zn^{2+} uptake and various stress resistance mechanisms. It was postulated that *Lc. lactis* utilizes Zn^{2+} to protect protein thiol groups from oxidation and that intracellular levels of this ion may act as a metabolite flux sensor for sensing and control of general stress responses (273).

Many regulators in streptococci modulate the interplay between oxidative stress and iron homeostasis, virulence, and competence (250). Iron is considered a key element in regulatory proteins sensing redox modifications in the presence of oxygen (274). This is illustrated by an *S. thermophilus* CNRZ368 mutant lacking the genes encoding an iron ABC transporter; the consequent imbalance in intracellular iron is the cause of an increase in superoxide anion radical sensitivity (145). In fact, control of iron homeostasis is considered to be coupled to ROS protection in streptococci (250). Also, the *S. pyogenes* Rgg regulator modulates the expression of general stress response proteins (275). Its involvement in the regulation of proteins related to oxidative stress was revealed when the *rgg* gene was inactivated (275). Inactivation

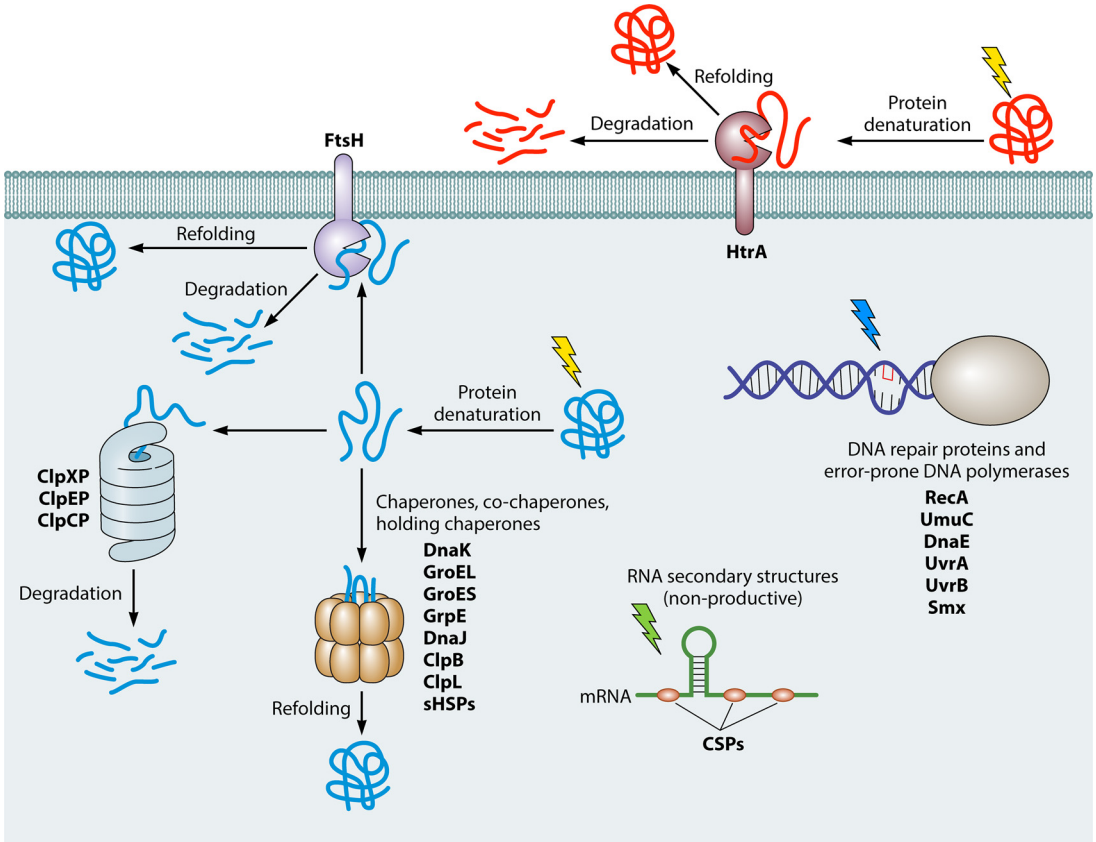

FIG 5 Schematic representation of the main molecular mechanisms repairing damaged macromolecules in LAB.

of an Rgg-like protein of *S. thermophilus* CNRZ368 produced a phenotype similar to that of *S. pyogenes rgg* (250).

Accumulation of compatible solutes. Osmotically active compounds serve a dual function in osmoregulation. In addition to maintaining a stable cell turgor, they also exert protective effects on biomolecules *in vitro* during stressful challenges. Various osmotically active compounds, such as proline and betaine, protect soluble and peripherally membrane-bound proteins from the denaturing effects of modifications in intracellular ionic strength and water availability. Osmotically active compounds can interact with proteins in several different ways, such as preferential exclusion, water replacement, hydration force, and vitrification of sugars (276). Betaine transport activity in LAB, such as *Lc. lactis* or various lactobacilli, increases with increasing osmotic stress and as a consequence of thermal insults (277, 278). Other osmotically active compounds, such as the disaccharide sucrose, protect *Lc. lactis* cells against pressure-induced inactivation of vital cellular components (279). The protective effect of ionic solutes has been ascribed to the intracellular accumulation of compatible solutes as a response to osmotic stress. Thus, ionic solutes (e.g., glycine betaine) provide only asymmetric protection in *Lc. lactis* cells; baroprotection requires a higher concentration of ionic solutes than of disaccharides (279).

(Multi)drug resistance systems. A large number of bacterial ABC-type drug export systems have been characterized (280). Most of these microbial drug extrusion systems are specific chemical or multidrug resistance (MDR) systems that remove various substances from the cytoplasm or membrane of a cell (281). Most of the currently known MDR systems use the PMF rather than ATP as the driving force and operate as drug/H^+ antiporters (282). A representative member of the MDR systems is the LmrA transporter of *Lc. lactis*, which shares both functional and structural features with the human MDR1 P-glycoprotein (283). Notably, LmrA expressed in human or insect cells efficiently exports drugs out of these cells and complements a lack of expression of the human MDR1 P-glycoprotein (284). A homolog of *lmrA* with highly significant structural and functional similarities to its lactococcal equivalent has been identified in *O. oeni* (282).

Treating Damaged Macromolecules

Major molecular chaperones and the Clp family of proteins. In bacteria, as in all living cells, the cellular proteome is in a constant state of flux. Protein quality control, including refolding or degradation of damaged proteins, plays an indispensable role under both stressed and nonstressed conditions. Similarly, the catalog of cellular proteins is continuously being modified to meet the challenges arising from changes in the environment. The synthesis of chaperones and proteases is quickly induced under stress conditions to combat the potentially deleterious aggregation of denatured proteins (Fig. 5). While the task of chaperones is to protect functional proteins and to refold misfolded ones, proteases provide the last line of defense by removing irreversibly damaged proteins, after which the amino acids are recycled. Under changing conditions, proteolysis also removes functional proteins for regulatory purposes or simply because they are no longer needed. It is obvious that the balance between refolding and proteolysis,

including the highly specific recognition of substrates, is crucial to avoid wasteful destruction (285, 286).

Chaperone proteins recognize stretches of hydrophobic amino acids typically exposed by nonnative proteins and bind to these regions to prevent aggregation. They can also actively assist protein folding in an ATP-dependent manner. One well-conserved bacterial chaperone system that can efficiently refold misfolded proteins is that of DnaK (Hsp70). The system consists of DnaK, its cochaperone (DnaJ), and a nucleotide exchange factor (GrpE) (286). Expression analyses conducted at the transcript and/or protein level revealed that production of DnaK is induced in different LAB under diverse stress conditions (287–290). A deletion in *dnaK* resulted in a thermosensitive phenotype for *Lc. lactis* (291) and *Streptococcus intermedius* (292). DnaK is essential in *S. mutans* (293). The inability of DnaK proteins of *S. intermedius*, *Tetragenococcus halophilus*, and *Lc. lactis* to complement the function of *E. coli* DnaK *in vivo* suggests that functional differences exist between the chaperone systems of LAB and the current models (292, 294). Since only a few reports deal with DnaK structure or biochemical function in LAB, several fundamental questions remain to be clarified. For example, to date, the cochaperone-dependent folding activity of DnaK has not been demonstrated for any LAB. A biochemical study using purified DnaK, DnaJ, and GrpE of halophilic *T. halophilus* did not reveal cooperation of DnaK with the cochaperones (294).

Another refolding system that is well characterized in model bacteria is the GroEL/GroES (Hsp60) chaperonin, comprised of the 14-mer GroEL protein and its heptameric cofactor GroES. In the absence of ATP, GroEL/GroES forms a macromolecular complex arranged as two stacked heptameric rings that can bind unfolded proteins via hydrophobic interactions (286). GroES is the most abundant protein in *B. subtilis*, with some 500,000 molecules per cell after 30 min of exposure to heat stress (295). The production of the highly conserved GroEL and GroES proteins is activated under various stress conditions in LAB (287–290, 296).

The bacterial Clp family of proteins and their yeast homologs Hsp104 and mitochondrial Pim1 protease belong to the HSP100 family of HSPs (297). The Clp machinery is probably the main system for general protein turnover in LAB, as is the case in other low-GC Gram-positive bacteria (298). The Clp family is a subgroup of the AAA^+ ATPase superfamily, whose members are characterized by an α-helical core domain and one or two Walker-type ATP-binding domains. The number and spacing of the ATP-binding sites in the proteins are the foundation for their division into various Clp ATPase families, including ClpA, ClpB, ClpC, ClpE, ClpL, and ClpX (299). The ATP-dependent ClpP protease is a two-component protease consisting of a *clpP*-encoded serine peptidase subunit and a Clp ATPase subunit. The central proteolytic core consists of 14 ClpP subunits stacked back to back in two heptameric rings, forming an internal chamber that encapsulates the active sites of the ClpP peptidases (300). The peptidase multimer associates with one or two hexameric rings of Clp ATPases to gain proteolytic activity. The Clp ATPases, together with the proteolytic core, are responsible for substrate recognition, unfolding, and translocation into the ClpP degradation chamber. In the absence of ClpP, threading of substrates through the Clp ATPase ring structures leads to disaggregation and refolding of protein substrates, which are activities typical of molecular chaperones (301). Some Clp ATPases are able to interact with ClpP, while others function as independent chaperones. ClpP-interacting Clp ATPases, which in LAB typically include the ClpX, ClpC, and ClpE proteins, can be distinguished by the presence of a ClpP recognition tripeptide (302). There are some distinctions between the catalogs of Clp ATPases in Gram-negative and Gram-positive bacteria, but ClpP and ClpX appear to be widely conserved. ClpE is absent in *E. coli* but present in many species of LAB, in conjunction with ClpC, ClpL, and ClpB (302). The ClpL and ClpB proteins lack the ClpP recognition tripeptide and are thus considered to be unable to interact with ClpP. With the exception of *clpX*, the genes encoding the Clp family of proteins are not essential for most bacteria, including LAB (303), which has facilitated the construction of knockout mutants and the study of the physiological roles of Clp proteins in a variety of LAB species. In contrast, ClpX is not essential in *S. mutans* (304). Phenotypic studies with mutant strains lacking functional ClpP, ClpL, ClpE, or ClpB revealed the central role of these proteins under stress conditions and, for pathogenic LAB species, in virulence (303, 305–308). In contrast to the situation in *B. subtilis*, LAB *clpC* mutants usually do not exhibit stress-sensitive phenotypes (306, 309). Phenotypic characterization of an *S. thermophilus clpC* mutant revealed that ClpC plays a role in the development of genetic competence in this organism (310). The alternative sigma factor ComX, which drives the expression of competence genes, was shown in *S. thermophilus* and *S. mutans* to be under posttranslational regulation by ClpCP through active degradation (310–313). This task is performed by ClpEP in *S. pneumoniae* (314).

Regulation of the major molecular chaperones and the Clp family of proteins. LAB lack the alternative sigma factor σ^B, a key element in the regulation of the general stress response in low-GC Gram-positive organisms, such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *B. subtilis*. The primary stress induction of molecular chaperones and Clp proteins in LAB occurs at the level of transcription and is regulated mainly by the transcriptional repressors CtsR and HrcA. HrcA recognizes the highly conserved CIRCE (control of inverted repeat for chaperone expression) operator sequence, while CtsR binds to a heptanucleotide repeat (A/GGTCAAA/T) located in the promoter regions of target genes (315). The *B. subtilis* CtsR and HrcA regulons are distinct from each other, while in LAB they sometimes overlap. One of the repressors is absent in some LAB species, and a single regulator controls expression of both regulons (315). The expression of *S. salivarius* and *S. thermophilus clpP* is under dual regulation by CtsR and HrcA (316). The HrcA and CtsR regulons may partially overlap in several other members of the *Streptococcaceae* (316, 317). Sequence analysis of *Lb. plantarum* revealed two CIRCE elements and a CtsR-binding site upstream of *hrcA-dnaK-grpE-dnaJ*, indicating dual control of this operon by both regulators (318). *O. oeni* was the first example of a low-GC Gram-positive bacterial species that lacks the widely conserved CIRCE-HrcA regulatory circuit. Its CtsR regulon comprises genes that encode the classical chaperone proteins DnaK, GroEL, and GroES and Clp proteins (317). An example of reductive evolution of stress repressors with a different outcome is provided by *Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lactobacillus johnsonii*, which all lack CtsR (201, 319, 320). The HrcA regulon in these species has expanded to include *clp* genes, in addition to genes encoding the *groEL* and *dnaK* operons. The molecular details behind the regulation of HrcA function have not been addressed for LAB. According to studies of other organisms, HrcA is prone to aggregation, a characteristic feature that is also central to its autoregulation

(321). HrcA is thought to be released from ribosomes in an inactive form. To become an active DNA-binding protein, it must interact with the GroEL chaperone. Under stress conditions, GroEL is titrated away from HrcA by accumulating denatured proteins, which drives the equilibrium toward aggregated/inactive HrcA (321). Thus, protein stress directly affects HrcA activity. Molecular studies have revealed several differences in the mechanisms of regulation of CtsR function between LAB and other bacteria. The *ctsR* gene is cotranscribed with *clpC* in all low-GC Gram-positive bacteria. In members of the *Bacillales* order, the *clpC* operon is tetracistronic and consists of genes encoding two modulators of CtsR activity (McsA and McsB) in addition to *ctsR* and *clpC*. McsA and McsB are lacking in LAB, and *ctsR* forms a dicistronic operon with *clpC* (315). McsB is not involved in the regulation of CtsR activity during heat stress *in vivo*, and CtsR itself acts as an intrinsic thermosensor whose DNA-binding activity gradually decreases with increasing temperature. The CtsR regulon is also derepressed under several environmental stress conditions other than heat shock, such as acid stress and oxidative damage. This implies that alternative mechanisms for CtsR inactivation must exist. Interestingly, in low-GC Gram-positive bacteria lacking McsA/McsB, such as *Lc. lactis*, ClpE activity appears to partially substitute for the function of McsA and McsB. *Lc. lactis* ClpE has been reported to contain a zinc finger domain similar to that of McsA and to undergo a thiol-dependent ClpP-mediated cleavage similar to that of McsA (298, 322). *Lc. lactis clpE* can complement a *B. subtilis mcsB* mutant in inactivating CtsR during oxidative stress (298). A recent report provided evidence that *S. mutans* ClpL interacts with CtsR both *in vivo* and *in vitro* and, by its chaperone activity, prevents CtsR from aggregating under ambient growth conditions (323).

FtsH, HtrA, and small HSPs (sHSPs). FtsH is a membrane-bound HSP with dual chaperone-protease activity (Fig. 5). The FtsH protein contains transmembrane segments in the N-terminal region and a main cytosolic region consisting of an AAA^+ ATPase and a Zn^{2+} metalloprotease domain (324). The functional roles of FtsH in LAB have not been widely studied. FtsH plays roles in protein quality control during heat shock in LAB, such as *Lc. lactis* (325), *O. oeni* (326), and *Lb. plantarum* (327). The FtsH proteins of *Lc. lactis* and *O. oeni* are both able to complement the growth defect of an *E. coli ftsH* mutant at 37°C, indicating that FtsH from these LAB can at least partially functionally replace *E. coli* FtsH. Inactivation of *ftsH* resulted in salt-, heat-, and cold-sensitive phenotypes of *Lc. lactis* (325), whereas reduced salt and heat tolerance as well as diminished biofilm formation were reported for an *Lb. plantarum ftsH* mutant (176). *Lb. plantarum ftsH* was shown to be a novel member of the CtsR regulon (327). The mechanisms controlling *ftsH* expression in other LAB have not been studied.

HtrA (high temperature requirement protein A), also known as DegP, is involved in degradation of extracellular polypeptides and misfolded proteins (Fig. 5). HtrA contains a chaperone and a proteolytic domain. An *Lc. lactis htrA* mutant is thermosensitive, and HtrA appears to play a fundamental role in the degradation of abnormal exported proteins (328). The *htrA* gene has been identified as a heat shock gene in *Lactobacillus helveticus* (329), and elevated HtrA levels have been detected in *Lb. casei* in response to phenolic compounds (330). However, the regulatory mechanism behind the stress-inducible expression of *htrA* in LAB remains to be elucidated.

The members of the sHSP family function as folding chaperones, assisting the protein folding process by stabilizing unfolded or partially folded proteins without actively promoting their remodeling (331). Furthermore, sHSPs appear to play an important role in maintaining membrane integrity, especially under stress conditions (332). The sHSP family is widely conserved, and its members have been identified in several LAB (302). In *O. oeni*, an sHSP named Lo18 was identified as a major stress protein whose synthesis is greatly increased under various conditions, including ethanol shock (333). Lo18 has been suggested to play a dual role in *O. oeni* by preventing protein aggregation and stabilizing the cytoplasmic membrane (334).

Proteins induced by cold shock. A sudden temperature downshift induces a cold shock response, during which growth of cells is first arrested for a period before reinitiation at a new and much reduced rate. The cellular effects of cold shock include the following: (i) a decrease in membrane fluidity, (ii) a reduced efficiency of mRNA translation and transcription through stabilization of secondary structures of DNA and RNA, (iii) ineffective or slow protein folding, and (iv) hindered ribosome function (335). In cold-shocked *E. coli*, overall transcription and translation are slowed considerably, and a set of approximately 26 cold shock genes is preferentially and transiently expressed (336). Members of the CSP family enhance the expression of cold shock-inducible genes by destabilizing nonproductive secondary structures in mRNA at low temperatures and therefore are often called RNA chaperones (336). While cold and heat shocks are considered highly separate processes in *E. coli* and *B. subtilis* (337), recent evidence indicates that these responses are interconnected in LAB (338). Cold shock of *S. thermophilus* is accompanied by increased production of GroEL and ClpL (296, 307). Furthermore, the *clpL* knockout mutant of *S. thermophilus* is cold sensitive (307). Cold shock conditions increase the synthesis of DnaK and GroEL in *Leuconostoc mesenteroides* (288) and of sHSPs in *Lb. plantarum* (339). Transcriptional analyses have indicated that *clpX* is induced by both heat and cold in *Lc. lactis* (340). The proposed cross talk between cold and heat shock responses in LAB is further supported by the improved cryotolerance seen after heat treatment of *Lb. johnsonii* and *Lc. lactis* (341, 342). Similar findings may link acid and cold stress responses in LAB; for *Lb. delbrueckii* subsp. *bulgaricus*, an increase in freezing tolerance was observed after acidification of the growth medium (343).

Spx governs the response to oxidative damage of macromolecules. Spx is a global transcriptional regulator of oxidative stress in several Gram-positive bacteria and plays important roles as both a positive and a negative modulator of gene expression (344). The LAB Spx homolog TrmA (now named SpxA2) was first identified via mutations that alleviate the heat-sensitive phenotype of *clpP* mutants and *recA* mutants of *Lc. lactis* (245, 345). Proteolysis of Spx is mediated by the ClpXP protease system, and Spx accumulates to high levels in *clpX* or *clpP* mutants (304). Studies of *B. subtilis* Spx activity revealed that it interacts directly with the α subunit of the RNA polymerase (RNAP) and thereby controls global transcription initiation, either negatively or positively, by a unique mechanism not involving initial contact between Spx and DNA (344). Negative regulation is mediated by binding of Spx to Tyr263 in the C terminus of the RNAP α subunit. This residue is highly conserved among RNAP α subunits of Gram-positive bacteria, and the binding blocks interaction between other transcriptional activators and RNAP (344). *Lc. lactis* encodes seven Spx

paralogs, among which SpxB has been shown to be involved in the cell wall stress response. DNA microarray work with *spx* mutants confirmed the function of Spx as a major oxidative stress regulator (346, 347). Streptococci harbor two *bona fide* Spx regulators, whereas enterococci have only one (346–349).

Repair of stress-induced DNA damage. According to the current DNA damage paradigm, cells undergo an inducible process called the SOS response during genotoxic stress. This response triggers the expression of genes involved in DNA replication, repair, and mutagenesis (350). In the case of severe DNA damage, high-fidelity DNA polymerase cannot replicate over the incurred DNA lesions and is replaced by a low-fidelity DNA polymerase(s). The process, called translesion synthesis, is performed mainly by SOS-induced error-prone polymerases of the Y family (Pol IV and Pol V) or the C family (DnaE) (350). The SOS response is repressed under physiological conditions by binding of the LexA repressor protein to operator sites in the promoter regions of SOS genes, including its own gene, *lexA* (350). While the bacterial DNA damage response is one of the most extensively studied stress responses, it has not been studied widely in LAB. LexA regulons have been identified in some LAB species, such as *Lb. plantarum* (351) and *E. faecalis* (352). The *uvrA* gene, which encodes a nucleotide excision repair protein and is a typical member of LexA regulons, has been characterized for *S. mutans* (353) and *Lb. helveticus* (354). An *S. mutans uvrA* mutant, in addition to being sensitive to DNA damage, was also sensitive to acidic conditions, indicating that UvrA plays a role in the repair of acid-induced DNA damage (353). Other DNA repair activities that have been implicated in the resistance of *S. mutans* to acidic pH include those of the Smx nuclease (355, 356). The *S. pyogenes* C family DNA polymerase DnaE was shown to be highly error-prone and produced frameshift and point mutations during replication of both damaged and undamaged DNAs *in vitro* (357). In *S. uberis*, the Pol V subunit UmuC is SOS induced and essential for UV-induced mutagenesis (49).

PROTECTING THE CELL ENVELOPE

The Cell Envelope of LAB

LAB have cell envelopes that are typical of Gram-positive bacteria. They consist of a cytoplasmic membrane and a cell wall that may additionally be surrounded by an external polysaccharide capsule and/or a proteinaceous S layer. The cell wall itself comprises a PG sacculus that is usually decorated with (lipo)teichoic acids, polysaccharides, and proteins (for a review, see reference 358). The cell wall provides a point of (non)covalent attachment of important extracellular enzymes and external structures, such as pili and flagella (359). It also participates in processes of adhesion, cell aggregation, and cell division (360). Most importantly for this review, the cell envelope acts as a diffusion barrier between the cell and its surroundings. It is crucial for maintaining the integrity and shape of the cell and for resisting changes in osmotic pressure.

Cell envelope stress. Many of the environmental or technological stressors that LAB can encounter have an impact on the cell envelope. This is why it is very difficult to arrive at a strict definition of cell envelope stress, as regulator systems that respond to stresses exerted on or sensed at the cell envelope often also play a role in the normal physiology of the cell (361).

(i) Antibiotic stresses. The cell wall is a major target in our fight against pathogenic bacteria, including pathogenic LAB. Cell wall synthesis can be inhibited by antibiotics that block certain enzymatic steps or capture one of the precursors (361).

β-Lactam antibiotics, such as penicillin and its analogs, block PG biosynthesis through inhibition of the activity of a group of penicillin-binding protein (PBP) transpeptidases that catalyze the polymerization of the glycan strand or the cross-linking between glycan chains (362, 363). Resistance of bacteria to β-lactam antibiotics is usually a physiological adaptation resulting from selective pressure. Resistant cells can express a mutated or low-affinity PBP obtained via horizontal gene transfer (HGT) (362, 364), produce β-lactamases (365, 366), or extrude the antibiotic through efflux pumps (367).

Antibiotics such as vancomycin, lantibiotics, or bacitracin target the translocation via lipid II of PG precursors across the cytoplasmic membrane (lipid II cycle) (368). Vancomycin binds to the D-alanyl–D-alanine ends of PG precursors, thereby inhibiting the formation of mature PG (369). Inducible vancomycin resistance was first described for clinical isolates of enterococci (370, 371). So far, eight enterococcal *van* gene clusters (*vanA* to *-E*, *vanG*, *vanL*, and *vanM*) have been described, and they encode ligases required for the synthesis of either D-Ala–D-lactate or D-Ala–D-Ser PG precursors for which glycopeptides, such as vancomycin, have low affinity (371, 372). The expression of enterococcal *van* genes is under the control of VanRS TCSs (370) (Fig. 1). The molecular mechanisms of regulation of vancomycin resistance gene expression have been reviewed thoroughly (370) and are not discussed here. The specific ligand recognized by the VanS sensor proteins has not yet been determined unequivocally. Two distinct models have been proposed: direct induction, in which the sensor kinase binds the antibiotic, and indirect induction, in which the sensor kinase binds a cell wall component that either is an intermediate of cell wall biosynthesis or accumulates as a result of antibiotic action (370).

Lantibiotics, such as nisin produced by *Lc. lactis*, are polycyclic peptide antibiotics containing the nonproteinogenic amino acid lanthionine. Nisin binds lipid II and thus has a double effect on the cell: it inhibits PG synthesis, and it forms pores in the cytoplasmic membrane through which essential components can leak out, killing the cell (368). The main resistance mechanism against positively charged molecules, such as lantibiotics, is a change of the overall negative charge of the cell wall by coupling the positively charged amino acid D-alanine to teichoic acids in the cell wall (373).

Bacitracin, a cyclic dodecylpeptide antibiotic that blocks the lipid II cycle by inhibiting dephosphorylation of isoprenyl pyrophosphate (374), is counteracted by the action of specific ABC transporters (BceAB) (see below). Other mechanisms, such as the synthesis of alternative isoprenyl pyrophosphate phosphatases or *de novo* synthesis of undecaprenyl phosphate, have also been described (375, 376).

(a) The BceRSAB system participates in the cell envelope stress response of LAB. BceRS was first described for *B. subtilis* as a TCS regulating the cognate ABC transporter BceAB, which conferred resistance against bacitracin (377, 378) (Fig. 1). BceRS-like TCSs consist of an intramembrane HK (IM-HK) (379) and an OmpR family RR. A remarkable characteristic of these systems is that IM-HKs are unable to function as sensors: this function is provided by the ABC transporter, with which they are usually genetically and functionally associated (380, 381). In effect, the BceRSAB-like modules are antimicrobial peptide (AMP) de-

toxification systems in which the ABC transporter may play a dual role in mediating AMP resistance/detoxification and sensing AMPs.

BceRSAB-like modules are present in many LAB, e.g., BceAB of *S. pneumoniae* (382) and its ortholog YsaBC in *Lc. lactis* (383), but they have been characterized only for *E. faecalis* (384), *Lb. casei* (385), *S. mutans* (386), and *S. thermophilus* (387). These studies revealed remarkable differences in the genetic organization and regulons. *Lb. casei* harbors two BceRS systems (TCS09 and TCS12), each with its own BceAB homolog, while a third BceAB homolog is not genetically associated with any TCS (orphan ABC) (385). Both TCSs constitute functional units with their corresponding ABC transporters, and transcriptional analyses showed that while TCS09 controlled the expression of the cognate ABC09 transporter, TCS12 controlled the expression of the orphan ABC as well as the *dlt* operon (involved in D-alanylation of teichoic acids) and the *mprF* gene (involved in L-lysinylation of phospholipids) (385). *E. faecalis* JH2-2 encodes a single BceRS homolog that controls the expression of two BceAB-like transporters. In this organism, TCSs and ABC transporters are not genetically associated. One of the ABC transporters is essential for sensing bacitracin and eliciting a response (384). In response to bacitracin, the BceABRS system of *S. mutans* (also named MbrABRS) induces its own expression and that of three additional uncharacterized genes (386). A later study identified four additional genes that belong to the MbrABRS regulon, including those for the cell surface antigen SpaP and for MurM and MurN, which are alanine-adding enzymes involved in the branched-peptide PG biosynthetic pathway. These observations suggest that MbrABRS plays an important role in the cell envelope stress response of *S. mutans* (388).

(b) BcrR, a membrane-bound one-component bacitracin sensor. Although most OCSs are predicted to be cytosolic proteins, some may actually be membrane bound (54). The BcrR bacitracin sensor of *E. faecalis* has been characterized in detail (389–391) (Fig. 1). *E. faecalis* BcrR activates the transcription of the bacitracin resistance operon *bcrABD* in response to Zn^{2+}-bacitracin. BcrR contains four predicted transmembrane helices and an N-terminal DNA-binding domain (389). It binds Zn^{2+}-bacitracin and not Zn^{2+}-free bacitracin. An auxiliary protein is not required for bacitracin sensing by BcrR (391). Deletion of the transmembrane domain abolished DNA-binding activity, suggesting that membrane localization is essential for BcrR activity (391). Binding of bacitracin did not alter the DNase I protection pattern or the apparent DNA-binding constant for binding of BcrR *in vitro* to the *bcrABD* promoter, but it did lead to induction of transcription of this promoter in an *in vitro* transcription assay (391). On the basis of these results, BcrR is proposed to always be bound to its target DNA sequence; binding of bacitracin would induce a conformational change facilitating a productive interaction with the RNA polymerase, through an as yet undetermined mechanism (391).

(ii) Physical stresses. One of the main functions of the cell wall is to maintain cell shape and to counteract the high internal osmotic pressure, which can reach up to 2,000 kPa in Gram-positive bacteria. The cell envelope plays a key role in the regulation of osmotic stress, as it is highly permeable to water, while the cytoplasmic membrane acts as a barrier for most solutes. The main response of LAB to osmotic stress is the intracellular accumulation of osmoprotectants, of which the most common are glycine betaine, choline, carnitine, and dimethylsulfonioacetate (392). Due to their reduced biosynthetic capabilities, LAB have to import these compounds from their (natural) environments, such as milk, meat products, or plant materials (393, 394). The ability of these osmolytes to reduce the inhibitory effect of hyperosmotic situations has been established for a large number of LAB (395–406).

Another mechanism for LAB to cope with osmotic stress is through regulation of the intracellular concentrations of certain amino acids. Intracellular concentrations of proline, glutamate, and sometimes alanine and aspartate increase when *Lb. plantarum*, *Lc. lactis*, or *T. halophilus* is grown under conditions of high osmotic pressure (399, 407, 408). Some amino acids reduced the impact of osmotic stress on growth when they were added exogenously to LAB cultured in chemically defined media. This was reported for the addition of proline to *Lc. lactis* and *Pediococcus pentosaceus* (397, 400) and of aspartate to *O. oeni* (401). Di- and tripeptides generated by the activity of cell envelope-associated proteinases can also act as osmoprotectants in LAB, as reported for *O. oeni* (401) and *Lactobacillus zeae* (409).

LAB species have developed efficient mechanisms for recruitment of osmoprotectants from their environment. Accumulation of osmolytes is mediated by transporters that move solutes across the membrane against their electrochemical gradients in processes that require metabolic energy (410). Interaction of the transporters with membrane lipids determines their activity in response to osmotic changes (410). Betaine and proline uptake in *Lc. lactis* is controlled by the *opu/bus* operon (411). This operon encodes a transport system of the ABC family that uses two ATP molecules per translocated molecule of glycine betaine (412). The *Lc. lactis* glycine betaine ABC transporter OpuA/BusA has been characterized in considerable detail (Fig. 1). OpuA detects changes in the intracellular ionic strength, and its activity depends on electrostatic interactions between lipid head groups and OpuA (413). Regulation is mediated by two tandem cystathionine beta-synthase (CBS) domains in the C-terminal end of the ATPase subunit OpuAA (414). In particular, sensing depends on the C-terminal tail of the CBS domains, which is negatively charged (414). A model was proposed in which the transporter switches from the active to the inactive state depending on the interaction of the CBS domains with lipid head groups in the membrane. Repulsion between the charged C-terminal tail and the anionic heads of the membrane lipids counteracts the interaction between the CBS domains and the membrane. In this way, the intracellular ionic strength and the concentration of anionic lipids in the cytoplasmic membrane determine the activation of the transporter (414). A later study showed that the interaction of the CBS domains and the membrane lipids depends on a surface-exposed cationic region in the CBS domain, in agreement with the proposed model (415).

OpuA/BusA expression is repressed by the regulator BusR, which binds to the *busA* promoter with dependence on the ionic strength: at an elevated cytoplasmic ionic strength, it dissociates from its binding site, resulting in the osmotic induction of *busA* operon expression (416). In this way, both OpuA/BusA activity and expression are regulated by the intracellular ionic strength. A genome-wide gene transcription analysis revealed that *Lc. lactis opu/bus* is induced up to 60-fold under conditions of osmotic stress (417). Genes for Opu family members are present in the genomes of all the pathogenic LAB, including *S. mutans*, *Streptococcus agalactiae*, *S. pyogenes*, and *E. faecalis*, and in a number of

cases induction of expression of the genes upon osmotic stress (addition of NaCl) has been observed (406, 418).

Another osmolyte transporter from the ABC family that is present in several LAB is ChoQS. In *Lc. lactis*, the *choS* gene was induced by osmotic stress, while both genes (*choQ* and *choS*) were induced by acid stress (417). The uptake of osmolytes by *Lb. plantarum* is mediated by the QacT system, a quaternary ammonium compound transporter. This system has a high affinity for glycine betaine and a low affinity for proline and is expressed semiconstitutively, i.e., osmolytes added to the culture medium did not increase *qacT* gene expression (407).

Mechanosensitive Channels

When bacteria are faced with a sudden drop of the external osmotic pressure, they use mechanosensitive channels (MSC) to counteract the effects. The MSC act as emergency release valves, allowing the expulsion of osmolytes and water. MSC were discovered in bacteria more than 25 years ago (419), and the two major MSC were named after their *E. coli* equivalents, i.e., MscS (small conductance) and MscL (large conductance). MSC share an organization of a C-terminal cytoplasmic domain and an N-terminal transmembrane domain formed by symmetrical helices that can change between open and closed states (420). Although their mechanism of sensing is still unclear, it is well known that MSC can sense changes in the tension of the lipid bilayer of the cytoplasmic membrane (421). Most microbes appear to possess members of one or both families of bacterial MSC (422). MSC in LAB have been studied only for *Lc. lactis* (423). Although *Lc. lactis* has genes encoding both MscS (*yncB*) and MscL (*mscL*) channels, only the latter is functional and is used as the main mechanosensitive solute release system to protect cells under conditions of hypoosmotic stress (423) (Fig. 1).

Stress on the cell membrane. LAB can counteract several stresses by modifying their cytoplasmic membranes. The regulation of membrane fluidity in bacteria is a rather complex process that often involves universal protein chaperones, such as GroEL and sHSPs (424, 425). The fluidity of the cytoplasmic membrane is highly influenced by the ratio of unsaturated to saturated fatty acids, and alterations in membrane fatty acids may represent a generalized response utilized by many bacteria to survive environmental stresses affecting the membrane. In *Lb. casei*, *S. gordonii*, and *S. salivarius*, the levels of monounsaturated membrane fatty acids increased in response to environmental acidification (426). The ratios of unsaturated to saturated and cyclic to saturated membrane fatty acids decreased in a strain of *Lb. delbrueckii* subsp. *bulgaricus* subjected to acid stress, resulting in cross-protection against cold and acid stress (427). The decrease in membrane fluidity in *Lactobacillus* could be compensated by other variations, such as an increase of cyclic fatty acids (428). Applying a cold, acid, or ethanol shock to *O. oeni* resulted in a rigidification of the cytoplasmic membrane, with various stress-specific effects on the viability of the cells (429).

Osmotic stress and the cell wall. When *Lb. casei* is placed under osmotic stress, its cell wall conformation changes. High salt concentrations led to an increase of the size of the cells and to sensitivity to antimicrobial peptides targeting PG, such as nisin (430). These effects were associated with a reduction of the cross-linking of PG molecules due to altered expression and activity of PBPs (430, 431). Osmotic stress induced by a high salt concentration in *Lb. delbrueckii* subsp. *lactis* led to an increase of autolytic activity and survival following lyophilization (432). *Lb. rhamnosus* also showed adaptations to technological stress conditions by decreasing the amounts of proteins responsible for cell wall biosynthesis (MurD, MurC, and UDP-GlcNAc-2-epimerase) (176). Exposure of *Lc. lactis* subsp. *lactis* to osmotic stress led to expression of the *murF* and *murG* genes, which are involved in PG biosynthesis (417).

Sensing of cell envelope stress. As described in previous sections, TCSs play key roles in the signaling of stress in LAB. The most-studied TCS related to the cell envelope stress response in *Firmicutes* is LiaFSR of *B. subtilis*, a system originally identified in a study of the response of *B. subtilis* to bacitracin (378). This system also responds to the presence of other antibiotics that interfere with the lipid II cycle (vancomycin and ramoplanin), to the action of detergents and organic solvents, and to other unspecific stimuli on the cell envelope (433–438). LiaFSR is a three-component system, as a third protein, LiaF, acts as a negative regulator of LiaRS-dependent signal transduction (439). The *B. subtilis lia* operon also specifies the phage shock proteins LiaH, LiaI, and LiaG, which are membrane proteins of unknown function. LiaFSR homologs are widely distributed in LAB, but they lack homologs of the *B. subtilis* LiaIHG proteins (25, 63, 440–442).

CesFSR was originally described as one of six seemingly complete TCSs present in *Lc. lactis*. Disrupting the gene for CesR led to a reduction in the ability to resist salt and osmotic stresses (66). The authors could not generate a CesS mutant, probably because that mutation was lethal. An *Lc. lactis* Δ*cesR* mutant was two times more sensitive to nisin, although this peptide did not activate the *cesFSR* promoter (441). Nevertheless, a nisin-resistant strain obtained through a nisin adaptation process overexpressed *cesFSR* and its regulon (383). The *cesFSR* system is also induced by vancomycin, bacitracin, and the bacteriocins plantaricin C and Lcn972 (441). Analysis of the transcriptome of *Lc. lactis* treated with Lcn972 revealed that at least 26 genes were upregulated. Many of the genes in the *cesFSR* regulon of *Lc. lactis* encode putative membrane- or stress-related proteins (441). One of the most upregulated genes is *llmg_0169*, encoding a small putative membrane protein of unknown function but with an essential role in protecting the cell against high temperatures (443). Among the most upregulated genes was the *llmg_2163-llmg_2164* operon, which encodes a protein of unknown function and a putative transcriptional regulator. The cooverexpression of these genes increased the resistance of *Lc. lactis* against Lcn972, while a mutant lacking *llmg_2164* suffered from a low resistance to high temperature and salinity (443). The *llmg_1155/spxB* gene encodes a regulator that stimulates the expression of *oatA*, a gene coding for a protein involved in the O-acetylation of PG and conferring a high resistance of the cells to autolysis and lysozyme treatment (444). Other notable *cesFSR* regulon members are *lmrA* and *rmaB*, which encode a multidrug ABC transporter and a transcriptional regulator from the MarR family, respectively; *llmg_2163*, specifying a putative transcriptional regulator with a PspC domain; and *llmg_2194* and *llmg_0165*, encoding proteins with protective roles in response to general envelope stressors (temperature, pH, and salt stresses).

The CesFSR three-component system is also activated when *Lc. lactis* is forced to overproduce membrane proteins, most probably through destabilization of the cytoplasmic membrane as a consequence of accumulation of misfolded proteins (445, 446). Growth of *Lc. lactis* and its ability to overproduce BcaP, an endogenous

membrane protein, are directly related to the CesFSR system; both are impeded when the genes of the CesFSR system or its operon are absent, while growth and BcaP production are improved when the *cesFSR* genes are overexpressed (446).

TCS systems homologous to LiaFSR have also been identified in other LAB species. In *S. mutans*, LiaFSR has been shown to regulate 174 genes, some of which specify surface proteins involved in biofilm formation, cell envelope biogenesis proteins, or cell envelope chaperones/proteases (442, 447). A 25-bp conserved motif essential for LiaR binding is present upstream of the genes directly controlled by the LiaFSR system (448). Disruption of this system in *S. mutans* led to augmented sensitivity to antibiotics interfering with lipid II cycling (442). A system homologous to LiaFSR has also been characterized for *S. pneumoniae*, in which it was shown to control the expression of at least 19 genes involved in protecting cells against murein hydrolases and lipid II-interacting antibiotics (449). In *S. agalactiae*, this system controls not only cell wall stress-related responses but also pilus expression and genes involved in the defense against host antimicrobial systems (440).

Deletion of the LiaR homolog in *E. faecalis* led to an increase in susceptibility to bacitracin (63). This deletion also restored susceptibility to daptomycin and telavancin, antibiotics that disrupt the cell membrane, in a multidrug-resistant *E. faecalis* strain (450).

All in all, the LiaFSR system homologs have a widely conserved cell envelope stress-sensing function in *Firmicutes*, although the regulons differ quite considerably among genera. The regulons in LAB are broader than that in *B. subtilis*, which may indicate a more prominent role of the system in the cell envelope stress response in LAB (25).

STRESS MECHANISMS IN PROBIOTIC LAB

LAB employed for potentially health-promoting applications are mainly lactobacilli. They encounter a variety of stresses during production and use. Before a probiotic bacterium begins to fulfill its biological role in the gut, it must survive a battery of environmental challenges (27). Initially, probiotic bacteria must be processed in a suitable product form to enable oral consumption in adequate numbers, and then the bacteria must survive the harsh conditions imposed during passage through the GIT (451). Although they are not strictly considered probiotics, increasing evidence has shown that dead bacteria can still have a health-promoting effect (452).

Stress Associated with Technological Production

Probiotics are normally grown to high numbers before undergoing a drying process to produce a high-cell-density dried powder formulation. The stresses encountered during this processing include extremes in temperature, from very high (up to 200°C) during spray-drying to very low (down to −196°C) during freeze-drying and storage. Such extreme temperatures can affect membrane fluidity and compromise cellular integrity and basic cell processes, such as ribosomal function, protein folding, and enzymatic activity (27). Some species of lactobacilli are relatively thermotolerant; for example, *Lb. paracasei* 8R2 has been shown to survive thermization (60°C, 5 min) of cheese milk (with 90% survival) (453, 454). The effect of heat shock and the induction of a stress response have been studied in a variety of lactobacilli (see above). In general, the resistance to stress is higher when cells are previously exposed and adapted to a sublethal treatment with a homologous stress. For example, enhanced heat tolerance was obtained when *Lb. rhamnosus* GG was preexposed to 60°C for 20 min (451). Similarly, treatment of *Lb. paracasei* NFBC 338 at 52°C for 15 min increased the survival of the strain 700-fold in reconstituted skim milk and 18-fold during spray-drying (27, 455). This improvement was associated with induction of the chaperone GroEL. Heat stress was previously shown to enhance the expression of the heat shock genes *hsp18.5*, *hsp19.3*, and *hsp18.55*, carried on the genome of *Lb. plantarum* NC8 (339, 456). As mentioned earlier, CSPs are induced as a result of cold shock pretreatment, and they have been associated with the stabilization of mRNA (457). Three induced CSPs were previously identified in *Lb. plantarum* following cold shock treatment, namely, CspL, CspP, and CspC (457).

In addition to temperature stress, spray-drying also exposes cells to osmotic stress, dehydration, and oxidative stress (27). A sudden increase in the environmental osmolarity (hyperosmotic stress) results in the movement of water from the cell to the outside, causing a detrimental loss of cell turgor pressure and changing the intracellular solute concentration and cell volume, which ultimately can seriously affect cell viability (458). Probiotic bacteria can develop significant physiological changes to respond to osmotic stress conditions. Expression of the *htrA* gene increased 8-fold in *Lb. helveticus* CNRZ32 in response to exposure to NaCl (160). Similarly, the classical GroES/GroEL chaperone was positively regulated in osmotically stressed *Lb. rhamnosus* HN001 cells (459). Probiotic LAB can also adapt to hyperosmotic conditions by accumulating compatible solutes, such as betaine, carnitine, and trehalose (160, 458, 459).

Probiotic LAB may use enzymes (NADH oxidase, NADH peroxidase, and SOD) or nonenzymatic compounds (Mn^{2+}, ascorbate, tocopherols, and glutathione) to reduce or eliminate the deleterious effects of oxygen radicals (16, 160). For example, *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 can reduce oxygen to H_2O_2 with an NADH-dependent oxidase to eliminate or lower the level of oxygen (460). The same was found for *Lb. plantarum* ATCC 8014 through the oxidation of NADH (160). In a study on *Lb. helveticus* CNBL 1156, the fatty acid composition of the cell membrane changed in response to oxidative stress (461). This change was explained in terms of an increased activity of the oxygen-consuming fatty acid desaturase system, which serves to reduce free radical damage in the cell.

Stress Associated with Intestinal Transit

The principal stresses encountered by probiotic bacteria during passage through the stomach and upper intestinal tract include the low pH in the stomach as well as the detergent-like properties of bile in the duodenum. Exposure to these stresses causes damage to the cell envelope, DNA, and proteins (16). The survival of probiotic lactobacilli in low-pH environments can be increased hugely (several orders of magnitude) in the presence of metabolizable sugars, such as glucose (462). It is thought that glucose, by providing ATP to F-ATPase, enables proton exclusion and hence protection (see above). Other systems to regulate internal pH also exist; for example, *Lb. reuteri* uses both the GAD system and the ADI pathway to combat acid stress (463, 464). Similarly, amino acid decarboxylation combined with an amino acid antiporter leads to the biochemical consumption of protons, thus contributing to acid tolerance during growth (218, 465). The exposure of *Lb. delbrueckii* cells to acidic conditions prevailing in the GIT (pH

3.5 for 60 min) was shown to result in increased expression of the *clpP*, *clpE*, *clpL*, and *clpX* genes. As detailed above, the Clp chaperones actively promote refolding or degradation of damaged proteins (466).

Acid stress can also cause molecular changes in the cell surface. Moreover, bacteria are able to change their membrane lipid composition in response to external stresses. The composition of the cytoplasmic membrane can have a dramatic effect on the ability of probiotic bacteria to survive stresses ranging from heat to low pH. For example, the membranes of acid-adapted cells of *Lb. casei* ATCC 334 and *Lb. johnsonii* NCC 533 showed a significant increase in the ratio of saturated to unsaturated membrane fatty acids (122, 467). In the latter strain, the membrane fatty acid composition (and in particular the saturation level) is associated with changes in cell survival during heat or acid shock.

Probiotic LAB can also sense and develop resistance mechanisms against bile salts. For example, an eight-gene operon encoding a TCS, a transporter, an oxidoreductase, and four hypothetical proteins has been implicated in sensing bile salt in *Lb. acidophilus* (468). Likewise, the genes encoding multidrug transporters driving extrusion of bile salts are upregulated in *Lb. casei* and *Lb. acidophilus* during exposure to subinhibitory concentrations of bile (235). Indeed, mutations in the *lr1584* gene (encoding an MDR transporter) impaired the ability of *Lb. reuteri* ATCC 55730 to reinitiate growth in the presence of bile and to survive in the human small intestine (469). Analogous results in terms of growth and survival were obtained when the LBA0552, LBA1429, LBA1446, and LBA1679 transporters were deleted in *Lb. acidophilus* NCFM (470). Exposure of *Lb. delbrueckii* to 0.1% bile salts increased the expression of *clpP* and *clpE*, which, as we have seen above, are involved in repair/degradation of damaged proteins (466). This is in agreement with the observation that mutations in *lr1864* (*clpL*) in *Lb. reuteri* were associated with reduced survival in the presence of bile (469). Some probiotic LAB can efficiently express a range of bile salt hydrolases (BSH) that confer protection against bile via bile salt deconjugation (471). The cellular response toward bile of the well-documented probiotic *Lb. rhamnosus* GG showed that it can also respond by changing cell envelope-related functions, such as altering pathways affecting fatty acid composition, cell surface charge, and thickness of the EPS layer (236). Production of external EPS layers can have a significant protective effect against bile and other harsh environmental conditions. Indeed, strains producing EPS exhibit significantly more growth in the presence of 0.3% bile than strains that do not (472). Although intrinsic bile tolerance appears to be strain dependent, probiotic strains can be made to adapt progressively to the presence of bile salts by subculture in gradually increasing concentrations of bile (473).

In considering probiotics, it is also important that stationary-phase cells are generally more resistant than log-phase cells to various stressors, since many stress resistance mechanisms are switched on when active growth ceases. This is an important consideration, since probiotic LAB are generally prepared from stationary-phase cultures.

Increasing the Stress Resistance of Probiotic LAB

Understanding the complex stress response networks of probiotic bacteria has allowed the development of a variety of methods to improve their technological and gastrointestinal performance to potentially produce "superfit" bacteria. Some of the approaches aim at improving culture viability under the conditions of spray-drying, a procedure increasingly employed to concentrate and preserve particular probiotic cultures used in functional foods and health supplements. Exposure of *Lb. johnsonii* NCC 533 to acid stress significantly increased ($P < 0.05$) the oleic acid content in the membrane when the growth medium was supplemented with linoleic or linolenic acid, indicating that saturation of the membrane fatty acids occurred during acid stress (467). Thus, supplementation of the growth medium with saturated fatty acids is likely to increase the acid and heat tolerance. Presumably, this effect is due to increased rigidity of the cytoplasmic membrane. Overexpression of HSPs or CSPs in probiotic LAB has also proved successful. The overexpression of the GroES/GroEL chaperone in *Lb. paracasei* increased the strain's ability to better survive both spray-drying and freeze-drying (27). Similarly, overexpression of HSPs in *Lb. plantarum* improved bacterial growth at high (37°C or 40°C) and low (12°C) incubation temperatures (474). Overexpression of CspL, CspP, or CspC improved the performance during temperature downshift of this strain (475). Interestingly, heterologous expression of the BilE transporter in *Lc. lactis* resulted in a 2.5-log enhanced resistance of the modified strains to 1% porcine bile (27). Other authors have emphasized the importance of preconditioning cells with homologous stressors, particularly heat shock prior to spray-drying (453). Bile-adapted strains usually display cross-resistance to a variety of other stress factors. Finally, culture reconstitution conditions should be optimized for each probiotic strain in order to achieve accurate viable probiotic numbers from dried cultures (476).

STRESS RESPONSES AND PATHOGENICITY IN LAB

To colonize and thrive within the mammalian host, bacteria must cope with a variety of environmental insults. These include fluctuations in temperature, pH, and nutrient source and availability, the presence of ROS, reactive nitrogen species (RNS), and antimicrobial peptides generated by professional phagocytic immune cells, and, eventually, the different types of stresses brought about by administered antibiotics. Given that the ability to sense and respond to environmental stresses is an essential survival trait in bacteria, it is not surprising that stress responses are often intertwined with virulence. In the case of pathogenic LAB, which belong mainly to species within the *Streptococcus* and *Enterococcus* genera, multiple examples of stress survival pathways that participate in virulence have emerged in the past 2 decades. They do so directly, by facilitating bacterial survival and dissemination, or indirectly, by controlling the expression of virulence factors. This section focuses on important paradigms of stress regulators as well as stress-adaptive mechanisms that have been linked to virulence in streptococci and enterococci (Table 1).

General Stress Responses and Virulence

As already discussed above, general stress responses are triggered by different stress conditions that result in the accumulation of misfolded proteins, inducing DnaK, GroEL, ClpP, and HtrA. The genes encoding the ClpP peptidase and the Clp ATPase partners are by far the best-studied streptococcal stress genes in terms of virulence potential. The virulence of *S. pneumoniae* Δ*clpP* strains was attenuated in mice, regardless of the administration method (477–479). The core genome of *S. pneumoniae* encodes four Clp ATPases (ClpC, ClpE, ClpL, and ClpX), although ClpL does not have the recognition tripeptide responsible for the interaction

TABLE 1 Stress genes affecting the virulence of pathogenic streptococci and enterococci

Pathogen	Mutation	Stress defense mechanism	Animal model/administration	Reference
Streptococci				
S. agalactiae	Δ*clpP*	Molecular chaperone/protease	Mouse/intravenous	485
	Δ*sodA*	Oxidative stress (ROS scavenger)	Mouse/intravenous	521
	Δ*nox*	Oxidative stress (O_2 reduction)	Mouse/intranasal	515
	Δ*cydA*	Oxidative stress (respiration)	Neonatal rat/intraperitoneal	517
S. mutans	Δ*fabM*		Rat caries/oral swab	542
	Δ*clpP* or Δ*clpX*	Molecular chaperone/protease	Rat colonization/oral swab	304
	Δ*spxA1*	Oxidative stress regulator	*G. mellonella*/systemic	346
	Δ*spxA1* Δ*spxA2*	Oxidative stress regulator	Rat colonization/oral swab	346
S. pneumoniae	Δrel_{Spn}	Stringent response	Mouse/intranasal	126
	Δ*codY*	Nutrient-sensing regulator	Mouse/intranasal	549
	Δ*htrA*	Molecular chaperone/protease	Mouse/intranasal	487, 488
	Δ*htrA*	Molecular chaperone/protease	Mouse/subcutaneous	489
	Δ*clpP*	Molecular chaperone/protease	Mouse/intranasal	479
	Δ*clpP*	Molecular chaperone/protease	Mouse/intratracheal and intraperitoneal	477
	Δ*clpP*	Molecular chaperone/protease	Mouse/intraperitoneal	478
	Δ*clpE*	Molecular chaperone/protease	Mouse/intraperitoneal	480
	Δ*nox*	Oxidative stress (O_2 reduction)	Mouse/intranasal	514
	Δ*sodA*	Oxidative stress (ROS scavenger)	Mouse/intranasal	519
	Δ*dpr*	Oxidative stress (Fe^+ chelator)	Mouse/intranasal	527
	Δ*tpxD*	Oxidative stress (ROS scavenger)	Mouse/intranasal	522
	Δ*ahpCF*	Oxidative stress (ROS scavenger)	Mouse/intranasal and intraperitoneal	523
	Δ*ccpA*	Nutrient-sensing regulator		545
	Δ*ccpA*	Nutrient-sensing regulator		546
S. pyogenes	Δ*gpoA*	Oxidative stress (ROS scavenger)	Mouse/subcutaneous and intraperitoneal	524
	Δ*perR*	Oxidative stress regulator	Mouse/subcutaneous	530
	Δ*perR*	Oxidative stress regulator	Mouse/subcutaneous and intraperitoneal	529
	Δ*perR*	Oxidative stress regulator	Baboon/posterior pharynx	531
	Δ*ahpC*	Oxidative stress (ROS scavenger)	Mouse/intranasal, intravenous, or subcutaneous	523, 529
	Δ*htrA*	Molecular chaperone/protease	Mouse/intraperitoneal	489
	Δ*ccpA*	Nutrient-sensing regulator	Mouse/throat swab	544
	Δ*ccpA*	Nutrient-sensing regulator	Mouse/vaginal swab	548
S. sanguinis	Δ*spxA1*	Oxidative stress regulator	Rabbit/intravenous (endocarditis)	348
S. suis	Δ*sod*	Oxidative stress (ROS scavenger)	Mouse/intraperitoneal	520
	Δ*perR*	Oxidative stress regulator	Mouse/intraperitoneal	532
	Δ*spxA1*	Oxidative stress regulator	Mouse/intraperitoneal	347
	Δ*ccpA*	Nutrient-sensing regulator	Mouse/intraperitoneal	547
	Δ*tig*	Molecular chaperone	Mouse/intraperitoneal	493
Enterococci				
E. faecalis	Δ*clpB*	Chaperone	*G. mellonella*/systemic	308
	Δ*gls24*	General stress protein	Mouse/peritonitis and endocarditis	494
	Δ*sodA*	Superoxide dismutase	Mouse macrophages and microglia	254, 525
	Δ*tpx*	Peroxidase	Mouse macrophages	248
	Δ*npr*, Δ*tpx*, or Δ*ahpCF*	Peroxidase	Mouse macrophages	248
	Δ*msrA* or Δ*msrB*	Methionine sulfoxide reductase	Mouse/systemic or urinary tract infection	526
	Δ*perR*	Regulator	Mouse/peritonitis	94
	Δ*hypR*	Regulator	Mouse macrophages or peritonitis	532, 533
	Δ*spx*	Oxidative stress regulator	Mouse/intraperitoneal	535
	Δ*asrR*	Regulator	*G. mellonella* or mouse macrophages	536
	Δ*ers*	Regulator	Macrophage infection	537
	Δ*relA* Δ*relQ*	Stringent response	*C. elegans*/bacterial lawn, *G. mellonella*/systemic, or rabbit abscess/intravenous	124, 551, 552
E. faecium	Δ*gls33-glsB* Δ*gls20-glsB1*	General stress protein	Mouse peritonitis	496
	Δ*nox*::Tn*917*	NADH oxidase	*C. elegans*	516
	Δ*ccpA*	Regulator	Rat endocarditis	550

with ClpP; thus, it functions mainly as a chaperone. For an *S. pneumoniae* mutant lacking ClpE, virulence was strongly reduced in a mouse peritonitis model (480). Transcriptomic and proteomic analyses indicated that ClpE affects pneumococcal pathogenesis by modulating the expression of important virulence determinants and metabolism-related factors (480). Despite participating in several important physiological processes (481, 482), ClpC is apparently not required for pneumococcal virulence

(477, 479), while inactivation of *clpX* is lethal (303). Although ClpL is required for growth at 43°C and for penicillin tolerance (478, 483), an *S. pneumoniae* Δ*clpL* mutant showed a level of virulence similar to that of the parental strain in a murine intraperitoneal infection model (478). Systematic deletion of *clpP* and the five Clp ATPase genes (*clpB*, *clpC*, *clpE*, *clpL*, and *clpX*) in the dental pathogen *S. mutans* revealed that the ClpXP proteolytic system modulates the expression of several virulence-related traits (304, 484) and that the infectivity of strains lacking *clpP* or *clpX* is slightly reduced in a rat model of oral colonization (304). Inactivation of the remaining Clp ATPases did not affect tooth colonization, but the role of the *S. mutans* Clp proteins in caries development or in infective endocarditis remains to be evaluated further. Virulence of a mutant GBS, *S. agalactiae* lacking *clpP*, was not attenuated in mice inoculated intravenously (485); the roles of ClpP and associated Clp ATPases in GAS virulence remain to be established. The involvement of Clp ATPases has not been studied extensively in enterococci. *E. faecalis* carries genes for ClpP, ClpB, ClpC, ClpE, and ClpX (214). Deletion of *clpB* reduced the ability of *E. faecalis* to acquire thermotolerance and for pathogenesis as assessed in *Galleria mellonella* larvae (308).

Another highly conserved bacterial protein associated with the pathogenic potential of streptococcal species is HtrA. Deletion of *htrA* in *S. pneumoniae* significantly reduced nasopharyngeal colonization (486) and virulence in mouse models of pneumonia and bacteremia (487, 488). The 50% lethal dose (LD_{50}) for an *S. pyogenes htrA* mutant in mice was 35-fold higher than that for the parent strain (489). A subsequent study revealed that HtrA is responsible for processing secreted virulence factors, such as the SpeB cysteine protease and streptolysin S (490). However, an HtrA-deficient GAS mutant was not attenuated in a murine model of subcutaneous infection (490).

Additional general stress proteins have been implicated in stress responses and virulence of LAB. The trigger factor protein, a ribosome-associated molecular chaperone functioning in cooperation with the DnaK and GroEL chaperones, has been associated with stress tolerance in different streptococcal species (491–493). For the zoonotic pathogen *S. suis*, a trigger factor-deficient strain was attenuated in a mouse peritonitis model (493). Two general stress proteins, Gls24 and GlsB, have been identified in *E. faecalis* (494). Deletion of *gls24* led to decreased lethality in a mouse peritonitis model and to sensitivity to bile salt. Deletion of *glsB* did not influence virulence but resulted in sensitivity to bile salt. Similar conclusions concerning the involvement of Gls24 and GlsB in pathogenicity were also reached in a mouse endocarditis model (495). Two paralogous *gls24-glsB* loci (*gls33-glsB* and *gls20-glsB1*) were identified in *E. faecium*. Only deletion of both loci resulted in reduced pathogenicity, while sensitivity to bile stress was observed in the double mutant and each of the single mutants (496).

Global Transcriptional Regulators and Virulence

Global transcriptional regulators, such as quorum sensing-activated regulators and serine-threonine protein kinases, which regulate a variety of cellular processes, including stress survival, have also been shown to mediate streptococcal virulence. The universal LuxS QS system, which produces the furanosyl borate diester AI-2, regulates a large panel of genes in a variety of microorganisms. By *luxS* inactivation in GAS, it was demonstrated that loss of AI-2 signaling leads to increased acid tolerance, increased cell invasion, and increased intracellular survival (152, 497). However, the exact role of the LuxS/AI-2 system in GAS pathogenesis remains to be elucidated. An *S. pneumoniae luxS* mutant could colonize the nasopharynx of mice as efficiently as its parent, but it was impaired in the ability to disseminate to distant sites, such as the lungs, and was less virulent in a peritonitis mouse model (208). Inactivation of the *luxS* gene in *S. suis* increased tolerance to H_2O_2 but negatively affected capsule production and adherence to epithelial cells (498). As a result, the *luxS* mutant was dramatically attenuated in a piglet model (498).

Transcriptome studies have highlighted the importance of several TCSs of GAS in coordinating the expression of genes involved in stress adaptation and virulence (499–502). In particular, the TCS CovSR was shown to regulate approximately 15% of the genome of *S. pyogenes* and is required for growth under general stress conditions (503). In a murine model of nasopharyngeal colonization, the CovSR system was shown to be required for *S. pyogenes* for optimal infection and transmission from the nasopharynx (504). Likewise, *S. pyogenes covR* mutants caused significantly less invasive disease and death in mice than those seen with the wild-type strain, although the local lesions produced by the *covR* mutants were more severe and purulent (505). A GBS mutant of the CovSR ortholog, also known as CsrS/CsrR, displayed impaired viability in human serum and attenuated virulence in murine infection models (506, 507). It has been hypothesized that the translocation of GBS from the acidic milieu of the vagina to neonatal tissues with a neutral pH signals the conversion from a colonizing to an invasive phenotype in a CsrRS-dependent manner (508). The CiaHR TCS of *S. pneumoniae* regulates many genes involved in competence development and virulence, including *htrA* (see above) (486). Virulence of an *S. pneumoniae ciaR* mutant in mice was significantly attenuated (509). As described above, HtrA has been implicated in virulence by influencing the ability of pneumococci to colonize the nasopharynx of rats (486, 487). Notably, the reduced virulence of the *ciaR* mutant could be restored by increasing the expression of HtrA (509). The *S. suis* CiaHR ortholog was also shown to contribute to virulence in mice and pigs (510).

In addition to TCS signaling systems, an alternative regulatory pathway involving reversible phosphorylation is controlled by the eukaryote-type serine/threonine kinase (Stk). Stk of *S. pyogenes* activates genes involved in osmoregulation, fatty acid and cell wall synthesis, and virulence (511). An *S. pyogenes stk* deletion mutant was more sensitive to penicillin and osmotic stress than the wild-type strain, and Stk was required for optimal virulence (511, 512). An *S. suis stk* mutant exhibited reduced tolerance to a multitude of stresses and showed attenuated virulence in animal models (513).

Oxidative Stress and Virulence

Bacteria are constantly exposed to oxidative stress of either an intracellular or extracellular origin. Of particular relevance to bacterial virulence is the organism's ability to evade the oxidative onslaught of phagocytic immune cells by using a combination of reducing enzymes, ROS scavengers, and protein and DNA repair enzymes. Thus, it is not surprising that the virulence of strains with defective oxidative stress responses is often attenuated or totally abolished. Streptococci and enterococci are generally considered unable to carry out oxidative phosphorylation. Instead, the bulk of oxygen metabolism in streptococci is due to the NADH oxidase Nox, which reduces oxygen, one electron at a time, to water through the oxidation of NADH to NAD^+. If not fully reduced by Nox, environmental oxygen can form toxic superoxide

and H_2O_2. Strains of GBS and pneumococcus lacking Nox were unable to grow under vigorous agitation conditions and showed attenuated virulence in animal models (514, 515). An *E. faecium* Tn*917* transposon insertion mutant of *nox* showed a 98% reduction of NADH oxidase activity (516). The mutated strain was defective in nematode killing as a result of less H_2O_2 production than that in the wild type. The possible involvement in the observed phenotype of the gene downstream of *nox* through a polar effect could not be ruled out given the inability to perform a complementation analysis of *nox*.

The genome of GBS encodes a cytochrome *bd* oxidase (CydABCD), and the organism can engage in respiratory metabolism when the environment provides quinone and heme (517). Inactivation of *cydA* negatively affected GBS growth in blood and strongly attenuated virulence in a neonatal rat sepsis model (517). The animal pathogen *S. uberis* also contains cytochrome *bd* oxidase; since it lacks quinone and heme biosynthesis pathways, it is thought to use a similar strategy to activate respiration. *E. faecalis* also carries a cytochrome *bd*-type respiratory oxidase (518), but its involvement in virulence has not been investigated.

Streptococci lack catalase but encode several other detoxification enzymes, including SOD and up to three peroxidases. For GBS, *S. pneumoniae*, and *S. suis*, *sod* mutants were more susceptible to oxidative stresses and showed attenuated virulence in different murine infection models (519–521). The contributions of alkylhydroperoxidase (AhpCF) and thiol peroxidase (Tpx) to streptococcal virulence have been assessed only for *S. pneumoniae*. Inactivation of *tpx* impaired pneumococcal virulence in mice infected intranasally but not in those in which the bacteria were administered directly into the bloodstream (522). These results most likely can be explained by the differences in oxygen tension in the nasopharynx (high) and blood (low). Loss of alkylhydroperoxidase activity attenuated pneumococcal virulence in cutaneous, pneumonia, and bacteremia models (523). The third characterized streptococcal peroxidase, glutathione peroxidase (GpoA), is essential for GAS pathogenesis in several murine models that mimic suppurative diseases but not in a zebrafish streptococcal myositis model characterized by the absence of inflammation (524). In contrast to streptococci, *E. faecalis* contains the heme-dependent catalase KatA, which is activated by heme taken up from the environment (259); its implication for virulence has not yet been investigated. Deletion of *E. faecalis sodA* attenuated resistance to H_2O_2 and survival in mouse macrophages (254) and microglia (525). *E. faecalis* also contains three peroxidases: Npr, AhpCF, and Tpx (248). All three are involved in resistance to H_2O_2, to various degrees, while survival in murine macrophages was mostly affected in the Δ*tpx* mutant and the peroxidase triple mutant. Two additional antioxidant repair enzymes, the methionine sulfoxide reductases MsrA and MsrB, have been characterized for *E. faecalis* (526). Mutants carrying a deletion in the respective genes exhibited sensitivity to H_2O_2 and attenuated virulence in systemic and urinary tract infection models.

In addition to reducing enzymes and molecular scavengers, the DNA-binding protein Dpr (also known as MrgA in GAS) is another major player in oxidative stress responses. Dpr provides protection against oxidative stress by binding to iron and thereby preventing the Fenton reaction. An *S. pneumoniae* Δ*dpr* strain displayed a reduced colonization ability and was more rapidly cleared from the nasopharynxes of infected animals (527). Despite the *in vitro* hypersensitivity to peroxide (528), loss of Dpr did not affect GAS virulence in zebrafish or in different murine infection models (529).

Two ubiquitous LAB oxidative stress regulators have been shown specifically to be required for streptococcal and enterococcal virulence. The PerR metalloregulator, also involved in iron homeostasis, has been studied extensively in GAS. Virulence of *perR* mutants was significantly attenuated in a variety of murine models as well as in a baboon model of GAS pharyngitis (529–531). Likewise, the pathogenicity of an *S. suis perR* mutant was attenuated in a mouse peritonitis model (532). Deletion of *perR* in *E. faecalis* increased resistance to H_2O_2 but did not cause major alterations in the expression profile of eight genes related to oxidative stress (94). The *perR* mutant survived as well as the wild type in murine macrophages, but it was less lethal in a mouse peritonitis model. These findings indicate that although PerR plays a role in the virulence of *E. faecalis*, its involvement in the oxidative stress response of this bacterium may be different from that in *B. subtilis* (94). *E. faecalis* HypR was identified as a regulator of the oxidative stress response and as a virulence factor (533). It is a transcriptional activator of several genes during oxidative stress, including *ahpCF*, *tpx*, *ef3270* (encoding a glutathione reductase), *sodA*, and *katA* (248, 534). An *E. faecalis hypR* mutant was highly sensitive to H_2O_2 and showed a reduced ability to survive in macrophages and a decreased lethality in a mouse peritonitis model (533, 534). Recent studies have shown that streptococcal genomes encode two copies of the redox-sensing Spx regulator, designated SpxA1 and SpxA2. In *S. mutans*, loss of *spxA1*, *spxA2*, or both attenuated virulence in the *G. mellonella* invertebrate model (346), while the ability to colonize the teeth of rats was significantly impaired in the Δ*spxA1* mutant and the double mutant but not in the Δ*spxA2* mutant (346). SpxA1 and SpxA2 were shown to modulate stress tolerance in *S. suis*, and loss of *spxA1* or *spxA2* significantly reduced the ability of *S. suis* to survive in pig blood and to disseminate to different tissues (347). In a rabbit model of infective endocarditis, a *Streptococcus sanguinis* Δ*spxA1* strain exhibited an approximately 5-fold reduction in competitiveness in a competitive index assay (348). Although SpxA2 has also been implicated in the stress tolerance of *S. sanguinis*, its relevance *in vivo* has not been investigated. *E. faecalis* carries only one *spx* gene, and when it was deleted, the mutant strain showed defective growth under aerobic conditions and sensitivity to oxidative stress agents (535). The deletion mutant was also susceptible to killing in murine macrophages and was less efficient at colonization and dissemination in a murine peritonitis model. AsrR (antibiotic and stress response regulator), an oxidative sensing regulator of the MarR family, was recently described for *E. faecium* (536). The AsrR regulon consists of genes involved in pathogenesis, antibiotic resistance, oxidative stress, and adaptive responses. An *E. faecium* Δ*asrR* strain showed greater persistence in *G. mellonella* colonization and mouse systemic infection models but a lower survival rate in murine macrophages. Deletion in *E. faecalis* of the gene encoding the PrfA-like regulator Ers (enterococcal regulator of survival) resulted in sensitivity to H_2O_2 and decreased survival in an *in vivo-in vitro* macrophage infection model (537).

Acid Stress and Virulence

Pathogenic streptococci must be able to withstand the low pHs encountered upon the formation of necrotic lesions or abscesses. In addition, cariogenic streptococci, such as *S. mutans*, need to cope with sustained acidification of their environment as a result

of the production of lactic acid from fermentable sugars by LAB residing in plaque. Because of the direct linkage between low-pH survival and dental caries and the ease of genetic manipulation, *S. mutans* has become the bacterial paradigm for LAB acid stress responses (538). Notwithstanding this, the number of investigations that directly associate *S. mutans* acid stress survival pathways with caries formation is still rather limited. In addition to the aforementioned *in vivo* studies with Clp chaperones/proteases and the Spx oxidative stress regulators, which are also important for acid stress survival (304, 346), several caries studies were carried out using *S. mutans* strains lacking different TCS regulators or the PknB serine/threonine kinase (539–541). An *S. mutans fabM* mutant did not produce unsaturated membrane fatty acids, was extremely acid sensitive, was poorly transmitted from host to host, and exhibited fewer and less severe carious lesions than those caused by the parent strain (542, 543).

Starvation and Virulence

Pathogenic LAB often have to face severe starvation or intermittent nutrient availability, depending on the infection site. As mentioned above, LAB have developed sophisticated regulatory networks that combine transcriptional regulation with allosteric modulation of enzymatic activities to coordinate nutrient biosynthesis and acquisition. They utilize nutrient-sensing regulators controlling the expression of genes involved in adaptation to starvation as well as those encoding *bona fide* virulence factors, such as toxins, proteases, and adhesins. The roles in streptococcal virulence of the key nutrient stress-responsive regulators CcpA and CodY and of the (p)ppGpp alarmone have been tested in *in vivo* models. The *ccpA* mutants of the GAS *S. pneumoniae* and *S. suis* were significantly less virulent than their parents in different mouse models of infection (544–548). To date, studies on the roles of CodY and (p)ppGpp in streptococcal virulence are restricted to *S. pneumoniae*. A CodY-deficient strain poorly colonized the nasopharynx of mice, a finding that was supported by its diminished ability to adhere to nasopharyngeal cells *in vitro* (549). An *S. pneumoniae* mutant lacking Rel, the major (p)ppGpp synthetase activating the stringent response, was severely attenuated and displayed a significantly altered course of disease progression in a murine pneumonia model (126). Deletion of *ccpA* in *E. faecium* also resulted in attenuated virulence in a rat endocarditis model (550). The levels of (p)ppGpp in *E. faecalis* correlated with stress responses, antibiotic resistance, and virulence (123, 124, 130). An *E. faecalis* mutant lacking Rel (RelA), the main one of the two (p)ppGpp synthetases in this organism (see above), exhibited altered behavior with respect to different stress conditions and antibiotic resistance, but its pathogenicity was similar to that of the wild type (123, 124). Only the double *relAQ* mutant showed diminished pathogenic potential in *Caenorhabditis elegans* and *G. mellonella* invertebrate models and impaired growth and survival in a rabbit subdermal abscess model (124, 551, 552).

PHAGE INFECTIONS AND LAB STRESS PHYSIOLOGY

Bacteriophage attacks represent a constant threat to the dairy industry and are the main cause of delayed, inferior, or even failed milk fermentations. The issue is exacerbated in modern dairy facilities, where a small number of defined starter cultures are responsible for the continuous transformation of large volumes of milk. Dairy phages may originate from endogenous or exogenous sources (553, 554). Notably, many LAB strains are lysogenic, i.e., they contain an integrated copy of one or more temperate phages (or prophages) in their genomes. These prophages may be activated or induced under particular conditions of physiological/environmental stress, resulting in prophage excision from the host chromosome followed by phage DNA replication and virion production, ultimately causing bacterial cell lysis.

Despite their undesirable properties, prophages are nonetheless important genetic elements that modulate bacterial genome evolution (555). In fact, it has been demonstrated that phage DNA represents a sizeable part of the bacterial genome, reaching up to 5% of bacterial DNA content in particular LAB species, such as *Lc. lactis* subsp. *cremoris* MG1363 (556). Furthermore, phages catalyze horizontal gene transfer between bacteria (555) and may be crucial providers of new genetic traits that increase the ecological fitness of the host (555, 557, 558). Host-parasite relationships constitute an arms race that forms an unstable genetic equilibrium where advantages based on host fitness-enhancing prophage genes are short-lived if the prophage ultimately kills its host (555, 559).

Phage Induction

Activation of the so-called SOS response causes induction of several lambdoid lysogens (560). Extrinsic stress factors, such as ROS, UV radiation, and various chemicals, all induce DNA damage and DNA replication arrest, thereby instigating the SOS response and, consequently, prophage excision and lytic activation. Much of the current literature on phage induction relates to phage lambda: when RecA becomes activated, it triggers the SOS response and catalyzes autoproteolysis of the lambda CI repressor, thereby causing a switch from a lysogenic to a lytic life cycle (561). As in lambda, genomes of temperate LAB phages carry a genetic switch region encompassing divergently oriented promoters that are controlled by analogs of the CI and Cro proteins (562–566).

Further factors that influence lysogenic maintenance or phage induction in LAB include various environmental stresses, such as pH, oxidative stress, temperature fluctuations, osmotic pressure, and low nutrient concentration, as well as the presence of toxic compounds, such as fluoroquinolones. The effects of the latter compounds were studied in *Streptococcus* spp., where they were shown to induce phages via an SOS-dependent, i.e., RecA-mediated, route due to a response to a topoisomerase IV-fluoroquinolone complex (567).

The effects of extracytoplasmic stress on prophage induction are, however, not well understood, especially for polylysogenic hosts (568). In this context, for phage LC3, which infects *Lc. lactis* strain 3107, it was shown that a decrease in pH caused a reduction in the level of phage induction (569). It has been postulated that changes in the intracellular pH affect the stability and binding features of LexA and CI repressors and thus influence the transcription of their target genes (570). In contrast, various other stressors (e.g., exposure to high temperature or high osmotic pressure or being in late stationary phase) did not provoke phage induction in *Lc. lactis* (571), suggesting that the control system that keeps lactococcal prophages in their lysogenic state is very tight and that stressors which commonly occur in the industrial environment of *Lc. lactis* do not influence phage induction (571).

Stress Mediated by Phage Infection

When a phage particle infects a bacterial cell, it causes major changes in the host transcriptome that are considered phage infection stresses. In this context, phage infection of *Lc. lactis* IL1403 is perceived as a

membrane stress by the host, triggering adjustments in the cell envelope (572). This is believed to be orchestrated by the action of membrane-associated, phage shock-mediated protein C-like homologs, the global regulator SpxB, and the two-component regulatory system CesSR (572). Analysis of the host transcriptome response to infection by the lactococcal phages Tuc2009 and c2 revealed the induction of genes involved in metabolic flux and energy production, in cell wall modification, and in the conversion of ribonucleotides to deoxyribonucleotides (572, 573).

STRESS PHYSIOLOGY OF LAB IN THE POSTGENOMIC ERA

The Genomic Acceleration

In 1995, the first genomic sequence of the free-living microbe *Haemophilus influenzae* was published (574), launching the genomic era of molecular microbiology. Although the initial focus of microbial genome sequencing was centered on pathogenic bacteria, advances in high-throughput and low-cost sequencing technologies over the past decade have expanded the genomic perspective to a large variety of microbes relevant to biotechnology as well, including LAB. The first LAB genomes were published in 2001 (575). The 2.4-Mbp *Lc. lactis* subsp. *lactis* strain IL1403 genome was predicted to encode 2,310 proteins. The observed close relatedness of *Lc. lactis* to the streptococcal genus was further supported by the determination of the genome of the yogurt bacterium *S. thermophilus* (576). A comparison of the genomes of representatives of the *Lactobacillus* genus, *Lb. plantarum* (213) and *Lb. johnsonii* (319), exemplified the high degree of diversity within this genus (577), while the genome of *Lb. acidophilus* (201) underlined the relatively high relatedness of subgroups within this genus, i.e., the "acidophilus complex."

Comparative genomics of LAB took a strong step forward by the comparative analysis of nine genome sequences of various LAB species, including the definition of the so-called LaCOGs, which represent an LAB-specific refinement of the existing categories of orthologous genes (COGs). The distributions of LaCOGs among the genomes of these nine LAB allowed analysis of their genome-wide evolutionary relatedness (6). Comparative genomics also enabled the comparative analysis of specific subsets of functions, e.g., the secretome, encompassing all proteins exported to the cell surface and thus playing a direct role in interaction with the environment (578–580) and contributing significantly to stress tolerance (see below).

These species comparisons were followed by comparative genomic analyses of strains within a species, initially employing comparative genome hybridization (CGH) approaches for different LAB. At present, it is quite common to determine genome sequences for multiple strains of an LAB species (581–583). As a consequence, the GenBank database contains more than 100 complete LAB genomes and an even larger number of partial genome sequences, representing LAB isolated from a variety of environmental sources and encompassing more than 50 LAB species. This wealth of information requires accurate and effective bioinformatic mining strategies to evaluate the relatedness and evolutionary relationships of strains, but also to analyze specific gene categories, such as those associated with stress responses and robustness. Notably, a variety of biotechnologically important functions are known to be encoded on plasmids (584–587), underlining that the evaluation of the genetic repertoire of LAB should pay appropriate attention to these extrachromosomal genetic elements.

Comparative Genomics of the LAB Stressome

The original LaCOG assignments enabled effective mining of LAB genomes for genes associated with stress response functions (i.e., the LAB stressome) and their regulators (588). Most LAB encode the typical HrcA regulator that acts as a repressor of the class I HSPs, encompassing the universally conserved chaperonin functions (GroELS, DnaJK, and GrpE) but in various LAB also including additional chaperones, such as HtpX and HSP20 (IbpA). As mentioned above, a notable exception is *O. oeni*, which appears to lack an *hrcA* gene, and class I HSP regulation in this species has been proposed to be controlled by complex regulatory networks involving multiple regulators (589). The involvement of CtsR in regulation of class III stress proteins, including the Clp proteases and related functions, is predicted for many LAB, although *ctsR* appears to be absent in several LAB genomes, such as those of *L. mesenteroides* and "acidophilus complex" lactobacilli. Genes for the class III-associated regulon Clp proteases (ClpC, -X, -Q, and -P) are present in most LAB genomes, whereas the presence of other Clp-encoding genes (*clpYQ*; also designated *hslUV*) is more variable (588). Protection against oxidative stress in LAB involves a variety of functions that are highly variable among the LAB species and strains (including many oxidases, peroxidases, catalases, and superoxide dismutases [see above]). Nevertheless, a central role in oxidative stress tolerance has been proposed for the strongly conserved thioredoxin system (TrxA and TrxB) (588, 590, 591). Many environmental stress conditions can lead to DNA damage, and DNA damage responses also appear to be strongly conserved among LAB, including functions associated with homologous recombination and double-strand-break repair (RecABDFJNOR, RuvAB, and Ssb), the homology-independent facilitator complex (GyrAB and TopA), and the pathway involved in base excision repair (Mfd, UvrABCD, and Xth), although the endonuclease IV (Nfo) that is important in this pathway is absent in many species (588). Notably, the highly conserved MutS and MutL functions that are involved in DNA mismatch repair appear to be lacking in *O. oeni*, which also lacks the related RecQ-encoding gene. The latter gene is also absent in *S. thermophilus*, which has been suggested to be related to the high frequency of pseudogenes and function loss observed in this species (250, 576). Notably, this typical genome decay is a characteristic that *S. thermophilus* shares with its protocooperative partner in yogurt fermentations, i.e., *Lb. delbrueckii* subsp. *bulgaricus*, and it has been proposed to be a consequence of their extensive adaptation to the nutrient-rich milk environment (320).

These examples illustrate the acceleration of our understanding that is promoted by (comparative) genomics, identifying both conserved and different mechanisms of stress responses in LAB that contribute to their robustness and survival under the challenging conditions encountered in industrial applications. However, an important restriction of these approaches is that genomic sequences provide no more than a blueprint of the encoded capacities. Experimental verification of predicted functions in stress responses is required to establish the roles of gene regulatory networks and the effector functions they control in stress tolerance and robustness.

Functional Genomic Approaches To Unravel the LAB Stressome

DNA microarrays have been employed intensively to identify genetic factors regulated during stress exposure of LAB. The major-

ity of studies have been performed under laboratory conditions in which a single stressor is applied. Recently, these transcriptome studies have been complemented with several proteome studies. Specific studies also report the responses to multiple individual stresses or combined stresses in single strains or several strains of an LAB species. Such multiple "stressomics" approaches are highly valuable for comprehensive stress response analyses, as they not only identify the genes directly involved in robustness and/or stress survival but also can reveal the regulatory networks involved. Moreover, the availability of several transcriptome profiles for a strain grown under different (stress-related) conditions (592, 593) allows identification of genes that are coregulated. This information can then be used to reconstruct gene regulatory (stress) networks and regulons (351). An alternative approach to identifying stress regulons is the transcriptome analysis of regulator mutants, with sometimes highly revealing results. For example, individual mutations of the regulator genes *hrcA* and *ctsR* of *Lb. plantarum* WCFS1 (see above) led to loss of regulation of their known regulon members but also significantly changed the expression of a variety of genes associated with transport and binding of sugars and other compounds, primary metabolism, transcription regulation, capsular polysaccharide biosynthesis, and fatty acid metabolism. These findings are in good agreement with global proteome analyses of *Lb. plantarum* WCFS1 and its Δ*ctsR* derivative under optimal and heat stress conditions (594). An even more pleiotropic effect on the genome-wide transcription profile was observed in a strain deficient for both *hrcA* and *ctsR*, which illustrates the complexity of and cross talk between the gene regulatory networks influenced by these stress-associated regulators (318).

Since HrcA and CtsR act as repressors of transcription of class I and class III stress responses, respectively, the deletion of their genes might be assumed to result in strains that are more stress tolerant. Indeed, a higher stress robustness of *ctsR* deletion mutant derivatives has been reported for several LAB. For example, compared to their cognate wild-type strains, an *S. thermophilus ctsR* mutant was more tolerant to heat stress during exponential growth (595), and a *ctsR*-deficient *Lb. plantarum* strain could resist higher levels of oxidative stress (318). On the other hand, mutation of *ctsR* in *S. thermophilus* increased osmotic and oxidative stress sensitivity (595, 596), while an *Lb. plantarum ctsR* mutant was more sensitive than its parent to ethanol and heat stresses (318). These results clearly illustrate the unpredictability of the impact in LAB of deregulating stress responses, especially multiple stress regulons at the same time. This is most likely related to the pleiotropic effects on gene transcription in these (multiple) regulator mutants, which may affect many phenotypes other than stress tolerance (318).

The unpredictable nature of the stress robustness phenotype with enhanced stress regulon expression (e.g., as seen in the *hrcA* and *ctsR* mutants) is in apparent contradiction with the observation that preexposure to specific sublethal stress conditions can induce adaptation responses that protect against various subsequent severe stresses (451). These cross-protective effects are probably related to similarities in the molecular damage caused by different stresses to DNA, protein, or cell envelope components. This assumption implies that cross-protection depends on the induction of generic damage protection and repair functions, such as those encoded within the class I and class III stress regulons. For example, the adaptation of *Lc. lactis* to sublethal acid stress induced improved robustness under subsequent heat, ethanol, oxidative, and osmolality stresses (597). Cross-protective stress responses can be employed to design preadaptation approaches for the production of robust LAB with improved survival and functional properties under industrial application conditions. Genomics-based approaches have started to shed light on the genes and regulatory mechanisms involved and may thereby accelerate the exploitation of such preadaptation strategies.

Near-zero growth rates can be induced by carbohydrate-limited cultivation in a retentostat (an adjusted chemostat that retains biomass by use of a cross-flow filter in the effluent line). This setup forces microbes into a state where cellular physiology and metabolic energy are reoriented from growth-associated processes toward those dedicated to maintenance (598, 599). Retentostat studies have been reported for LAB, for example, *Lc. lactis* (600) and *Lb. plantarum* (601). Transcriptome studies of *Lc. lactis* adaptation to retentostat conditions not only confirmed that the expression of metabolic genes was adjusted to sustain zero-growth physiology (600, 602) but also revealed the induction of general stress responses, including derepression of the HrcA and CtsR regulons. These results showed that a tight correlation exists in this organism between growth rate and stress robustness (603). Stress exposure experiments employing severe heat, acid, and oxidative stress treatments confirmed that near-zero-growth cultures of *Lc. lactis* displayed strongly enhanced stress robustness compared to faster-growing cells. Moreover, mathematical modeling defined quantitative relationships between growth rate, stress robustness, and expression of stress genes (603). This work illustrated the congruency of the intrinsic stress regulation network in *Lc. lactis* with the generic evolutionary trade-off between growth and survival (604). In this context, it is important that protein turnover is considered one of the most important metabolic costs in bacterial cells, including *Lc. lactis* (605), and that generic stress responses play important roles in the capacity of cells to maintain or restore the functionality of cellular components, for example, by the refolding capacity of chaperonin complexes, such as GroELS and DnaKJ. This consideration explains the biological relevance of stress response induction in near-zero-growth cultures of *Lc. lactis*, which are entirely focused on maintenance-associated processes and benefit from these stress responses by reducing protein turnover costs. Although the activation of general stress responses under near-zero-growth conditions appears to be conserved among some microorganisms, it appeared to be absent in others, e.g., *Lb. plantarum*. In the latter, predominantly SOS responses are induced, which may enhance mutation rates in these microbes rather than their intrinsic robustness (606). These recent studies illustrate how an improved (genomics-driven) understanding of the regulatory networks involved in stress response regulation can help in the design of industrial approaches to harness the intrinsic adaptive responses of LAB for enhanced industrial robustness.

Applications of Functional Genomic Technologies *In Situ*

The data described above were generated with simplified laboratory models that do not fully mimic the physicochemical complexity encountered during industrial fermentation and processing (607, 608) or the multitude of stresses encountered in the GIT (609). Therefore, *in situ* transcriptome and proteome profiling studies are valuable, complementary approaches to identify factors contributing to the functionality of LAB under these circumstances. A proteomic approach was used to unravel, e.g., the adap-

tive behavior of *S. thermophilus* during milk fermentation. That study revealed a strong regulation of sugar metabolism pathways and induction of the transport and biosynthetic pathways for sulfur-containing amino acids (610). Despite the insight generated, the study did not reflect the most predominant industrial application of *S. thermophilus*, namely, coculture with *Lb. delbrueckii* subsp. *bulgaricus* during yogurt production. Therefore, the same group also reported on the *S. thermophilus* gene expression profiles in milk during coculture with *Lb. delbrueckii* (611), whereas a complementary transcriptome study proposed that formic and folic acid production by *S. thermophilus* and the proteolytic capacity of *Lb. delbrueckii* provide the metabolic dependencies that stabilize this classic industrial coculture fermentation system (612). Analogously, recent studies reported on the global transcriptome and proteome profiles of *Lc. lactis* and *Lb. helveticus* during growth in milk (613, 614). The response of probiotics to GIT conditions is also highly relevant. Notably, *in situ* transcriptomes of *Lb. johnsonii* (615) and *Lb. plantarum* (616, 617) residing in the GIT have been reported. These studies illustrate the complex molecular adaptations of these LAB to the many physicochemical challenges encountered during mammalian intestinal tract transit. Many of the elicited transcriptional responses were associated with metabolic flexibility and energy metabolism, emphasizing the competitive nature of this habitat that is densely colonized with microbes. Also, significant modulations of cell envelope-associated functions were seen, illustrating the importance of cell surface architecture in this challenging niche.

Experimental evolution strategies are very well suited to investigate the molecular adaptations enabling the enhancement of complex phenotypes. Low-cost next-generation sequencing has drastically boosted the downstream genomic analysis of experimentally evolved variants. Experimental evolution has been used to study the adaptation of LAB to novel or altered environmental conditions. An elegant example is the adaptation of the plant isolate *Lc. lactis* KF147 to growth in milk. Accumulation of mutations that enhanced the capacity of the variants to harvest amino acids from milk was observed, while other mutations suppressed the broad-spectrum carbohydrate utilization capacity that is typical for plant isolates of this species. Since the transcriptome signatures of the milk-adapted variants were significantly more similar to those of typical *Lc. lactis* dairy strains, these adaptations were referred to as domestication signatures, based on the assumption that historically, initial milk fermentations involved plant-derived strains (618). Experimental evolution strategies to enhance LAB stress robustness have also been reported. For example, *Lc. lactis* variants with enhanced heat resistance contained single amino acid substitutions in a membrane-bound stress signaling protein of the GdpP family that was predicted to exhibit c-di-AMP-specific phosphodiesterase activity, and these elicited altered stress responses in these variants. Notably, the heat-resistant variants displayed hypersensitivity to salt stress compared to the wild-type strain, which may illustrate the existence of an evolutionary tradeoff between these two phenotypic traits (146) but may also be a consequence of the specific experimental setup employed for variant selection (619). An experimental evolution approach to adapt *Lb. plantarum* to the complex conditions of the mammalian GIT has also been reported (620). Repeated exposure to the mouse intestinal tract by three consecutive rounds of (re)feeding and isolation of colonies that displayed the longest persistence in the system allowed isolation of independent intestine-adapted variants with a >5-fold prolonged gut residence time compared to that of the original strain. The variants had accumulated mutations that could predominantly be assigned to functions associated with energy metabolism and the synthesis of cell envelope components (620). This study corroborates the importance of the latter functions in survival and persistence in this complex habitat deduced from *in situ* transcriptome signatures of wild-type strains (see above). Intriguingly, cell envelope-associated functions have also been reported to be the main effectors driving the strain-specific host interactions that are likely to support the health benefits of probiotic products, exemplifying the dualistic nature of these functions in the context of probiotic functionality (621–623).

Gene Function Discovery by Phenotype Diversity Mining Strategies

Although stressome-related functions appear to be quite conserved among strains of a species and even among species (see above), the stress robustness of strains of a species is quite variable. This phenotypic variation has a major impact on survival and performance under industrial conditions. It is not restricted to stress tolerance but encompasses many other aspects of *in situ* functionality of LAB. For example, different *Lc. lactis* strains have different effects on flavor formation in cheese production (624, 625). Phenotypic strain variation is at least partially caused by gene content differences, which implies that mining the data from genotype-phenotype matching (GPM) approaches may reveal novel gene functions. The validity of the GPM approach for LAB was first illustrated by the discovery in *Lb. plantarum* of the gene encoding the mannose-specific adhesin (Msa), which was proposed to play a role in the probiotic competitive exclusion of enterotoxic *E. coli* in the human intestine (626). Subsequent comparative analysis of the *msa* gene and the Msa protein in various strains of this species revealed strain-specific domain compositions of Msa, which were correlated with strain-specific mannose adhesion capacities (627). Advanced GPM approaches based on the Random Forest algorithm for correlation analysis (628, 629) identified or verified the roles of several genes in sugar metabolism in *Lc. lactis* (628) and various metabolic capacities in *Lb. plantarum* (630). However, while GPM approaches employing strain-specific heat and oxidative stress robustness phenotype variations in *Lc. lactis* or intestinal robustness phenotype variations among strains of *Lb. plantarum* did identify genes with correlated gene presence-absence patterns, subsequent mutational analyses did not verify their function in the respective phenotypes (631, 632).

Clearly, many phenotypes may not be determined by the "simple" presence or absence of one or more genes but may depend on strain-specific levels of expression of conserved genes. An example of such transcriptional diversity leading to phenotypic diversity has been described for *Lc. lactis*, where the activity levels of five enzymes involved in flavor formation were shown to be subject to highly strain-specific transcriptional regulation (633). The results suggest that transcript level diversity is the predominant driver of the observed variation of this industrially relevant phenotype (633). Analogously, because many genes involved in stress tolerance may be conserved among strains of a species, strain-specific diversity of this phenotype most likely also depends on the relative expression levels of genes rather than their presence or absence. This notion was recently confirmed using the two model systems described above, i.e., heat and oxidative stress robustness in *Lc. lactis* MG1363 (593) and intestinal robustness in *Lb. plantarum*

WCFS1 (592, 634). Both studies combined differential fermentation conditions with transcriptome profiling and stress tolerance analyses to identify robustness effector molecules on the basis of transcriptome-phenotype matching (TPM). Fermentation conditions were identified that induced increased or reduced stress tolerance levels compared to those under reference fermentation conditions. For example, aerobic fermentation of *Lc. lactis* MG1363 stimulated enhanced heat stress tolerance, whereas fermentation at elevated temperatures enhanced the survival of the cultures under oxidative stress conditions (593). Increased NaCl concentrations during fermentation induced a reduction of intestinal stress tolerance in *Lb. plantarum* WCFS1, while growth at a low pH enhanced the survival when the strain was exposed to stomach conditions (i.e., pH 2.5) (634). TPM using random forest-based correlation analysis allowed identification of transcripts that are quantitatively correlated with enhanced or suppressed stress tolerance, suggesting a role of the encoded functions in the studied phenotypes. For example, elevated expression of the *metC-cysK* operon, involved in sulfur-containing amino acid metabolism in *Lc. lactis* MG1363, correlated with increased oxidative stress tolerance. Subsequent experiments using cysteine-free medium, known to enhance *metC-cysK* expression (635, 636), confirmed a role for these genes and the corresponding metabolic pathway in oxidative stress robustness (593). Similarly, TPM of the *Lb. plantarum* WCFS1 data sets uncovered several candidate genes whose expression levels were predicted to contribute to GIT survival. This TPM-predicted role could be confirmed for three of the candidate genes by robustness analysis of mutants of these genes. The identified intestinal robustness factors included Pbp2A and a Na^+/H^+ antiporter (NapA3) that not only contributed to overall intestinal stress robustness but also played a role in bile acid and osmotic stress tolerance in *Lb. plantarum* WCFS1. The third robustness gene encoded a transcriptional regulator of the AraC family that was shown to affect the expression of cell envelope-associated genes in *Lb. plantarum* WCFS1, in particular the expression of a capsular polysaccharide synthesis gene cluster. Thus, all three identified robustness effectors affected cell envelope-associated functions, supporting the importance of this functional category in stress robustness, particularly for intestine-related environmental conditions, such as low pH (stomach) and bile acid exposure (small intestine) (634).

CONCLUSIONS

As may be clear from the data presented above, LAB have over the years received increased attention with respect to their behavior and robustness under stressful conditions. This is not at all surprising considering that they are an extremely important group of industrially and medically relevant bacteria. Solving the mysteries of stress responses in LAB, and ultimately applying the obtained know-how in biotechnology or in medicine, is a research quest for a double-faced grail. What is required for and often needs to be increased in food-related/probiotic bacteria (robustness) has to be combatted in the pathogenic LAB, although stress sensitivity, e.g., leading to cell lysis, may also be a desired attribute of LAB in some food applications, such as cheese ripening.

All species of LAB are subject to various intrinsic and extrinsic stresses and have developed intricate mechanisms to cope with these insults. The responses to several of the stressors follow paths that look quite similar to those that have been examined and described in great detail for the bacterial model organisms *E. coli* and *B. subtilis*. However, there are also several clear differences between these paradigms and the LAB discussed here that warrant the further examination of the mechanisms in the latter from a fundamental point of view.

The rapid advances in next-generation nucleotide (DNA/RNA) sequencing have also accelerated research in LAB genomics. To date, a host of LAB genomes have been sequenced to (near) completion. Although the first nucleotide sequences were from the few plasmid-free laboratory model LAB, increasingly more sequences appear from the full genomes (chromosomes plus plasmid complements) of LAB from many different natural sources. Several LAB species carry various numbers of different plasmids. Some of these, as it appears now, carry genes that may have an impact on strain robustness and survival under certain stress conditions, as discussed above. A nonexhaustive list of such genes includes those specifying several metal transporters, Cu^+ resistance, phage resistance, citrate metabolism, HSPs, sHSPs, and antibiotic resistance. Both conjugative and mobilizable plasmids have been described for LAB. The genome plasticity provided by these plasmids and also by other mobile elements, such as transposons, IS elements, and (pro)phages, which may introduce additional genes or (in)activate existing genes, adds another layer of complexity to the understanding of the full breadth of stress responsiveness of LAB.

The recent genomics-based advances, in combination with both GPM and TPM approaches, are starting to shed even more light on the genes and regulatory mechanisms involved in, among other things, LAB stress responses and stress tolerance. The expansion of our understanding of the complexity of the gene regulation networks and their components in the adaptation of LAB to their challenging environments is crucial for the translation toward industrial improvement strategies. These include an enhancement of robustness without a loss of functional properties that are relevant for their roles in product formation (e.g., acidification rate and flavor and texture formation), or in gastrointestinal survival and health stimulation in the case of probiotics. For instance, probiotic tolerance can be improved through (i) genetic manipulation and overexpression of chaperones, (ii) preexposure to an equivalent stress (adaptation), (iii) EPS production, and (iv) modification of the fatty acid composition of the cell membrane. But while all these mechanisms might improve survival and facilitate the delivery of more viable cells to the GIT, it is not known how they might affect the ability of the organism to impart a positive health effect on the host. Such knowledge can be generated only through *in vivo* trials where the modified/improved strains are tested with respect to their probiotic mechanisms.

For obvious reasons, (initial) studies of responses to stresses are often performed under laboratory conditions that try to mimic the real condition as much as possible, be it the industrial process in which the food/feed LAB are used or the actual situation in the host upon infection with a pathogenic LAB, or after ingestion in the case of probiotics. The complexity of the *in situ* responses of the various LAB species underpins the importance of combining these investigations with information from simplified *in vitro* models. For the industrial LAB, the combination of *in vitro* and *in situ* studies can provide the knowledge framework to, for instance, refine preadaptation strategies during production and preculture conditions. The occurrence of cross-protective stress responses may potentially be employed to design successful preadaptation schemes.

What is clearly lagging in LAB research, especially for the food

and probiotic LAB, is knowledge of the possible roles that small RNAs (sRNAs) may play in LAB stress responses. This may be due in part to choices in research focus and to a technical hurdle: until recently, genome-wide transcription analyses of LAB were performed using DNA microarray technology employing amplicons or oligonucleotides designed based on the known genes of the species or strains under study. This obviously restricted the analyses to these mostly protein-encoding genes. The reduction of the costs and, with that, the accessibility of high-throughput RNA sequencing strategies now offer new possibilities. Although the knowledge about the role of sRNAs in LAB is still limited, genome-wide screens for sRNAs were recently performed on some species (637–641). These data are also the basis of the first functional studies with regard to the possible roles of these important regulatory molecules in stress responses (642). In a recent examination by RNA sequencing of the transcriptome landscape of an *Lc. lactis* batch culture in rich medium, sampled at six different points, over 200 sRNAs were uncovered (643). The immediate, 5-min response of *Lc. lactis* to various stresses was also determined via RNA sequencing, and the stress-specific induction of several sRNAs was observed (643). It is now evident that sRNAs play an essential role in gene regulation under various conditions of growth, stress, and survival in bacteria. Thus, it is crucial that we uncover this important regulatory layer in the LAB, as this will certainly lead to new insights and refine our understanding of the way in which LAB defend themselves against (potentially lethal) stresses.

Another issue that requires attention is the importance of cell individuality in responding to stresses. Cells in a bacterial population, even in a very uniform environment, may differ considerably with respect to the genetic program that is operative under these conditions (51, 52). Not a lot is known about such flexibility in the behavior of LAB, but it is highly likely that it also occurs in these bacteria. In a recent study of diauxy during growth on two different sugars, Solopova and coworkers showed that different and stable metabolic phenotypes can exist next to each other in an *Lc. lactis* culture (644). The ratio between the two observed metabolic phenotypes depends on epigenetic cues and on the level of carbon catabolite repression. Most importantly with respect to this review, it also relies on the metabolic state-dependent induction of the stringent response, an important stress response pathway. All in all, the occurrence of distinctive metabolic phenotypes might represent a bet-hedging strategy. It would be interesting and important to find out whether culture heterogeneity also plays a role in the responses of LAB toward certain stresses.

One huge new development facilitated by genomic technology and next-generation sequencing power is that of experimental evolution, in which carefully chosen and controlled selection schemes are used to select certain advantageous mutants from a population of cells. Subsequently, the mutation(s) can easily be recognized through full-genome resequencing, allowing a quick and detailed understanding of the mechanisms involved in the adaptation. Importantly, experimental evolution strategies hold promise for the direct application of the adapted (improved) food, feed, and probiotic LAB, as they would be non-genetically modified organisms (non-GMO).

Overall, it is expected that investigations of the stress physiology of LAB will continue to be central to understanding their behavior. Novel experimental approaches available today will lead to important discoveries that can be applied in the food industry or in a medical setting.

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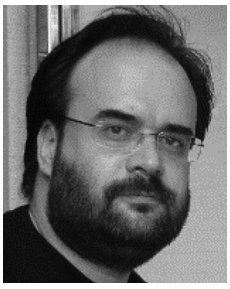


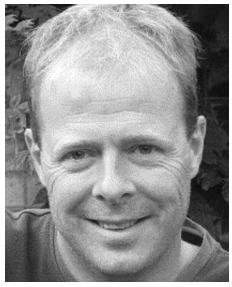
Konstantinos Papadimitriou was awarded his Ph.D. degree on the stress physiology of lactic acid bacteria in 2006, with distinction. Since then, Dr. Papadimitriou has worked on different projects, funded by the European Union or national funds, as a research associate in the group of Effie Tsakalidou. His main research interests include the microbiology of milk and milk products, the physiology, genetics, and genomics of lactic acid bacteria, the metagenomics of food ecosystems, single-cell microbiology, plasmid biology, and applied bioinformatics. Dr. Papadimitriou has published 21 original research articles and has received more than 200 citations. Dr. Papadimitriou and Prof. Tsakalidou are the editors of *Stress Responses of Lactic Acid Bacteria*, published by Springer (2011). Furthermore, Dr. Papadimitriou is a member of the editorial board of *Applied and Environmental Microbiology*, and he has served as an *ad hoc* reviewer for more than 20 research journals.

Ángel Alegría received his degree in biology at the University of Oviedo in 2008. He completed his Ph.D. (cum laude) in food biotechnology in 2013 at the Dairy Institute of Asturias (IPLA), which belongs to the Spanish National Research Council (CSIC). During his Ph.D. studies, he focused on the dynamics of traditional fermentations and the development of new starter cultures. In 2013, he received a FEMS research fellowship to perform a brief postdoctoral stay at the University of Verona, Italy, centered on the genome analysis of *Bifidobacterium pseudocatenulatum*. After that, he was awarded a Marie Curie fellowship to study the cell envelope stress response of *Lactococcus lactis* in the group of Molecular Genetics at the University of Groningen in The Netherlands, where he is currently a postdoctoral researcher. He is a coauthor of 14 publications in international journals and 2 book chapters (h-index = 10).

Peter A. Bron initiated his research career in 1999 as a Ph.D. student within WCFS (nowadays known as TI Food & Nutrition [TIFN], Wageningen, The Netherlands), studying the molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. He has been working on host-microbe interactions ever since and succeeded his career with a 2.5-year postdoctoral position in Cork (Ireland) investigating *Listeria monocytogenes* temporal gene expression profiles during infection. Subsequently, he returned to TIFN in 2007 as a postdoctoral researcher on a project on fermentation-enhanced probiotic function. Nowadays, he holds a senior scientist position within the fermentation workgroup at NIZO Food Research (The Netherlands). He was also one of the principal investigators that in 2014 cofounded Flagship 10 within the BE-Basic Consortium, which aims to develop natural methods for strain improvement for the food industry. His work has so far resulted in over 40 publications in peer-reviewed journals (h-index = 25, according to Google Scholar) and 5 patent filings.

Maria de Angelis received a degree in agricultural sciences from the University of Perugia, with good marks, in 1996. She completed Ph.D. studies in food biotechnology and was a researcher at the Institute of Sciences of Food Production, CNR, Bari, Italy. In 2005, she became a researcher for the Biotechnology Faculty of the University of Bari. From 2011 to the present, she has been an Associate Professor at the University of Bari. She is in charge of courses on microbiology, microbial biotechnology, and functional foods. Her main research work is focused on food microbiology, especially on genomics and proteomics of lactic acid bacteria, functional foods, and the human microbiota in response to diseases and food intolerance. She is an author or coauthor of ca. 225 papers, 11 chapters, and 10 patents. As reported by the ISI Web of Science, the publications reviewed received an average of 27.11 citations each, with an h-index of 35.

Marco Gobbetti graduated from the University of Perugia, Agriculture Faculty. Since 2000, he has been a Full Professor of Food Microbiology at the Department of Soil, Plant and Food Sciences, Aldo Moro University of Bari, Italy. He is the author of about 400 publications related to food microbiology, most of them (ca. 270) in international journals and reviewed by the Institute for Scientific Information (ISI). As reported by the ISI Web of Science, the publications reviewed were cited ca. 8,000 times, with an h-index of 49. Dr. Gobbetti's main areas of expertise are the molecular microbiology and biotechnology of sourdough, cheese, and vegetable/fruit lactic acid bacteria and the proteomics of lactic acid bacteria in response to environmental stresses and the human intestinal microbiome in response to diseases and food intolerance.



Michiel Kleerebezem studied biochemistry at the University of Groningen, where he obtained his university degree in 1989. In 1990, he started his Ph.D. studies in molecular microbiology at the University of Utrecht, and he graduated in 1995. From 1995 until 2015, he was employed at NIZO Food Research as a principal scientist, dedicating part of his time to TI Food & Nutrition, where he was appointed Scientific Director in 2011. Since 2007, he has held a position as Professor of Bacterial Metagenomics and Host-Microbe Interactions at Wageningen University, where he has been appointed full time since 2015. Since 1995, he has specialized in the genomics, molecular biology, and physiology of bacteria, with a special focus on lactic acid bacteria, probiotics, and the intestinal microbiota. In recent years, he has expanded his field to the (postgenomic) molecular analysis of mechanisms of communication between intestinal bacteria and the host mucosa.

José A. Lemos obtained his Ph.D. in microbiology and immunology from the Federal University of Rio de Janeiro (Brazil) in 2000. He did postdoctoral research with Robert Burne at the University of Rochester when he became interested in studying the stress response mechanisms of low-GC Gram-positive bacteria. In 2003, he became a Research Assistant Professor in the Department of Oral Biology at the University of Florida. In 2007, he joined the faculty of the University of Rochester in the Center of Oral Biology. Recently, he returned to the University of Florida, where he is an Associate Professor of Oral Biology. His laboratory investigates the stress survival mechanisms of Gram-positive pathogens, with a particular focus on *Streptococcus mutans* and *Enterococcus faecalis*.

Daniel M. Linares finished his Ph.D. work (2007) in the molecular microbiology lab of the Instituto de Productos Lacteos de Asturias-Consejo Superior de Investigaciones Cientificas (IPLA-CSIC) in Spain, having worked on the physiology and genetics of biogenic amine (BA) biosynthesis by lactic acid bacteria (LAB). After that, he was a postdoctoral researcher in the Biochemistry Department of the University of Groningen (The Netherlands), where he engineered *Lactococcus lactis* host strains suitable for efficient overexpression of functional recombinant membrane proteins. In 2011, he returned to IPLA-CSIC to complete research on BA and the cytotoxicity associated with their ingestion. He is now a Senior Postdoctoral Researcher in the Food Bioscience Department of Teagasc Food Research Centre (Ireland) and a member of the APC Microbiome Institute, where he studies the relationship between diet, microbiota, and health.

Paul Ross has been Head of the College of Science, Engineering and Food Science at University College Cork (UCC) since August 2014. Prior to that, he was Head of the Teagasc (National) Food Research Programme (Moorepark & Ashtown Food Research Centres). Paul is also a Principal Investigator in the Alimentary Pharmabiotic Centre (APC) and was the first Research Professor appointed by University College Cork (in 2012). He graduated with a B.Sc. in microbiology/biochemistry in 1984, with a Ph.D. in microbiology in 1989, and with a D.Sc. based on published work in 2009 (all from UCC). He is a member of The Royal Irish Academy and a Fellow of the American Academy for Microbiology. His main research interests are the gut microbiota, antimicrobial peptides, bacteriocins, probiotics, and anti-infectives, and he has worked on lactic acid bacteria for over 30 years.

Catherine Stanton graduated from University College Cork with B.Sc. and M.Sc. degrees in nutrition and food chemistry, and she earned a Ph.D. (1988) in biochemistry from Bournemouth University, United Kingdom. She was awarded a D.Sc. from the National University of Ireland in 2009, based on published work. She is a Principal Research Officer at Teagasc, Moorepark Food Centre, Fermoy, County Cork, and a Principal Investigator in the APC Microbiome Institute. She has an h-index of 43, having published over 250 papers. She was a joint recipient of the Elie Metchnikoff Award 2010, along with colleagues Paul Ross, Colin Hill, and Gerald Fitzgerald, for research on the application of LAB in fermented dairy products to improve health and on the mechanistic basis of LAB and probiotic functionality. She was appointed Adjunct Professor of UCC, College of Medicine and Health, in 2012.

Francesca Turroni is Assistant Professor at the University of Parma, Italy. She received her Ph.D. in food sciences and technology at the University of Parma, Italy, in 2010. She did postdoctoral research work at the Department of Microbiology, National University of Ireland, Cork (Ireland), before joining the faculty at the University of Parma in 2015. Her research is focused on the microbe-microbe as well as host-microbe interactions occurring in the human gut.

Douwe van Sinderen obtained his B.Sc. and M.Sc. degrees, in biochemistry and molecular biology, and his Ph.D. degree at the University of Groningen, The Netherlands. He then moved to the National Food Biotechnology Centre at University College Cork, where he worked as a postdoctoral researcher for 2 years, before he joined the School of Microbiology at the same institution as an academic staff member in 1998. Since 2004, he has been a Principal Investigator at the APC Microbiome Institute at University College Cork. For more than 20 years, he has been studying the molecular biology of selected lactic acid bacteria and bifidobacteria and their infecting phages, being inspired by the intricacies of microbe-host interactions and by an interest in their fascinating adaptive abilities as well as their biotechnological applications and purported health-promoting properties.

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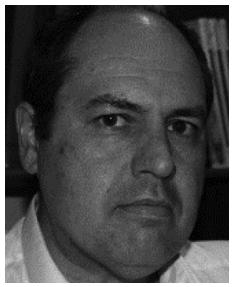
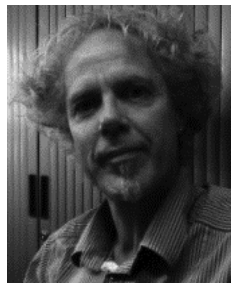
Pekka Varmanen obtained a Ph.D. in food microbiology (1997) from the University of Helsinki, Finland, and held an industrial postdoctoral position at Valio Ltd., Finland, in 1997 and 1998. He then moved to Denmark and was a Research Assistant Professor at the Royal Veterinary and Agricultural University (KVL, Copenhagen, Denmark) from 1998 to 2001. In 2001, he moved to the University of Helsinki, where he has led his own research group since 2004 and has held positions as a Postdoctoral Scientist (2001 to 2004), Academy Research Fellow (2004 to 2009), and University Lecturer (2009 to present). He first became intrigued by bacterial stress physiology while he was a postdoctoral researcher in the laboratory of Finn K. Vogensen (KVL) in 1998 to 2001, working on a project focusing on stress response mechanisms of *Lactococcus lactis*. His current research interests include stress response mechanisms in nonpathogenic and pathogenic bacteria adapted to the milk environment.

Marco Ventura is an Associate Professor at the University of Parma, Italy. He received his Ph.D. in natural sciences at the Swiss Federal Institute of Technology (ETH), Zurich, Switzerland, in 2003. He did postdoctoral research work at the Department of Microbiology, National University of Ireland, Cork (Ireland), before joining the faculty at the University of Parma in 2005. His research is focused on the molecular analysis of bifidobacteria as well as the diversity and host significance of the gut microbiota.



Manuel Zúñiga has been a Tenured Scientist of the Spanish National Research Council since 2005, working at the Instituto de Agroquímica y Tecnología de Alimentos (IATA). He obtained his Ph.D. at the University of Valencia (Spain) in 1994, focusing on the genetics of *Oenococcus oeni*. After receiving his Ph.D., he moved to the IATA to work on the genetics of the arginine deiminase pathway of *Lactobacillus sakei*. From 1997 to 1999, he stayed at the Molecular Genetics Department of the University of Groningen, working on the replication of the *Lactococcus lactis* bacteriophage r1t. Back at the IATA, he has been working on the physiology and genetics of *Lactobacillus casei*, the molecular evolution of metabolic pathways, and the characterization of the intestinal microbiota of rabbits. Since 2005, his research has been focused on signal transduction two-component systems of *L. casei* and their roles in stress responses.

Effie Tsakalidou graduated with a degree in chemistry from the Aristotle University of Thessaloniki, Greece. She then moved to Germany for postgraduate studies with a scholarship from the A. S. Onassis Foundation, and she received her Ph.D. in biochemistry from the University of Cologne. In 1987, she joined the Agricultural University of Athens, Greece, as a researcher in the Laboratory of Dairy Research. Currently, she is Professor of Food Biochemistry in the Department of Food Science and Human Nutrition. Her research interests lie in the field of lactic acid bacteria, with emphases on taxonomy, metabolism, physiology, genetics, bioinformatics, antimicrobial peptides, probiotics, and technological performance. She has coauthored over 280 publications in the above-mentioned fields, including papers in peer-reviewed international journals, book chapters, and papers at international and national conferences. She has also participated as a coordinator in more than 40 national and European Union research projects.



Jan Kok obtained his Ph.D. in molecular genetics at the University of Groningen, The Netherlands, in 1987. Since then, he has been investigating many aspects of the molecular biology of lactic acid bacteria, with a special emphasis on *Lactococcus lactis*. These studies were conducted both from a fundamental point of view and for more applied interests. The research has been focused on the responses of *L. lactis* to various stressors. A seminal paper detailing the nucleotide sequence of the entire genome of the model strain of *L. lactis* opened up the full power of "-omics" technologies to the study of these important microbes and led to a large body of papers on the life and functioning of *L. lactis*. Dr. Kok is a full Professor at the Department of Molecular Sciences of the Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, The Netherlands. He has supervised 25 Ph.D. students, (co)authored over 190 papers in peer-reviewed journals and 14 book chapters, and is an inventor on 18 patent applications. He is an ISI Highly Cited Microbiology Researcher, with an h-index of 53.