Review

Oxidative stress in *Lactococcus lactis*

Anderson Miyoshi[1], Tatiana Rochat[2], Jean-Jacques Gratadoux[2], Yves Le Loir[3], Sérgio Costa Oliveira[4], Philippe Langella[2] and Vasco Azevedo[1]

[1]Department of General Biology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[2]Unité de Recherches Laitières et de Génétique Appliquée, INRA, Jouy en Josas, France
[3]Laboratoire de Microbiologie, INRA, Rennes, France
[4]Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
Corresponding author: V. Azevedo
E-mail: vasco@mono.icb.ufmg.br

Abstract. *Lactococcus lactis*, the most extensively characterized lactic acid bacterium, is a mesophilic- and microaerophilic-fermenting microorganism widely used for the production of fermented food products. During industrial processes, *L. lactis* is often exposed to multiple environmental stresses (low and high temperature, low pH, high osmotic pressure, nutrient starvation and oxidation) that can cause loss or reduction of bacterial viability, reproducibility, as well as organoleptic and/or fermentative qualities. Among these stress factors, oxidation can be considered one of the most deleterious to the cell, causing cellular damage at both molecular and metabolic levels. During the last two decades, considerable efforts have been made to improve our knowledge of oxidative stress in *L. lactis*. Many genes involved with both oxidative stress resistance and control mechanisms have been identified; functionally they seem to overlap. The finding of new genes, and a better understanding of the molecular mechanisms of stress resistance in *L. lactis* and other lactic acid baterium, will lead to the construction and isolation of stress-resistant strains. Such strains could be exploited for both traditional and probiotic uses.

Key words: *Lactococcus lactis*, Oxygen, Stress

INTRODUCTION

Lactic acid bacteria (LAB) comprise a group of Gram-positive and facultative anaerobic microorganisms able to convert carbohydrates into lactic acid via homo- or heterofermentative metabolism (Stackebrandt and Teuber, 1988; Thompson, 1988). Due to this latter feature, some food-grade LAB species (*Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp., *Streptococcus* sp., and *Lactococcus* sp.) are widely used in the production of fermented food (vegetables, milk and meat), where these bacteria are responsible for both preservation and sensory characteristics, such as color, flavor and texture. Moreover, probiotic properties are attributed to some species of lactobacilli (Seegers, 2002).

Lactococcus lactis is a mesophilic- (optimal growth temperature around 30°C) and microaerophilic-fermenting LAB (Duwat et al., 2000) with a relatively small genome (2.5 Mbp; Bolotin et al., 2001). This bacteria is commonly found in nature, on plant and animal surfaces and is widely used in the dairy industry for the production of fermented products, like cheese and buttermilk. Since over 10^7 tons of cheeses are annually produced worldwide, *L. lactis* is of great economic importance (Henriksen et al., 1999).

Until the late 1970s, *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* were empirically used by the dairy industry (Davidson et al., 1996; Henriksen et al., 1999). Thereafter, impelled by the advent of recombinant DNA technology, our knowledge about many biological aspects (e.g., microbiology, physiology, biochemistry, and mainly genetics) of these bacteria, and of other LAB, has greatly improved. Since then, the use of genetic tools has enabled the construction of many genetically modified strains that exhibit better controlled and/or improved growth and acidification, bacteriophage resistance and improved proteolytic properties (de Vos, 1999). Today, *L. lactis* is the most extensively characterized LAB (Bolotin et al., 2001), and it has been used to produce heterologous proteins of high biotechnological and medical interest, such as enzymes and antigens; it also has potential as a live vaccine (Langella and Le Loir, 1999).

However, despite all the available data, the loss of some bacterial phenotypes, affecting viability, reproducibility, organoleptic, and/or fermentative capabilities, remains difficult to circumvent. In most cases, these problems are caused by the exposure of LAB to a range of stressful environmental conditions in both natural and/or industrial habitats. Industrial processing exposes *L. lactis* to multiple stress conditions, such as low and high temperature, low pH, high osmotic pressure, nutrient starvation, and oxidation.

During the last two decades, several research groups have intensively studied stress responses in *L. lactis*. We describe general and specific aspects of oxidative stress in *L. lactis* and current insights related to the construction and isolation of both recombinant and non-recombinant *L. lactis* oxidative stress-resistant strains.

OXIDATIVE STRESS

Bacterial stress can be defined as a physiological perturbation, caused by environmental modifications (physical, chemical and/or nutritional) that can have many consequences for the bacteria, such as retarded growth and cell death (Farr and Kogoma, 1991; Fridovich, 1998; Duwat, 1999). Oxidative stress can cause several types of damage to the bacterial cell, including: i) metabolic pathway disruptions, ii) spontaneous mutations, and iii) bacteriostatic and bactericidal effects (Berlett and Stadtman, 1997; Fridovich, 1998).

Oxygen by itself is unable to cause any damage to the cell; however, during the cellular processes (metabolic pathway), O_2 is partially reduced to water, leading to the formation of reactive O_2 species, which are the superoxide anion radical (O_2^-), the hydroxyl radical ($OH^\bullet$), and hydrogen peroxide (H_2O_2). These intermediates have a high oxidizing potential and thus are responsible for cellular oxygen toxicity (Farr and Kogoma, 1991; Fridovich, 1998; Storz and Imlay, 1999).

OXIDATIVE STRESS IN *LACTOCOCCUS LACTIS*

Described as a microaerophilic-fermenting LAB, *L. lactis* can, under certain conditions, tolerate and even use O_2 (Condon, 1987; Duwat et al., 2001). Nevertheless, O_2 consumption results in an altered redox state, and consequently greater NADH oxidase activity. Sugar fermentation is shifted towards mixed fermentation (Figure 1); hence, O_2 participates in the oxidoreduction steps of NADH to NAD^+. Through the action of NADH oxidases, H_2O_2 is formed (Table 1; Condon, 1987). The O_2^- can be generated through the following reaction: $NADH + 2O_2 \rightarrow NAD^+ + H^+ + 2O_2^-$; which occurs because the flavin group of NADH oxidase carries out single-electron transfer, as well as the transfer of two or four electrons (Thomas and Pera, 1983; Imlay and Fridovich, 1991). The $OH^\bullet$ can be formed during the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow OH^\bullet + H_2O + Fe^{3+}$) (Duwat et al., 1995) or by spontaneous reactions ($O_2^- + H_2O_2 \rightarrow OH^- + OH^\bullet + O_2$) (Condon, 1987).

Oxidative stress damages at the molecular level

At the molecular level, O_2^-, $OH^\bullet$ and H_2O_2 can react with cellular targets, such as proteins and nucleic acids. O_2^- has a moderate oxidizing potential and can attack compounds such as polyphenols, ascorbate and catecholamines (Farr and Kogoma, 1991; Fridovich, 1998). H_2O_2 can directly oxidize protein cysteinyl residues, thus inactivating enzymes (Storz and Imlay, 1999). It can also react with cations, such as Fe^{2+} and Cu^+, and give rise to $OH^\bullet$, through the Fenton reaction (Farr and Kogoma, 1991; Duwat et al., 1995; Fridovich, 1998). $OH^\bullet$ is a strong oxidant agent that can attack most organic compounds and can cause strand breaks and a wide spectrum of base modifications in DNA (Czapski, 1984; Farr and Kogoma, 1991; Fridovich, 1998). Another type of damage is the peroxidation of membrane lipids and membrane protein alterations, affecting cell permeability and osmoregulation (Harley et al., 1978; Kong and Davison, 1980).

Oxidative stress damages at the metabolic level

Many effects of O_2 are also observed at the metabolic level. Under anaerobic conditions, *L. lactis* has a fermentative metabolism that enables the transformation of various types of carbohydrates into lactic acid (Figure 1) (Holt et al., 1994; Lopez de Felipe et al., 1997, 1998). In this case, 2 NADH molecules, generated from the oxidation of glyceraldehyde-3-phosphate, are reoxidized to favor the reduction of pyruvate to lactic acid by the action of lactate dehydrogenase (LDH). However, the NADH/NAD^+ ratio plays a determinant role in the shift control from homolactic acid to mixed-acid fermentation in *L. lactis* (Garrigues et al., 1997). Under aerobic conditions, increased expression and activities of NADH oxidase and NADH peroxidase (Table 1) compete with LDH for NADH molecules (Murphy and Condon, 1984). Conse-
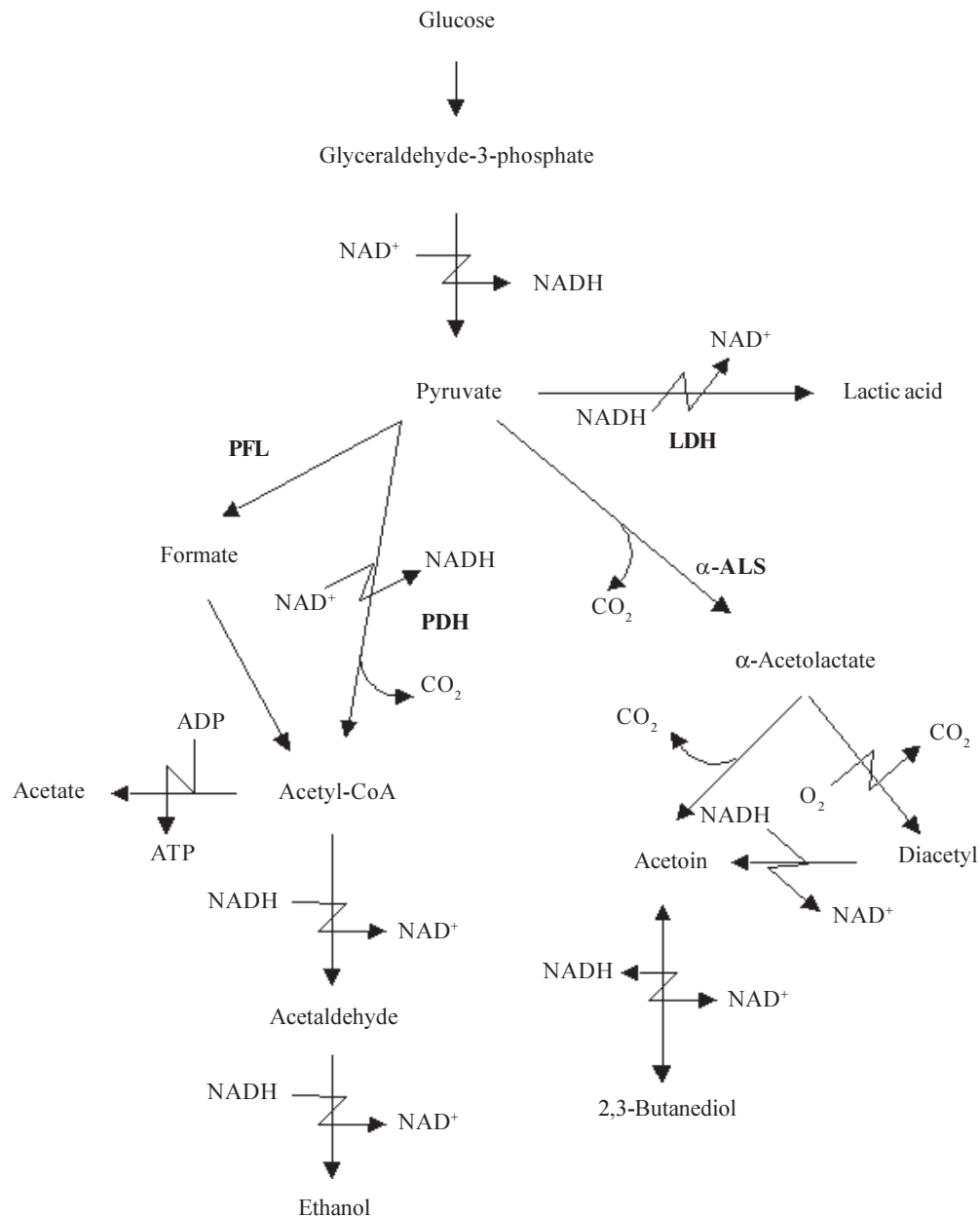
Glucose

Glyceraldehyde-3-phosphate

NAD^+ → $NADH$

Pyruvate

PFL

NAD^+ → $NADH$ Lactic acid

$NADH$ **LDH**

Formate

NAD^+ → $NADH$

PDH

CO_2 α-**ALS**

CO_2

α-Acetolactate

ADP

Acetate   Acetyl-CoA

ATP

CO_2 CO_2

$NADH$ O_2

Acetoin   Diacetyl

$NADH$ → NAD^+

NAD^+

$NADH$ → NAD^+

Acetaldehyde

$NADH$ ← NAD^+

$NADH$ → NAD^+

2,3-Butanediol

Ethanol

Figure 1. Schematic pathway of glucose metabolism in *Lactococcus lactis*. Intermediate and final glucose metabolism products are indicated by arrows. Catalytic enzymes are abbreviated in bold (LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PFL: pyruvate-formate lyase; α-ALS: α-acetolactate synthase).

quently, lactic acid production is reduced and glycolytic flux is shifted towards the production of acetate, ethanol, acetoin, diacetyl and CO_2 (mixed-fermentation) by the action of pyruvate dehydrogenase (PDH), pyruvate-formate lyase and α-acetolactate synthase (Figure 1). These changes also lead to the formation of H_2O_2, which causes a reduction of the growth rate of *L. lactis*, and even its death. Concentrations of around 0.2 mM H_2O_2 inhibit the growth of this

Table 1. Enzymatic reactions utilizing oxygen in lactic acid bacteria and the respective catalytic enzymes (after Condon, 1987).

Enzymatic reactions	Catalytic enzymes
$NADH + H^+ + O_2 \longrightarrow NAD^+ + H_2O_2$	NADH: H_2O_2 oxidase
$2NADH + 2H^+ + O_2 \longrightarrow 2NAD^+ + 2H_2O$	NADH: H_2O oxidase
pyruvate + phosphate + $O_2 \longrightarrow$ acetylphosphate + CO_2 + H_2O_2	Pyruvate oxidase
α-glycerophosphate + $O_2 \longrightarrow$ dihydroxyacetone phosphate + H_2O_2	Alpha-glycerophosphate oxidase
$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$	Superoxide dismutase
$NADH + H^+ + H_2O_2 \longrightarrow NAD^+ + 2H_2O$	NADH peroxidase

bacterium by 50% and concentrations >1.15 mM H_2O_2 can compromise cell viability (Anders et al., 1970; Duwat et al., 1999). Although NADH peroxidase (Table 1) contributes to cellular H_2O_2 detoxification, its activity is generally low (10 to 30 times lower than that of NADH oxidase) and endogenous or exogenous H_2O_2 overcomes the cell's capability to deal with this oxygen species (Anders et al., 1970; Condon, 1987).

OXIDATIVE STRESS RESISTANCE MECHANISMS IN *LACTOCOCCUS LACTIS*

How does *L. lactis* overcome damage caused by active oxygen species? Experimental data and genomic analyses indicate that *L. lactis*, similar to *Escherichia coli* and *Bacillus subtilis*, is equipped with general and specific stress response mechanisms (Duwat et al., 1999). Several genes (see below) that participate in these mechanisms have been identified, and the respective encoded proteins have been shown to contribute to oxidative stress resistance. Moreover, induction of some of these genes is growth phase-dependent (exponential or stationary), and their products confer multi-stress resistance (Farr and Kogoma, 1991; Kjelleberg et al., 1993; Walker, 1996; Duwat et al., 2000).

NADH oxidase/NADH peroxidase

The most common oxidative stress resistance mechanism found in *L. lactis*, as well as in other LAB, is performed by a coupled NADH oxidase/NADH peroxidase system (Table 1; Condon, 1987). Initially, intracellular O_2 can be eliminated by NADH oxidase activity, which utilizes O_2 to oxidize NADH into NAD^+, thereby giving rise to H_2O_2. Furthermore, NADH peroxidase reduces H_2O_2, generating H_2O. However, NADH peroxidase activity is low, and considering that *L. lactis* does not possess any catalase activity, cellular H_2O_2 detoxification is inefficient. It was recently proposed that at high glycolytic fluxes, *L. lactis* ATCC19435 (*L. lactis* subsp. *lactis*, lacking both NADH peroxidase and superoxide dismutase (Sod) activities) metabolically produced pyruvate reacts nonenzymatically with H_2O_2 to form H_2O and acetate (van Niel et al., 2002), thus providing the cell with an alternative mechanism of protection against H_2O_2.

Superoxide dismutase

A second common resistance mechanism is provided by the action of a Sod that re-

moves O_2^- radicals (Table 1; Condon, 1987). In prokaryotes, the classification of these enzymes depends on the metal cofactor content (Cu-Zn, Fe or Mn). Most LAB have Sod activity. A unique manganese-containing Sod (MnSod) has been identified in *L. lactis*, during an analysis of acid stress-induced protein expression (Sanders et al., 1995). The growth rate of *sodA* mutants, under anaerobic conditions, is similar to that of the wild type strain, whereas under aerobic conditions the growth rate of this mutant strain is reduced (Sanders et al., 1995). Moreover, under anaerobic conditions, *L. lactis sodA* has a low initial expression, which is shifted to a high gene expression level under aerated conditions. An alternative mechanism to eliminate O_2^- radicals, which could compensate the low Sod activity, can be provided by high levels of intracellular glutathione (Fahey et al., 1978; Wiederholk and Steele, 1993).

RecA

Another oxidative stress resistance mechanism is supported by RecA activity. This protein is commonly found in bacteria, such as *E. coli*, where it plays a key role in the SOS response and homologous recombination (Miller and Kokjohn, 1990; Walker, 1996). Duwat et al. (1995) constructed a *L. lactis recA* mutant strain, and showed that RecA is involved in resistance to oxidative and thermal stresses. They observed that during exponential and stationary growth phases, *recA* mutants are highly sensitive to aeration, resulting in a reduction in growth rate and viability. They also postulated that in the absence of RecA activity, the OH• radical, generated during the Fenton reaction, is responsible for damage to DNA. To test this hypothesis, an iron chelator was added to aerated cultures of *L. lactis recA* mutants; bacterial doubling time was restored close to that of anaerobic conditions, leading to the observation that i) O_2 radicals are responsible for oxygen toxicity and that ii) RecA plays an essential role in the repair of DNA damage caused by these compounds. They also studied the function of RecA under thermal stress. Under both oxidative and thermal stress, *recA* mutants have an elevated expression of HflB protein, which transcriptionally down-regulates heat-shock proteins, such as DnaK, GroEL and GrpE, and thus keeping these heat-shock proteins from being induced under elevated temperature conditions (above 37°C). Moreover, *recA* of *L. lactis* is co-transcribed with another DNA repair gene, formamidopyrimidine DNA glycosylase (*fpg*) (Duwat et al., 1992, 1995). In conclusion, activation of *recA* by oxidative stress can confer cross-protection against thermal stress. A single stimulus can activate various stress resistance mechanisms, conferring protection against various types of stress in *L. lactis*.

Cytochrome *d* oxidase (*cydA*)

Another way to cope with oxidative stress would be efficient O_2 uptake and utilization, as in the case of bacteria able to respire. Despite its fermentative metabolism classification, there is evidence that *L. lactis* can undergo respiration in aerated culture media in the presence of heme (an iron-containing porphyrin molecule that forms the oxygen-binding portion of molecules such as hemoglobin) (Sijpesteijn, 1970). More recently, based on *L. lactis* IL1403 genome data, it was found that this bacterium possesses some of the genes needed for respiration, such as *cydA*, which encodes for cytochrome *d* oxidase (Bolotin et al., 1999). During respiration, CydA directly participates in the electron transfer chain, where O_2 is the terminal electron acceptor, thus generating ATP. To test these possibilities and the respiration capacity of *L.*

lactis, a *cydA* mutant strain was constructed (Duwat et al., 2001). Under aerated conditions, in the presence of heme, it was observed that i) *L. lactis cydA* expression is induced during the stationary growth phase; ii) this bacterium has an extended growth period and improved long-term survival, and iii) its metabolism is shifted from fermentation to respiration, as demonstrated by reduced lactic acid production and elevated acetate, acetoin and diacetyl formation. Based on this evidence, it is clear that *L. lactis* is able to respire, and probably this ability enables it to overcome oxidative stress injuries.

Adaptation

Another resistance mechanism in *L. lactis* is adaptation. It is known that in response to environmental changes, bacteria can trigger adaptive mechanisms that enable cells to tolerate harsh conditions (Condon, 1987; Woojin et al., 1999, 2002). Previously, it was observed that lactococci exposed to sublethal concentrations of H_2O_2 became able to grow in the presence of lethal concentrations (≥ 1.15 mM) of H_2O_2 (Anders et al., 1970; Condon, 1987). More recently, it has been shown that adaptation to a specific stress, such as UV irradiation, starvation or high temperature, can lead to multi-stress resistance acquisition, probably due to the induction of genes that have functions in common in the resistance mechanisms against various types of stress (Duwat et al., 1995; Hartke et al., 1996, 1997; Duwat, 1999).

CONTROL OF OXIDATIVE STRESS RESPONSES IN *LACTOCOCCUS LACTIS*

Oxidative stress resistance mechanisms in *L. lactis* seem to be overlap, and thus cells under one specific stress condition can trigger different stress responses. Some studies have provided us with interesting clues concerning the control of oxidative stress responses in *L. lactis* (Duwat et al., 1999, 2000; Rallu et al., 2000; Scott et al., 2000; O'Connell-Motherway et al., 2000).

Sigma factors

Control at the transcriptional level, by sigma (σ) factors would be an efficient way to modulate stress responses. However, whereas *E. coli* and *B. subtilis* have stress specific σ-factors (Lange and Hengge-Aronis, 1994; Herman et al., 1995; Hecker et al., 1996), *L. lactis* only has three σ-factors: RpoD, ComX and SigX (Araya et al., 1993; Bolotin et al., 1999, 2001). The vegetative σ-factor, RpoD (Araya et al., 1993), allows the binding of RNA polymerase and the initiation of transcription at the so-called "vegetative promoters", whereas ComX function is likely to induce competence genes (Bolotin et al., 1999, 2001). Although SigX, an extracytoplasmic function (Helmann, 2002) RNA polymerase σ-factor in *B. subtilis* has been associated with heat stress resistance (Huang et al., 1997), no function for the lactococcal SigX has been proposed.

Metabolite flux sensors

The cAMP receptor protein, the fumarate and nitrate reduction regulator (FNR) and the FNR-like proteins (Flp) are members of a superfamily of structurally related transcriptional

factors that could be involved in the control of stress responses (Green et al., 2001). Commonly found in a wide range of bacteria, these proteins possess structural properties that provide cells with versatile sensory (transducing environmental and metabolic signals) and DNA/RNA-regulatory mechanisms (DNA recognition motifs and RNA polymerase interaction) that trigger general and specific responses to particular physiological conditions, such as stress. In both *E. coli* (Jordan et al., 1997) and *Lactobacillus casei* (Gostick et al., 1998), FNR and Flp play a key role in oxidative stress by interaction with an oxygen-labile [4Fe-4S] cluster. *L. lactis* possesses two Flp encoding genes, *flpA* and *flpB*, located in two paralogous operons (*orfX-orfY-flp*), where the proximal genes appear to encode potential metal ion transport systems (Gostick et al., 1999). To further characterize the *L. lactis* Flp proteins, single and double mutants of *flpA/B* were constructed (Scott et al., 2000). No phenotypic effects were observed between the single mutant and the wild-type strain when submitted to oxidative stress conditions (H_2O_2). However, under the same conditions the double mutant was more affected, as evidenced by i) an increased lag phase, ii) an altered polypeptide profile, and iii) a lower intracellular zinc [Zn(II)] content. This indicates that at least one of the *L. lactis* Flp must be functional in order to be able to cope with oxidative stress. Based on these observations, it was suggested that FlpA/B may be involved in the control of Zn(II) uptake and stress resistance mechanisms. It was also postulated that *L. lactis* uses Zn(II) to protect protein thiol groups from oxidation and that intracellular levels of this compound may be functioning as a "metabolite flux sensor" (MFS), sensing and controlling general stress responses.

An example of how MFS can control stress responses was given by the construction of *L. lactis recA*-derived mutant strains. The *L. lactis recA* strain is sensitive to both oxidative and thermal stresses. Therefore, in order to identify other mechanisms related to this sensitivity, *recA*-derived strains were isolated by random insertional mutagenesis (Duwat et al., 1999). Most mutated genes were found to encode proteins involved in metabolic pathways for the biosynthesis of guanosine phosphate (GP) (*deoB*, *guaA* and *tktA*) or in phosphate uptake (*pstS* and *pstB*). These mutations conferred resistance to multiple stresses and it was postulated that low intracellular levels of GP and/or phosphate are functioning as MFS, controlling general stress responses. This hypothesis was later confirmed by Duwat et al., 2000, who showed that depletion of GP in growth media of wild-type strains was able to induce general stress resistance; otherwise, the addition of guanine (over 15 µg/ml) to the growth medium from a *guaA*/ *recA*-derivative mutant strain resulted in complete loss of the stress-resistant phenotype. The involvement of phosphate pools was also demonstrated with the *pstB*/*recA*-derivative mutant strain (Duwat et al., 2000). Similar results were obtained during an independent selection of acid-resistant mutants of *L. lactis*, which were also found to be resistant to oxidative stress caused by H_2O_2 (Rallu et al., 2000).

Two-component regulatory systems

Control of stress responses could also be achieved by cellular systems that sense and transmit environmental signals into the cell, thereby modulating gene expression and physiological changes. These systems have been described in both prokaryotic and eukaryotic organisms, and are known as "two-component regulatory systems" (Chang and Stewart, 1998). Basically, these systems are composed of a membrane-anchored sensor protein, usually a histidine protein kinase, and an intracellular response regulator. In *L. lactis*, six of these systems have been

identified (O'Connell-Motherway et al., 1997) and three of these (systems B, D and F) are involved in specific susceptibility to acid, osmotic and oxidative stress (O'Connell-Motherway et al., 2000). In the latter (system F), the *L. lactis* mutant strain, obtained by insertional mutagenesis, has greater H_2O_2 sensitivity than the wild-type strain (O'Connell-Motherway et al., 2000). At least in this case, it seems that the control of stress responses are stress specific.

In summary, many questions remain and it is possible that control of stress resistance mechanisms in *L. lactis* operates through pleiotropic effects or even through multiple regulatory mechanisms, some of which may be unique to this bacterium.

PRESENT AND FUTURE DIRECTIONS

Despite all the efforts that have been made to identify and improve our knowledge about oxidative stress resistance and its control mechanisms in *L. lactis*, a number of questions remain unanswered. Many aspects of general and specific stress in this bacterium differ from model microorganisms, such as *E. coli* and *B. subtilis*. Thus, it becomes an important challenge to isolate and construct new stress resistant strains that can help to answer these questions.

Most studies concerning stress in *L. lactis* concern the production of stress-resistant mutant strains; depending on the method employed to obtain these strains, they can be classified as: i) "non-genetically modified mutant strains" (non-GMM) or ii) "genetically modified mutant strains" (GMM). In the former, stress resistant strains are acquired through screening in selective media, or are isolated from industrial sources or from their natural habitat. Nevertheless, these types of mutants are not very informative, due to difficulties in identifying possible genes and/or metabolic pathways involved in stress resistance. Alternatively, such genes or pathways can be characterized by the identification and analysis of stress-induced proteins in two-dimensional polyacrylamide gel electrophoresis (Giard et al., 2002). GMM strains can be constructed by site-directed mutagenesis (homologous recombination utilizing non-replicative plasmids; Leloup et al., 1997; O'Connell-Motherway et al., 2000) or by random insertional mutagenesis (transposition elements; Schafer et al., 1991; Maguin et al., 1992). One of the most powerful tools that has been developed is the thermo-sensitive plasmid pG+host:IS*S1* (Maguin et al., 1996), which permits insertional gene inactivation and subsequent determination of its involvement in bacterial stress.

In addition to basic knowledge of the *L. lactis* stress response, obtained through the construction of stress resistant strains, new information about oxidative and other types of stress has opened new possibilities for their practical application. Today, similar findings have been applied to the metabolic engineering of sugars and other molecules (Lopez de Felipe et al., 1997, 1998; Hugenholtz et al., 2002; Koebmann et al., 2002; Cocaign-Bousquet et al., 2002), in the development of new expression systems (Sanders et al., 1998; Madsen et al., 1999), and to develop new anti-oxidant strains of *L. lactis* that could become useful in medicine and industry.

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