# Chapter 6
# Responses of Lactic Acid Bacteria to Oxidative Stress

**Bénédicte Cesselin, Aurélie Derré-Bobillot, Annabelle Fernandez, Gilles Lamberet, Delphine Lechardeur, Yuji Yamamoto, Martin Bastian Pedersen, Christel Garrigues, Alexandra Gruss, and Philippe Gaudu**

## 6.1 Introduction

Lactic acid bacteria that are generally recognized as safe (we used the term $LAB_{GRAS}$ when discussing dairy bacteria because they also belong to the large family of bacteria producing lactic acid; some of them are pathogens) are widely used in dairy and other food fermentations and are therefore naturally present in the human gastrointestinal tract (GIT). Named for their capacity to degrade lactose or other carbohydrates into lactic acid, $LAB_{GRAS}$ are used to promote milk coagulation and to contribute to the complex flavors, tastes, and textures of dairy products, for example, through the production of diacetyl and small peptides. They also may secrete small antimicrobial peptides (bacteriocins), which can limit the development of food contaminants like *Staphylococcus aureus* and *Listeria monocytogenes* and further improve food quality (Corr et al. 2007; Galvez et al. 2007). The "safe" and even "beneficial" status of $LAB_{GRAS}$ is attested to by their widespread consumption in foods. In the last two decades, researchers aimed at developing new applications of $LAB_{GRAS}$ in the area of biotechnology. For example, overproduction systems were developed in $LAB_{GRAS}$ for large-scale recombinant protein purification and for protein delivery. The systems make use of natural promoters that respond to diverse molecules for induction, such as nisin (Mierau and Kleerebezem 2005), acid (Madsen et al. 2005), or metals (Morello et al. 2008). Additionally, the overproduced proteins can be targeted to the cytoplasm or directed into the medium (although high-efficiency secretion remains a bottleneck). The production of immunomodulators (interleukin) or enzymes was successfully reported in $LAB_{GRAS}$ and might be useful for vaccine strategies to limit infection or in the treatment of cancer (Bermudez-Humaran et al. 2008).

P. Gaudu (✉)
Unité Bactéries Lactiques et Pathogènes Opportunistes, INRA UR888,
Jouy en Josas 78352, France
e-mail: philippe.gaudu@jouy.inra.fr

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Environmental pressures, either in industrial fermentations or in the host, can have marked consequences on bacterial gene expression, growth, and survival. This may limit the uses and applications of $LAB_{GRAS}$. Major stress factors are acid, salt, heat, and oxygen, which are relevant to bacterial starter preparation, to dairy fermentations, and to $LAB_{GRAS}$ life in the GIT, where cells are exposed to nutritional competition with other bacteria of the microbiota. To survive, bacteria have, as a nearly unique option, detection of the stress, which would lead to reprogramming of the gene expression and a consequent physiological adaptation to the new environment. $LAB_{GRAS}$ that are used industrially, or are present in the GIT, grow via sugar fermentation. In view of the importance of sugars in the $LAB_{GRAS}$ environment, this chapter focuses on aspects of oxidative stress mainly in *Lactococcus lactis* related to carbon metabolism. More recently, in addition to growth under fermentation, several LAB, including *L. lactis*, have been shown to undergo a respiration metabolism when heme, or heme and menaquinone, is available in the medium; heme is needed to activate a terminal cytochrome oxidase, while menaquinones serve as electron transfer molecules in the electron transport chain (Poole and Cook 2000).

## 6.2 Oxygen Species

### *6.2.1 Oxygen*

Oxygen is a biradical molecule (˙O-O˙), because of its small size, it can easily access the catalytic site of proteins. Published analysis has established that $O_2$ reacts with certain metals, in particular with iron (present in heme-dependent cytochrome oxidase, for instance), and with activated amino acids, like the glycyl radical in some enzymes. In contrast to cytochrome oxidases that use $O_2$ as a substrate, pyruvate formate lyase (PFL), an anaerobic enzyme bearing a glycyl radical, is extremely sensitive to $O_2$ (Zhang et al. 2001). This reaction leads to the cleavage of the protein into two fragments and furthermore to irreversible inactivation of activity (Melchiorsen et al. 2000). However, reduction of $O_2$ generates products that are far more toxic than $O_2$ itself, such as superoxide anion radicals ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (HO˙) (Imlay 2008). These molecules are physiologically more important in the oxidative stress than $O_2$, since for example *Escherichia coli* cells that are aerobically grown maintain concentrations of $O_2^{\cdot -}$ and $H_2O_2$ at 0.0001 and 0.1 μM, respectively, compared to an intracellular $O_2$ concentration of 210 μM (Imlay and Fridovich 1991; Gonzalez-Flecha and Demple 1995).

### *6.2.2 Superoxide*

$O_2^{\cdot -}$ is the product of a one-electron reduction of $O_2$ (Imlay 2008). It is generated as a consequence of the oxidation of dihydroflavins ($FADH_2$, $FMNH_2$, Riboflavin $H_2$) or quinols (demethylmenaquinol, menaquinol, ubiquinol) (Huycke et al. 2001;

Rezaiki et al. 2008). The reason is that the radical forms of flavins and quinones are relatively stable, which then facilitates the one-electron reduction. Thus, quinones belonging to the respiratory chain, and flavoproteins were often supposed to be the major generators of $O_2^{\cdot -}$ in bacteria, including *Enterococcus faecalis*, *E. coli*, and *L. lactis* (Imlay and Fridovich 1991; Huycke et al. 2001; Korshunov and Imlay 2006; Rezaiki et al. 2008). Inactivation of the quinone biosynthesis pathway or NADH dehydrogenases of the respiratory chain significantly reduced $O_2^{\cdot -}$ production in *E. coli* (Korshunov and Imlay 2006), while cyanide, an inhibitor of cytochrome oxidases, strongly led to an increase in $O_2^{\cdot -}$ and $H_2O_2$ levels, as reflected by the overproduction of superoxide dismutase (SOD) and catalase (Hassan and Fridovich 1979).

$O_2^{\cdot -}$ is not considered a strong oxidant or reducer, and the presence of SOD remained an enigma for a long period. The construction of a strain deficient in SOD activity in *E. coli* finally helped to reveal the mechanism of $O_2^{\cdot -}$ toxicity (Farr et al. 1986). This mutant does not grow in chemical-defined medium in the presence of air and is strongly sensitive to $O_2^{\cdot}$-producing agents like paraquat (a catalyzer of $O_2^{\cdot -}$) in rich medium. The phenotypes result from oxidation of certain dehydratases containing particular [4Fe-4S] clusters, such as aconitase A or fumarase A and B (Imlay 2008). This [4Fe-4S] cluster is linked to proteins via only three irons, while the last one binds to the substrate (citrate and fumarate for aconitase and fumarase, respectively). This labile iron is attacked by $O_2^{\cdot -}$, leading to destruction of the cofactor and concomitant enzyme inactivation. This inactivation also coincides with increased free iron in cells according to the reaction $[4Fe\text{-}4S] + O_2^{\cdot -} + 2H^+ \rightarrow [3Fe-4S] + Fe + H_2O_2$. It was also demonstrated in *E. coli* that $O_2^{\cdot -}$ reacted with an activated amino acid (tyrosyl radical) in proteins, as exemplified by aerobic deoxyribonucleotide di-phosphate reductase, a key enzyme of DNA synthesis (Gaudu et al. 1996).

In an acidic environment, the protonation of $O_2^{\cdot -}$ leads to a more reactive species, hydroperoxyl radical ($HOO^{\cdot}$). Due to the absence of charge, $HOO^{\cdot}$ can pass through the membrane and then return to its anionic state in the cytoplasm, as the intracellular environment is less acidic than the outside. Thus, bacteria that encounter acidic environments, for instance, the stomach or fermented products, might be particularly exposed to oxidative stress. In *L. lactis* MG1363, SOD was overproduced about fourfold after exposure of cells to acid stress (Budin-Verneuil et al. 2005). Thus, the presence of SOD should provide an advantage for colonization and survival in an acidic environment. Note that *Lactobacillus plantarum* species do not have SOD; they compensate for this absence by incorporating high concentrations of manganese (Archibald and Fridovich 1981a, b).

### *6.2.3 Hydrogen Peroxide and the Hydroxyl Radical*

$H_2O_2$ is a neutral molecule that crosses the membrane barrier of the cell but is not reactive by itself. It is issued from two-electron reduction by flavoproteins, quinols, and metabolic enzymes (e.g., NADH peroxidase, pyruvate oxidase, and

lactate oxidase) present in several $LAB_{GRAS}$, and in particular in lactobacilli (Lorquet et al. 2004; Goffin et al. 2006; Barre et al. 2007). Additionally, SOD or manganese produce this molecule from $O_2^{\cdot -}$. In some lactobacillus species, $H_2O_2$ can reach millimolar concentrations in some environments, which is high enough to stop growth (Ocana et al. 1999). As LAB have peroxidases and/or catalases (Kono and Fridovich 1983; Frankenberg et al. 2002) to eliminate peroxide, the high levels of peroxide they produce suggest that these enzymes might require specific conditions for activity and/or expression. For instance, many catalases contain heme as a cofactor in prokaryotes, although a manganese-dependent catalase has been reported in *L. plantarum* (Kono and Fridovich 1983). In the case of catalases using heme as a cofactor, *E. faecalis* would need to scavenge it from their environment, as it lacks the complete biosynthesis pathway like other LAB (Kono and Fridovich 1983; Duwat et al. 2001; Frankenberg et al. 2002). Peroxidase activity is mainly assured by the AhpF-AhpC complex. AhpF (also named Nox-1) is a dehydrogenase that drives electrons from NADH to AhpC in *Streptococcus mutans* (Higuchi et al. 1999, 2000). Furthermore, AhpC catalyzes the reduction of peroxide into water or alcohol via the oxidation of its cysteine residues. So a depletion of NADH due to the lactate dehydrogenase activity (or $H_2O_2$-producing NADH oxidase) probably limits the activity of AhpF-AhpC. Besides the aforementioned activity, AhpC can switch to a chaperone function as it forms oligomeric complexes as described in *Helicobacter pylori* and thus might stabilize the protein folding during stress conditions (Chuang et al. 2006). Interestingly, we recently found that AhpC is also a heme-binding protein, which is needed for full respiration activity of the opportunist pathogen *Streptococcus agalactiae* (Lechardeur et al. 2010).

$H_2O_2$ is activated by ultraviolet light or in the presence of metals and is converted into $HO^{\cdot}$, the most reactive species of $O_2$ byproducts. Nonheme iron was estimated to be around 10 μg/g of cell dried weight in *L. lactis* MG1363 (Gostick et al. 1999), under some conditions. While similar measurements are unavailable for $LAB_{GRAS}$, it is likely that iron is present, as since (1) some housekeeping enzymes require iron, such as dihydroorate reductase (Rowland et al. 2000), (2) $LAB_{GRAS}$ carry genes for potential iron transporters, (3) $LAB_{GRAS}$ genome analysis revealed genes coding for enzymes involved in iron utilization (Gostick et al. 1999, Thibessard et al. 2004), and (4) an iron chelator (like Desferal) protected DNA integrity when *L. lactis* was exposed to $O_2$ (Duwat et al. 1995; Rezaiki et al. 2004).

$HO^{\cdot}$ reacts at its site of production and oxidizes all biomolecules present in its vicinity. The steady-state concentration of this molecule is thus considered very low compared to $O_2^{\cdot -}$ and $H_2O_2$. In the case of DNA, the damage incurred suggested that iron (or other redox metals) is probably sequestered in the phosphate backbone of the DNA (Ouameur et al. 2005). To strengthen this, the family of Dps/Dpr proteins, which are used in iron storage, prevented $HO^{\cdot}$ formation and further protected the DNA (Yamamoto et al. 2002; 2004). Interestingly, Dps from *E. coli* and *L. lactis* bind to DNA (Martinez and Kolter 1997; Stillman et al. 2005), probably highlighting the importance of this class of proteins in DNA integrity in bacteria.

## 6.3 Target and Defense: Glyceraldehyde-3-Phosphate Dehydrogenase, Pyruvate Formate Lyase, and Respiration as General Barriers Against Reactive Oxygen Species

Besides some proteins that protect DNA from damage, others have recently been characterized for their sensitivity to oxidative stress in *L. lactis*, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PFL.

### *6.3.1 Glyceraldehyde-3-Phosphate Dehydrogenase*

GAPDH, called GapB in *L. lactis* MG1363, catalyzes the conversion of glyceraldyde-3-phosphate to glycerate-1,3-biphosphate in glycolysis. A decrease in *gapB* gene expression resulted in a dramatic reduction in growth, suggesting that this enzyme, not surprisingly, is essential in lactococci (Solem et al. 2003). The *gapB* gene is highly expressed, and GapB is among the most abundant proteins in the *L. lactis* glycolytic pathway. It is readily detected on 2D gels, and appears as two spots with different isoelectric points but the same molecular weight, indicating that GapB is not cleaved, in contrast to PFL, when *L. lactis* cells are exposed to $O_2$ (Melchiorsen et al. 2000). The relative levels of these two spots were observed to change in *L. lactis* under two conditions: One is in a thioredoxin reductase mutant (*trxB1*), and the other is in respiration metabolism. This led us to consider that these two forms were related to oxidative stress (Vido et al. 2004, 2005). Using mass spectrometry, the two spots were analyzed in the *trxB1* background, and the sole difference observed was oxidation of the catalytic cysteine. The thiol group of this amino acid was converted into the sulfinic ($SO_2H$) or sulfonic ($SO_3H$) form, resulting in irreversible oxidation. To date, no reducing system to reverse this oxidation has been described in prokaryotes, while the reduction of sulfenic acid (SOH) and sulfoxide methionine has been reported. For instance, methionine sulfoxide reductase restores oxidized methionine in proteins (Boschi-Muller et al. 2008). Thus, to avoid cysteine oxidation in GapB, enzymatic reducing systems like thioredoxin-thioredoxin reductase systems are required to eliminate reactive oxygen species (ROS) before they attack the proteins. The large amount of GapB in *L. lactis* MG1363 may also be a means to avoid a decrease in the glycolytic flux occurring through oxidation of GapB, as a threefold lower GapB still maintained a fully active glycolysis (Solem et al. 2003). Moreover, the growth defect of the *trxB1* mutant is alleviated by the presence of metabolites like glutathione, cysteine, and pyruvate, indicating that these small molecules can protect GapB against ROS (Vido et al. 2005). *L. lactis* contains a second GAPDH, GapA. Like GapB, GapA is also present as two isoforms on 2D gels, indicating that this protein is also subject to oxidation (Willemoes et al. 2002). GapA is not overproduced in the *trxB1* mutant, suggesting that this paralog does not compensate for the *gapB* inactivation and may not be involved in the stress response (Vido et al. 2005).

### 6.3.2 Pyruvate Formate Lyase

Under anaerobiosis, PFL catalyzes the conversion of pyruvate into acetyl-CoA. During the shift from anaerobiosis to aerobiosis, PFL is cleaved into two fragments, resulting in its irreversible inactivation. One strategy to avoid the cleavage is to reduce the glycyl radical into glycine by the PFL deactivase (Melchiorsen et al. 2000). Otherwise, the induction of an alternative metabolic pathway is essential to maintain the production of acetyl-CoA under aeration. In this latter growth condition, genes encoding the pyruvate dehydrogenase complex (PdhABCD, PDHc) are overexpressed (Jensen et al. 2001). This complex also catalyzes the conversion of pyruvate into acetyl-CoA, but unlike PFL, the second end product of this reaction is NADH. As the lactate dehydrogenase recycles NADH into NAD but also consumes pyruvate, it cannot assist PDHc in its activity. Thus, other NADH oxidoreductases, such as the NADH:$H_2O$-forming oxidase (NOX), are required to recycle NAD independently of pyruvate. NOX catalyzes the four-electron reduction of $O_2$ into water. In some bacteria, this flavoprotein plays an important role in growth under aeration. It contributes both to the reduction of ROS formation through $O_2$ scavenging activity (Yamamoto et al. 2006) and to the production of acetyl-CoA coupled with PDHc. In *L. lactis*, an NADH:$H_2O$-forming oxidase overproduction produced a large amount of acetate, and acetoin, at the expense of lactate, indicating that this enzyme contributes significantly to the conversion of pyruvate into acetate (Lopez de Felipe et al. 1998; Hoefnagel et al. 2002), but the effect of NOX deletion on *L. lactis* growth or metabolic pathways remains unknown. In contrast, other *Streptococcaceae* may have less redundancy in this function. It is notable that NOX inactivation can be compensated for in respiring LAB by the NADH oxidase activity as part of the electron transport chain (Yamamoto et al. 2006). Interestingly, an *S. agalactiae nox-2* mutant failed to grow under aeration due to acetyl-CoA deficiency (its production requires NAD, which is produced less in the NOX mutant)**.** As an acetyl-CoA is a precursor of fatty acids, the addition of lipids or serum restored the *S. agalactiae* growth defect (Yamamoto et al. 2006).

### 6.3.3 Respiration as a General Barrier Against Reactive Oxygen Species

$LAB_{GRAS}$ species like *L. lactis* are traditionally considered to be obligate fermentative bacteria because even in aerobiosis they use sugar degradation for substrate-level phosphorylation, that is ATP production. However, experimental studies revealed that *L. lactis*, *E. faecalis*, and other LAB are capable of activating a heme-dependent cytochrome oxidase and of establishing a complete respiration chain (Winstedt et al. 2000; Duwat et al. 2001). In *L. lactis*, the respiratory chain comprises (1) an NADH dehydrogenase (likely to be encoded by *noxA* and/or *noxB*),

which drives electrons to menaquinone (Gaudu et al. 2002; Brooijmans et al. 2009a; Tachon et al. 2009), (2) menaquinones (synthesized in *L. lactis* and *E. faecalis*, but lacking in other respiration-competent LAB), which transfer the electrons to the terminal acceptor (Huycke et al. 2001; Rezaiki et al. 2008; Brooijmans et al. 2009a; Tachon et al. 2009), and (3) heme-dependent cytochrome oxidase CydAB, the terminal electron acceptor, which finally reduces $O_2$ into water (Duwat et al. 2001) (Fig. 6.1). The heme biosynthesis pathway is incomplete in all LAB that have been sequenced. Some bacteria, like *L. lactis* and *E. faecalis*, encode a ferrochelatase (called HemH), which catalyzes the incorporation of iron in protoporphyrin-IX (PPIX), to generate heme (Duwat et al. 2001).

An unexpected finding for respiration-grown *L. lactis* was that the cells survive much better at 4°C than under fermentation once they attain the stationary phase (Duwat et al. 2001). For instance, after 2 weeks of storage, a $10^6$-fold increase in the viable population was observed for respiration-grown cells compared to fermentation-grown cells. This effect is due to at least two factors: One concerns the higher pH of respiration cultures, due to less organic acid accumulation. Nevertheless, fermentative cells resuspended in medium buffered at pH 7.0 did not survive as well as cells cultured under respiration conditions, indicating that the pH difference is only part of the answer. The second and primary reason for the improved survival is therefore attributed to the $O_2$ scavenging properties of CydAB (Rezaiki et al. 2004). This class of oxidases is generally reported to have more affinity for $O_2$ than other

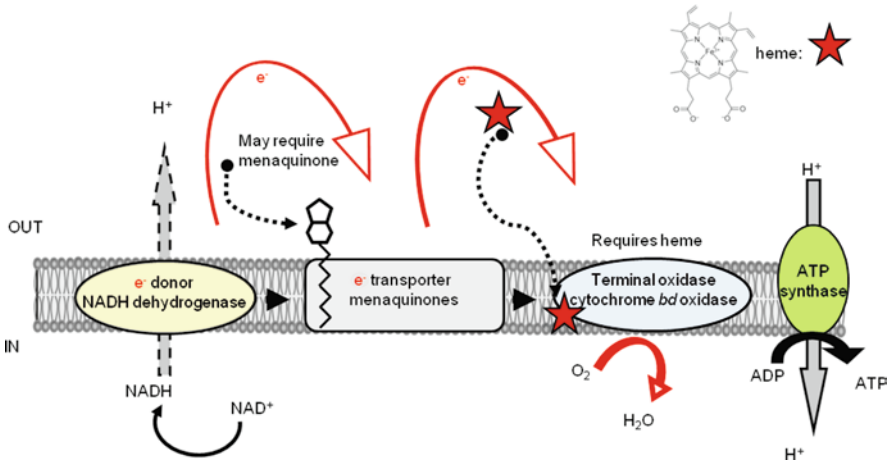


**Fig. 6.1** A respiratory chain in LAB. Heme uptake probably involves a specific transporter(s), including the system encoded by the *fhu* operon in *L. lactis* (not schematized) (Gaudu et al. 2003). The mechanism of incorporation into cytochrome oxidase, CydAB, is still unknown. Electrons from NADH are transferred to CydAB via NADH dehydrogenase (electron donor) and menaquinone (electron transfer intermediate). Genes for menaquinone synthesis are present only in some of the LABs (see Fig. 6.2). The activated respiratory chain reduces $O_2$ to water and concomitantly extrudes protons from the cytoplasm. ATPase makes use of a pH gradient to produce extra ATP (Koebmann et al. 2008). To avoid a toxic heme overload, putative efflux systems, including YgfBA, are produced (not schematized) (Pedersen et al. 2008)

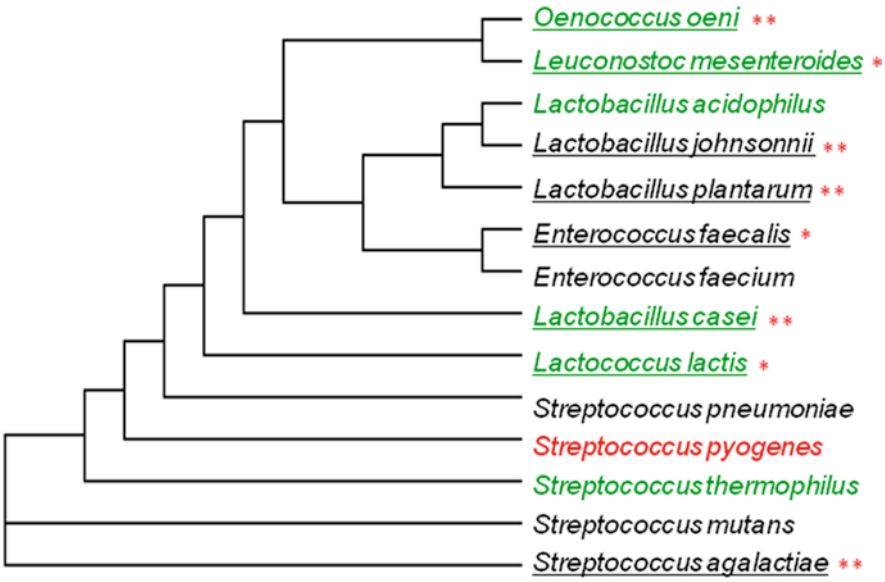


**Fig. 6.2** Distribution of respiration capacity in related $LAB_{GRAS}$ and other LAB. Related lactic acid bacteria (based on similarities of the conserved *recA* Orfs using ClustalW alignment) are presented. Bacteria are classified as food (*green*), commensal or opportunist pathogens (*black*), or pathogens (*red*). All these LAB lack heme biosynthesis genes. Among them, those encoding *cydABCD* genes are underlined. Those that require only heme to activate respiration metabolism are shown with one *red star*. Those that require heme and a menaquinone to activate respiration metabolism are shown with two *red stars*. Respiration metabolism was demonstrated experimentally for *L. lactis*, *E. faecalis*, *S. agalactiae*, *Lb. plantarum*, *L. mesenteroides* (Duwat et al. 2001; Huycke et al. 2001; Yamamoto et al. 2005; Brooijmans et al. 2009a; b); for *L. mesenteroides* (C. Foucaud, Alexandra Gruss and Philippe Gaudu, unpublished data)

cytochrome oxidases. Thus, $O_2$ consumption in the membrane reduces $O_2$ levels in the cytoplasm and in turn limits ROS production. In addition, *L. lactis* and other respiration-permissive LAB must strictly manage their heme pools to limit their reactivity (heme is redox-active, and iron can catalyze the Fenton reaction, leading to cell damage), yet allowing sufficient heme availability to activate the CydAB cytochrome oxidase (Rezaiki et al. 2004). Recently, we identified factors involved in intracellular heme management: AhpC was found to bind heme, and the YgfCBA proteins were found to be involved in efficient heme efflux (Pedersen et al. 2008; Lechardeur et al. 2010).

Several LAB encode the *cydABCD* genes, which are needed to produce the cytochrome bd oxidase (*cydCD* encodes functions implicated in cytochrome oxidase assembly) (Huycke et al. 2001; Yamamoto et al. 2005; Brooijmans et al. 2009a, b). These are summarized in Fig. 6.1. We consider this feature to be a reliable indication to identify which LAB have the potential to undergo a respiratory metabolism when heme, and in some cases a menaquinone source, is provided. For instance, *S. agalactiae*, an opportunistic human and bovine pathogen and a lactic acid bacterium, activates its respiratory chain once heme and menaquinone are supplied in

aerobic cultures (Yamamoto et al. 2005). More recently, the respiration capacity of *cydABCD*-encoding organisms was also validated in *L. plantarum*, giving similar phenotypes as above (Brooijmans et al. 2009b). When heme (or PPIX) is supplied to *L. lactis* under aerobic conditions, carbon metabolism is dramatically altered: Acetate and acetoin are produced in substantial amounts at the expense of lactic acid (Kaneko et al. 1990; Duwat et al. 2001; Pedersen et al. 2008). This change is possibly regulated through the NADH/$NAD^+$ ratio through changes in gene expression in enzymes around pyruvate metabolism, as indicated for both transcriptome and proteome analysis (Vido et al. 2004; Pedersen et al. 2008). Note that respiratory chain activity expulses protons to the outside of the cells, which is taken up by the ATPase to produce ATP (Blank et al. 2001; Koebmann et al. 2008). Under the energetically favorable respiration conditions (i.e., in aerobic growth with heme), *L. lactis* displays a gain of biomass compared to fermentation growth (Duwat et al. 2001; Pedersen et al. 2008). Unexpectedly, in contrast to *L. lactis*, *L. plantarum* displayed an additional respiratory metabolism with nitrate as the electron acceptor. This nitrate respiration also required heme and menaquinone for activity (Brooijmans et al. 2009b).

## 6.4 Regulation of Oxidative Stress Response: Evidence and Models

Although several stress-defense proteins, in addition to the respiratory chain, have already been identified in $LAB_{GRAS}$ (e.g., SOD, thioredoxin reductase, peroxidase, glutathione reductase, and RecA; see van de Guchte et al. (2002); and Hols et al. (2005) for a review) the mechanisms involved in their regulation remain widely unknown. Their genes are often induced under air or oxidative stress, as observed in other species like *Bacillus subtilis* or *E. coli*, suggesting that some strategies are common among bacteria. This hypothesis is supported by the presence of several potential regulators in LAB that are homologs to those characterized in *B. subtilis*, for instance. To date, only a few regulators have been identified in $LAB_{GRAS}$, and they can be classified in two categories: (1) transcriptional regulators that specifically recognize a motif in the promoter region of stress-response genes, to facilitate the recruitment of RNA polymerase, (2) proteins that modulate the activities of RNA polymerase.

### *6.4.1 Two-Component Systems*

Among the six two-component systems (TCSs) identified in *L. lactis* MG1363, only two were found to be involved in oxidative stress: LlrF (DNA-binding regulator) and LlkinF (sensor) (O'Connell-Motherway et al. 2000). The inactivation of LlrF exhibited a greater sensitivity to peroxide: After 20 min of exposure to 4 mM of $H_2O_2$, only 9% of the mutant population remained viable compared to

70% in the control. The genes that are under control of this TCS, as well as the detected stress signal, remain to be identified in *L. lactis*. In *S. mutans*, ScnRK, a TCS, appears to regulate several genes related to stress, including *tpx*, encoding a thiol peroxidase (Chen et al. 2008). As this is also present in *L. lactis, tpx* might be controlled by LlrF/LlkinF.

### 6.4.2 PerR

Genome analysis in *L. lactis* revealed the presence of several homologs of *B. subtilis* PerR and OhrR, which are involved in the peroxide stress response. In *B. subtilis*, PerR is related to the ferric-uptake repressor family of proteins (Lee and Helmann 2006). It is a dimer and contains two metal ions per subunit: Zinc is coordinated to the protein via four cysteine residues, while ferrous iron or manganese ion is likely linked via histidines (H37, H91, and H93) and aspartic acids (D85 and D104). The $PerR_{Zn/Fe}$ form reacted via iron with peroxide by Fenton reaction, and furthermore, $HO^{\cdot}$ oxidized histidine at position 37 or 91 in the peptide sequence (Lee and Helmann 2006). This oxidation modified the affinity of PerR for the test promoter region of *mrgA* (encoding a Dps-like protein). In contrast, no oxidation occurred with the $PerR_{Zn/Mn}$ form, indicating that PerR is likely to use iron and zinc as metal cofactors in vivo. The *L. lactis* MG1363 homolog of PerR is named Fur (llmg_1023). In *E. faecalis*, a PerR-like regulator has been identified and found to contribute in oxidative stress response (Verneuil et al. 2005). Unexpectedly, in contrast to PerR, a new regulator HypR, belonging to the LysR family, has been shown to respond to peroxide stress in *E. faecalis*. Nevertheless, the roles of HypR versus PerR in stress regulation are not yet clearly understood in this bacterium (Verneuil et al. 2004).

### 6.4.3 OhrR

*B. subtilis* OhrR protein is a homodimer that belongs to the multiple antibiotic resistance (MarR) family. It regulates the expression of the *ohr* genes encoding thiol peroxidase, which reduces hydroperoxide to the corresponding alcohols. OhrR responds to hydroperoxide stress via the oxidation of its unique cysteine residue, located at position 15 in the polypeptide (Fuangthong and Helmann 2002). During oxidative stress, cysteine is oxidized to sulfenic acid (RSH $\rightarrow$ RSOH) or to more oxidized forms. The oxidized protein does not bind to the promoter region of *ohrA*, encoding an organic peroxide resistance protein. A closer homolog of OhrR is present in *L. lactis* IL1403 (RmaJ), but seems to be absent in MG1363. This indicates that the latter cannot cope with organic peroxide stress, or that the signaling pathway differs from OhrR.

### 6.4.4 *Rex*

Unlike OhrR and PerR, which detect stress by the oxidation of an amino acid, the Rex protein governs the expression of the cytochrome oxidase gene (*cydAB*) in *B. subtilis* and *Streptomyces ceolicolor* via the pool of NADH (Wang et al. 2008). This protein is a homodimer containing two domains: The N-terminal domain binds to the promoter region of target genes such as *cydABCD*, while the C-terminal recognizes the ligand, NADH. One model is that when cells reach the stationary phase (or when the $O_2$ tension decreases due to high cell density), the NADH pool increases, leading to its binding to Rex. The Rex-NADH-DNA complex becomes unstable, and thus the repression of genes controlled by Rex is off. In *L. lactis* MG1363, the homolog of Rex is llmg_1514. However, *E. faecalis* contains in its genome two ORFs similar to Rex, suggesting that one might respond to other signals like NADPH instead of NADH, for instance.

### 6.4.5 *Factors Modulating RNA Polymerase*

Modification of RNA polymerase activity is reportedly involved in the regulation of stress responses in *L. lactis*. Two candidates have been identified recently in this bacterium: ppGpp and a new class of proteins, Spx.

#### 6.4.5.1 ppGpp

ppGpp is produced during the stringent response, and probably in all conditions that lead to growth arrest (Magnusson et al. 2005). Cross-linking experiments indicated that it interacted with the two β-subunits of RNA polymerase, close to the catalytic site (Chatterji et al. 1998). In *L. lactis*, inactivation of the high-affinity phosphate transporter, encoded by the *pst* locus, increased resistance to multiple stresses as reported for an *relA* mutant. The latter was shown to produce more ppGpp than the parent strain, indicating that the multiresistance observed in these mutants was correlated to ppGpp levels (Rallu et al. 2000). We developed a strategy to reveal *L. lactis* factors responding to oxidative stress, in which we disabled part of the oxidative stress response by growing cells in the presence of a reducing agent, dithiothreitol (DTT) (Cesselin et al. 2009). This molecule did not allow the production of SOD, AhpC, and TrxB1, confirming the use of a redox control in their respective gene expression. Unexpectedly, mutants isolated as resistant to stress in the presence of DTT were also inactivated in the *pst* locus. *Pst* mutants displayed an accrued resistance to copper, zinc, and tellurite (Turner et al. 2007; Cesselin et al. 2009). Copper and zinc bind to cysteine residues in proteins and, in the case of copper, also catalyze the Fenton reaction (Stadtman and Oliver 1991; Barre et al. 2007; Cesselin et al. 2009; Macomber and Imlay 2009). These observations suggest

a probable link between ppGpp levels and metal homeostasis, as reported in *E. coli*. In *E. coli*, a metal limitation (iron) stimulated ppGpp production (Vinella et al. 2005). Note that two FNR-like proteins, containing redox cysteine residues, are involved in stress sensitivity and were shown to modulate zinc homeostasis (Gostick et al. 1999). These results all seem to point to the important role of metals in bacterial stress responses.

#### 6.4.5.2 Spx

Spx proteins interact with the C-terminal domain of the RNA polymerase α-subunit in vitro and are thus a modulator of the transcriptional regulator – RNA polymerase interaction (Zuber 2004). Spx proteins are ubiquitous in Gram-positive bacteria, and the numbers of paralogs differ according to the species. For instance, *L. lactis* contains seven paralogs: The SpxB protein is involved in cell wall stress response (Veiga et al. 2007). TrmA (SpxA) inactivation leads to temperature resistance and compensates for defects due to *clpP* or *recA* inactivation (Duwat et al. 1999; Frees et al. 2001; Veiga et al. 2007). Functions of other Spx paralogs are under investigation in our laboratory (S. Kulakauskas, INRA). Interestingly, five of seven Spx proteins contain a CXXC motif in the N-terminal domain. This suggests that they might be under redox regulation. As this motif – present also in peroxiredoxin proteins such as thioredoxin – is highly conserved, it is tempting to propose that it might form disulfide bonds under certain conditions and thus might modulate Spx activity and its interactions with RNA polymerase. However, the work of You *et al.* suggests that this may not be the case (You et al. 2008). The addition of DTT, an agent that reverses disulfide bonds, had no effect on oxidized Spx after treatment of *B. subtilis* by paraquat. This observation suggests that cysteines of Spx proteins were probably oxidized in an irreversible form as observed in GapB (Vido et al. 2005). Moreover, the total absence of cysteine in the two other Spx proteins in *L. lactis* (llmg_1130 and llmg_1155) reveals a strong diversity in the activities of these proteins.

## 6.5 Concluding Remarks

In *E. coli*, $O_2^{\cdot -}$ reacts with particular iron-sulfur clusters present in some dehydratases (Keyer and Imlay 1996). As of now, no dehydratase containing the [4Fe-4S] cluster with a labile iron has been reported in $LAB_{GRAS}$, suggesting that the toxicity of $O_2^{\cdot -}$ might pass through a different mechanism of what was elucidated in the *E. coli* model. There are, nevertheless, *L. lactis* Orfs involved in amino acid synthesis, corresponding to dehydratases (like IlvD and SdaA), which likely involve Fe-S clusters. As the roles and requirements of iron in $LAB_{GRAS}$ have not yet been firmly established, many lines of research remain to be explored to understand the toxicity of ROS in $LAB_{GRAS}$.

Similarly, the identification of stress-response regulators and signaling pathways in $LAB_{GRAS}$ is incomplete, and most signals and targets are still unknown. Deeper characterizations of targets, for example, of the two-component LlrF-LlkinF system and of the roles of different Spx proteins in oxidative stress in $LAB_{GRAS}$ are likely to bring information that cannot be transposed from studies of aerobic model bacteria. The impact of central metabolism on stress responses is still preliminary in LAB; for instance, the stress response under respiration conditions may be radically different from that under fermentation conditions. Global and modeling approaches will be valuable in obtaining an integral view on the stressed cell and should complement more in-depth studies of specific factors, which have led to some of the fundamental findings reported here.

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