Molecular Mechanisms of Stress Resistance in *Lactococcus lactis*

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Lactococcus is an economically important starter culture bacterium extensively used in the manufacture of the both soft and hard cheeses. During its growth and storage, and throughout cheese processing, it encounters a variety of stresses including osmotic, oxidative, temperature, acid and cell envelope stress. These stressors can cause damage to DNA, proteins, lipid membranes and peptidoglycan and can lead to reduced growth and acidification rates and if severe enough, cell death. Despite its small genome (~2.5Mbp) *Lactococcus* is equipped to withstand stress through a number of specific and non-specific mechanisms. Genes involved in stress resistance and/or responding to stress have been identified using a variety of approaches including site-directed mutagenesis, random mutagenesis, microarrays, proteomics and bioinformatic methods. Further research utilising current and novel methods will yield a better understanding of how *Lactococcus* deals with stress and lead to innovations in industrial fermentation processes.

Keywords *Lactococcus*; starter culture; stress; oxidative; acid; heat; genes

1. Introduction

The preservation of milk by microbial fermentation is thought to date back 8000 years to the time the first animals (probably sheep and goats) were domesticated and, with the exception of wine production, represents one of the oldest forms of food preservation known to man [1]. Since that time many different cheese, yoghurt and liquid fermented milk products have arisen and similarly, the microbial flora used for the generation of these products has become highly diverse. Most fermentations used for cheese production can be regarded as either lactic, mold-lactic or yeast-lactic fermentations [2]. Lactic acid bacterial starter cultures, such *Lactococcus lactis*, are common to most modern cheeses. They are used primarily to acidify milk which causes the milk substrate to clot, forming the curd. Rennet, a proteolytic enzyme originally derived from the abomasum of the calf, but now obtained from recombinant sources, is often added to facilitate proper curd formation. Acidification is also important for the preservation of cheese, which if left as milk, would have a much shorter shelf life [1]. In addition to acidification, *L. lactis* also contributes to the flavour of cheese.

During cheese manufacture *L. lactis* is exposed to a variety of environmental stresses and suboptimal growth conditions. Starter cultures prepared for long term storage are subject to lyophilization or spray drying leading to osmotic and oxidative stresses [3]. Salt is commonly added to cheese for flavour and to control bacterial growth and acidification, also causing osmotic stress. Low temperatures are used during storage of frozen starter cultures (-20^{o}C) and cheese maturation (8-16^{o}C) and in addition, high temperatures (~ 40^{o}C) are used during the cooking of cheese to limit growth. Carbohydrate starvation and acid stress occur simultaneously as milk lactose is metabolized to lactate during fermentation [3]. Finally, during cheese maturation, enzymatic hydrolysis of the cell wall initially causes stress and ultimately results in cell lysis [4].

Characterization of the molecular mechanisms underlying stress responses of *L. lactis* has received increasing attention in recent years. A similar trend can also be seen throughout microbiology and general cell biology research. This can be at least partially attributed to the technical development and reducing costs of transcriptome- and proteome-wide screening techniques such as microarrays and 2D-gel electrophoresis respectively. The over-arching results from this research is that cells sense stress and respond to it based on the macromolecular damage caused, rather than the type of stress the damage is caused by [5]. For this reason, many proteins have been implicated in response to multiple types of stress. A more detailed picture of how *L. lactis* responds to various stresses is illustrated herein.

2. Oxidative Stress

Reactive oxygen species (ROS) from the environment or generated within the cell, have a major impact on cell physiology. Oxidation damages most types of biomolecules and as such, a highly complex and integrated system has been developed by *L. lactis* to combat this type of stress. Interestingly, recent work has alluded to the fact that oxidation is exacerbated by other stresses such as high and low temperature and acid pH.

2.1 Reactive Oxygen Species (ROS)

The hyrdoxyl free radical OH• is responsible for most, if not all, oxidative reactions with biomolecules [6]. Its production occurs via the Fenton reaction.

$H_2O_2 + Fe^{2+} \rightarrow OH^- + FeO_2 + H^+ \rightarrow Fe^{3+} + OH^- + HO\bullet$ [6]

Fe^{2+} plays a key role in facilitating H_2O_2 reactivity. H_2O_2 interacts with the d-orbitals of Fe^{2+} to lower the activation barrier required to break the dihydrogen bond, making H_2O_2 a far more efficient electron donor [6]. The availability of other ROS such as molecular oxygen O_2, the superoxide radical O_2^- and its protonated form H_2O^- are required to generate hydrogen peroxide (H_2O_2) and to determine the mechanism of oxidation [7].

2.2 Protein Oxidation

Cellular proteins are sensitive to oxidation. Oxidation modifies the structure and function of proteins beyond their native state. Oxidation of the protein backbone can lead to either protein hydrolysis or protein-protein cross linkages depending on the reactive oxygen species available [7]. Amino acid side chains are also vulnerable to oxidation, which can result in numerous oxidation products. The sulfur containing amino acids cysteine and methionine are the most sensitive to oxidation, forming disulfides and methionine sulfoxide respectively. Interestingly, most organisms (including *L. lactis*) produce reductase enzymes capable of reducing disulfides and methionine sulfoxide back to their native state [7]. Cysteine and methionine are the only amino acids that can be reduced following oxidation and as such, are preferentially oxidized to protect the other amino acids [7]. Iron-sulfur clusters employed at the active site of many enzymes and cofactors are also targets for oxidation [6].

2.3. Lipid Oxidation

Lipid oxidation in mammalian cells has been extensively studied and causes severe cytotoxic effects. Poly-unsaturated phospholipids, glycolipids and cholesterol are the primary targets of lipid oxidation in mammals [8]. Because bacteria produce mono-unsaturated fatty acids rather than poly-unsaturated fatty acids, ROS cause only moderate damage to bacterial lipid membranes [6].

2.4 DNA Oxidation

DNA is sensitive to oxidation and the effects of which are often deleterious or lethal. Fe^{2+} readily binds to both DNA and RNA, and with the availability of H_2O_2, facilitates localized production of OH• via the Fenton reaction. OH• can interact with the sugar and base moieties of DNA. The resulting DNA radicals can be resolved by the DNA repair machinery however, base mutations are often introduced into the DNA sequence [6].

2.5 Integrated Response to Oxidation

Research on *Bacillus*, *Staphylococcus*, *Streptococcus* and *Lactococcus* forms the basis of current insight into oxidative stress responses of Gram positive bacteria. Table 1 lists some recent research identifying important genes from *L. lactis* involved in the oxidative stress response. General stress resistance genes such as the so called heat shock proteins (HSPs) have been associated with oxidative stress [9]. Similarly, *spx* genes that function as global regulators have also been implicated in the response to oxidative stress [10, 11]. Both HSPs and Spx proteins are discussed in greater detail in sections 8.2 and 8.3 respectively.

In *L. lactis* molecular oxygen (O_2) can be removed from the environment via conversion to water by water-forming NADH oxidase (NoxE). An alternative route for the consumption of molecular oxygen is by peroxide-forming NADH oxidase (AhpF) and conversion of peroxide to water by an alkyl hydroperoxide reductase (AhpC). Dismutation of superoxide (O_2^-) by superoxide dismutase (SodA) is also a major contributor to H_2O_2 production. It has been demonstrated that each of these enzymes are functional in *L. lactis* subsp. *lactis* IL1403 [12]. Contrary to this, under aerobic conditions accumulation of H_2O_2 in *L. lactis* subsp. *lactis* ATCC19435 was not alleviated by enzymatic reduction (alkyl hydroperoxide reductase), but was found to be relieved non-enzymatically by pyruvate (the end product of glycolysis) particularly under conditions of high metabolic flux [13]. The observed deficiency of alkyl hydroperoxide reductase activity appears to be dependant on gene expression rather than the functionality of the enzyme [12].

The role of NADH oxidases (NoxE and AhpF) and components of the electron transport chain (MenC and NoxAB) in modifying the redox potential of milk has been investigated [14]. Under normal conditions milk has a redox potential of 400mV which can be attributed to dissolved oxygen and other oxidizing compounds found normally in milk. After inoculation and growth of *L. lactis* subsp. *cremoris* TIL46 the redox potential of milk drops to -220mV. This reduction of the culture medium was also observed in a large percentage of industrial *L. lactis* subsp. *cremoris* strains. In strain TIL46 a *noxE* mutant failed to reduce the redox potential of milk, suggesting that the removal of oxygen from milk by NADH oxidase activity is integral in generating a reducing environment. Mutation of the genes *menC*, and *noxAB* also impaired the ability of *L. lactis* to generate a reducing environment. It was also hypothesized that menaquinones located in the cell membrane may reduce external oxidized sulfur groups and metal ions to generate such a reducing environment [14]. Recently, and in partial agreement with this, removal of dissolved oxygen was found not to play a role in reducing the redox potential of MRS broth to values below zero; however, exposed thiol groups of membrane

and cell wall bound proteins were responsible for generating redox potentials as low as -200mV [15]. In addition to this, no accumulation of reducing metabolites were observed, which is in contrast to other bacteria [15].

Table 1 Genes identified in the *L. lactis* oxidative stress response

Source	Main Identification Methods	Gene/s	Function
[16]	Proteomics, gene inactivation	*nrdHIEF* *flpA,B*	Ribonucleotide reductase system Regulators of *nrdHEIF* operon
[17]	IS*S1* insertion	*pstFEDCBA* *phoU*	Phosphate transport operon Phosphate regulator
[18]	Restriction mapping	*fpg (mutM)*	Formamido-pyrimidine DNA glycosylase (DNA repair)
[9]	Sequence homology, Gene inactivation, Western blots	*recA* *groEL* *dnaK*	DNA repair Heat shock proteins, (Protein refolding) Heat shock proteins
[10]	IS*S1* insertion	*trmA*	Gene regulation (*spx* homolog)
[19]	Gene inactivation	*ccpA*	Catabolite control protein
[12]	Sequence homology, Protein expression, Purification	*ahpF* *ahpC* *noxE*	Peroxide-forming NADH oxidase Peroxidase Water-forming NADH oxidase
[20]	Proteomics, Bioinformatics, Gene inactivation	*copR* *copZ* *copA*	Regulator of Cu^{2+} homeostasis Cu^{2+} chaperone Cu^{2+} export ATPase
[21]	Microarrays, Gene inactivation	*ygfCBA*	Regulation of intracellular heme pools
[22]	IS*S1* insertion	*pstB,S* *arl1, arl2* *guaA*	Phosphate transport Unknown Purine nucleotide biosynthesis
[23]	Sequence homology	*hexA*	DNA mismatch repair
[24]	Phenotype testing	*flpA,B* $orfW_{A/B}$ $orfX_{A/B}$ $orfY_{A/B}$	Regulation of Zn^{2+} uptake and Storage Zn^{2+} uptake Zn^{2+} transporter Zn^{2+} storage, DNA binding
[25]	Sequence homology, Structure determined	*dpsA,B* *($orfY_{A/B}$)*	DNA binding proteins – directly protect DNA from oxidation (also bind Zn^{2+})
[14]	Gene inactivation	*noxE* *ahpF* *menC* *noxAB*	Water forming NADH oxidase Peroxide forming NADH oxidase Menaquinone synthesis Membrane NADH dehydrogenase
[26]	Protein purification	*nrdDG*	Anaerobic ribonucleotide reductase
[11]	IS*S1* insertion	*mntH* *trmA* *choQ* *pstD*	Manganese/iron transporter Gene regulation (*spx* homolog) Proline transport ATPase Phosphate transport
[13]	Enzyme activity assays	*sodA* *noxE*	Superoxide dismutase NADH oxidase
[27]	Gene inactivation	*trxB1*	Thioredoxin reductase

Defence against oxidative stress via regulation of metal ion uptake has been suggested by a number of researchers. Insertional mutation of the manganese/iron transporter *mntH* of *L. lactis* subsp. *cremoris* MG1363 conferred resistance growth on tellurite (an oxygen radical generating compound) containing media and in aerated cultures [11]. Inactivation of *mntH* partially prevented the accumulation of Fe^{2+} within the cell, thus potentially limiting the production of OH• from H_2O_2 via the Fenton reaction. Copper homeostasis by CopR has been characterized in *L. lactis* subsp. *lactis* IL1403 [20]. A copper chaperone *copZ* and a copper exporter *copA* were also identified as well as a number of other relatively uncharacterized genes. The two oxidation states of copper (Cu^{+} and Cu^{2+}) facilitate its use by enzymes in redox reactions, but also in the generation of ROS, placing importance on its tight regulation.

Intracellular zinc concentration has been implicated in oxidative stress. A *L. lactis* subsp *cremoris* MG1363 *flpA/flpB* double mutant displayed hypersensitivity to H_2O_2 and was found to have reduced intracellular Zn^{2+} pools. It was proposed that the $flp_{A/B}$ controlled operon encoded proteins for Zn^{2+} uptake ($OrfW_{A/B}$), Zn^{2+} transport ($OrfX_{A/B}$) and

Zn^{2+} storage ($OrfY_{A/B}$) [24]. More detailed research into the function of *orfY*$_{A/B}$ showed that the two gene products bind Zn^{2+}, but function as DNA binding proteins that physically protect DNA from oxidation [25].

Phosphate has been suggested to play a role in the homeostasis of both copper and zinc. A *pstA* (phosphate transport) mutant was more resistant than wild-type *L. lactis* subsp. *cremoris* MG1363 to 0.5mM copper and 7mM zinc [17]. The same study showed that mutations within the phosphate transport operon (*pstFEDCBA*) and the phosphate regulator *phoU* are primarily responsible for resistance to dithiothreitol at high temperature (37^{o}C). Sensitivity of wild type *L. lactis* to dithiothreitol was not observed at the optimal growth temperature of 30^{o}C, but only at 37^{o}C under aerobic conditions, indicating for the first time in *L. lactis* that high temperature exacerbates oxidative stress [17]. Previous research had also shown that phosphate plays a role in defense against oxidative stress [11] and other stresses such as heat and acid [22].

The protection and efficient repair of DNA is essential for combating oxidation. The central enzyme in DNA repair is the recombinase RecA. Under stress conditions RecA is activated by binding to single-stranded DNA, which can then induce the expression of other DNA repair enzymes [28]. A *recA* mutant was constructed in *L. lactis* subsp *cremoris* MG1363 and displayed sensitivity to oxidative and heat stresses [9]. In cultures grown aerobically, wild type *L. lactis* had a doubling time of 32-34 minutes as opposed to the *recA* mutant which had a doubling time of 70-90 minutes. The *recA* mutant had an impaired ability to produce heat shock proteins DnaK, GroEL and GrpE. The Fpg protein (formamido-pyrimidine DNA glycosylase) of *L. lactis* has also been identified for its role in repairing DNA damaged under oxidative stress conditions [18].

L. lactis possess two ribonucleotide reductase systems [16, 26] which employ activator proteins containing thiol active redox centers that interact with catalytic reductase proteins to effectively reduce the target ribonucleotides. These proteins facilitate the synthesis of DNA from RNA and also the reduction of oxidized DNA. As mentioned in section 2.2, preferential oxidation of sulfur containing molecules is an integral part of oxidative defense. The thioredoxin reductase system reduces undesirable disulphide bonds of cellular proteins to thiols. A *trxB1* (thioredoxin reductase) mutant was constructed in *L. lactis* subsp. *cremoris* MG1363 and grew significantly slower than wild type *L. lactis* under low O_2 conditions, but was completely unable to grow under oxidative stress conditions. Proteomic analysis comparing the *trxB1* mutant to wild type *L. lactis* revealed a number of proteins with altered expression including well established oxidative stress responsive genes: superoxide dismutase (SodA), alkyl hydroperoxide reductase (AhpC) and menaquinone biosynthesis (MenB, MenD) [27].

2.5.1 Heterologous Gene Expression and Addition of Co-factors for Enhanced Oxidative Stress Defense

The oxidative stress resistance of *L. lactis* can be enhanced by heterologous expression of oxidative stress resistance mediators. Glutathione has been successfully expressed in *L. lactis* subsp. *cremoris* NZ9000 [29]. Wild type *L. lactis* can not synthesize glutathione as it does not possess the biosynthesis genes *gshA* and *gshB*. The glutathione positive strain showed a 2.5-fold higher resistance to 150mM (relatively high concentration) H_2O_2 when compared to the glutathione negative strain. In the same study, heterologous glutathione expression also relieved oxidant hypersensitivity of a *sodA* (superoxide dismutase) mutant [29].

Catalases are commonly found in aerobic organisms, but as may be expected of a microaerophilic bacterium, *L. lactis* does not possess a catalase gene. The *Bacillus subtilis* catalase (*katE*) was successfully cloned into *L. lactis* subsp *cremoris* NZ9000 [30]. Catalase is an antioxidant metalloenzyme that reduces toxic H_2O_2 to two H_2O molecules and O_2. The catalase positive strain displayed survival 300- and 800- fold in excess of the control strain when incubated for 1 hour in the presence of 2mM and 4mM H_2O_2 respectively [30].

The addition of hemin to aerobically grown cultures of *L. lactis* subsp. *cremoris* MG1363 improves growth and survival compared to typical anaerobically grown cultures [31]. *L. lactis* genome sequence analysis revealed the presence of genes necessary for electron transport and the later stages of hemin biosynthesis, however the biosynthesis genes *hemABCD* were notably absent. Cytochrome d oxidase (*cydA*) was found to be essential for hemin mediated respiration and it was proposed that its activity resulted in an oxygen trap, thus preventing the formation/accumulation of ROS [31]. Deregulation of heme uptake is detrimental to cell survival. A catabolic control protein (*ccpA*) disruption mutant in *L. lactis* subsp *cremoris* MG1363 revealed that heme addition during lag phase resulted in growth arrest and cell mortality [19]. CcpA is proposed to be an activator of FhuR, the repressor of the *fhuBGD* operon which is required for heme transport. Utilizing microarrays, the *yjgCBA* operon was found to be up-regulated in response to heme, but genes for heme biosynthesis, electron transfer and electron acceptors were not induced by respiratory metabolism compared to aerobic fermentative metabolism [21]. In wild type cells CcpA is responsible for the switch from fermentative metabolism to respiration in response to glucose depletion. Heme mediated respiratory metabolism is advantageous over fermentative metabolism due to decreased oxidation of proteins and damage to DNA [32].

3. Heat Stress

Denaturation of macromolecules is the primary result of heat stress. According to Le Chatlier's principle, an increase in temperature leads to an increase in enthalpy and a corresponding increase in entropy (disorder). In the case of proteins, disruption from their native structure by high temperature is a frequently observed and perhaps obvious effect. In addition to this, protein structure can also be disrupted by low temperature. At sufficiently low temperatures, protein non-polar groups (at the core of globular proteins) interact with water molecules to become hydrated, resulting in unfolding [33].

L. lactis is a mesophilic bacterium with an optimal growth temperature of 30°C and a maximal growth temperature of 40°C. Many genes have been associated with the response to elevated temperatures (Table 2). The *recA* gene of *L. lactis* is significantly involved in the response to heat stress [9] and is found in many other species of bacteria including *Escherichia coli* [34]. RecA facilitates repair to damaged DNA by binding to single-stranded DNA. Although RecA responds to DNA damage, its expression recruits other general stress defense mechanisms such as the heat shock proteins, mediating resistance to high temperatures and oxidative stress [9]. A later study identified another 6 genes unassociated with RecA conferring resistance to heat stress, the majority of which are linked to general stress responses [35]. The *pnpA* gene encodes a polynucleotide phosphorylase which according to the authors may be involved with the regulation of (p)ppGpp and the stringent response [35]. Similarly, mutations within the *pst* operon (phosphate transporter) of *L. lactis* have also been associated with acid [22] and oxidative stress resistance [11]. Mutation of *trmA* (thermo-resistant mutant A) has been associated with resistance to high temperature by a number of researchers [35], [10] and has since been identified as a component affecting oxidative stress resistance [11]. The mechanism of TrmA activity and its interaction with ClpP is discussed further in section 8.3

Table 2 Genes of *L. lactis* involved in the heat stress response

Source	Main Identification Methods	Gene	Function
[9]	Sequence homology, Gene inactivation, Western blots	*recA* *groEL* *grpE* *dnaK* *hflB*	DNA repair, heat shock protein induction Heat shock protein (protein folding) Heat shock protein (protein folding) Heat shock protein (protein folding) Regulatory protease
[35]	IS*S1* insertion	*deo* operon *guaA* *pnpA* *tktA* *pst* operon *trmA*	Nucleotide recycling GMP synthase Polynucleotide phosphorylase transketolase Phosphate transport Gene regulation (*spx* homolog)
[36]	Sequence homology	*clpP*	House-keeping protease
[10]	IS*S1* insertion	*trmA*	Gene regulation (*spx* homolog)
[22]	IS*S1* insertion	*relA* *recN* *deoB* *guaA* *htp* *pst*	Synthesis/degradation of (p)ppGpp DNA metabolism purine salvage purine synthase Purine salvage Phosphate transport
[37]	Sequence homology	*hdiR*	SOS response regulator
[38]	Sequence homology Gene inactivation	*ctsR*	Negative transcriptional regulator of the *clp* house-keeping protease gene
[39]	Sequence homology	*cspA,B,C,D,E*	Adaptation to cold shock

Growth at sub-optimal temperatures results in a dramatic slow down of metabolism. *L. lactis* subsp. *lactis* grown at 30°C has a doubling time of 48 min compared to a doubling time of 3 hr 30 min, 57 hr, and 7 days at 16°C, 8°C and 4°C respectively [40]. A genetic basis for cold shock adaptation in *L. lactis* subsp *cremoris* MG1363 has been investigated [39]. A five gene operon (*cspABCDE*) was characterized and was strongly induced by a temperature shift from 30°C to 10°C. The *csp* genes encode small proteins with a molecular weight range of 7.1 – 7.6 kDa and are expressed maximally 2 – 4 hr after the cold shock event [39]. The exact mechanism of Csp activity still remains elusive.

4. Acid Stress

L. lactis frequently encounters acid stress during fermentation as sugars are metabolized primarily to lactate during fermentative growth, which lowers the pH. Differences exist between the two *L. lactis* sub-species *lactis* and *cremoris* in their capacity to deal with acid stress [41]. Various industrial *L. lactis* subsp. *lactis* strains can retain limited viability after incubation for 3 hours at pH 2.5, whereas *L. lactis* subsp *cremoris* strains only retain viability as low as pH 3. After incubation for 3 hours at pH 4.5, which is relevant to cheese making (casein precipitation to form the curd) *L. lactis* subsp *lactis* shows higher viability ($10^{7.8}$) compared to *L. lactis* subsp *cremoris* ($10^{6.5}$) [41].

The genetic factors leading to acid tolerance have been investigated in *L. lactis* and Table 3 gives an overview of the genes identified thus far. The primary affecter of intracellular pH is the H^+ ATPase first identified in *L. lactis* subsp *lactis* (*Streptococcus lactis* at the time of discovery) [42]. The H^+ ATPase functions as a proton pump, transporting H+ against the concentration gradient at the expense of ATP from within the cell into the extracellular environment. The arginine deiminase (ADI) pathway contributes to acid stress resistance by the production of ATP, which can be used by the H^+ ATPase [43]. The ADI pathway regulates the intracellular ratio of arginine to ornithine. Arginine is immediately converted to ornithine upon uptake, liberating NH_3 which has a neutralizing effect, and converting ADP to ATP. The 3 enzymes catalyzing this reaction are arginine deiminase (*arcA*), ornithine carbamoyltranferase (*arcB*) and carbamate kinase (*arcC1, arcC2*), all of which are located within the cytoplasm. The uptake of more arginine is facilitated by the arginine-ornithine antiporter (*arcD1*), allowing ornithine into the external environment in exchange for arginine [43].

More recently, mutations conferring tolerance to acidic culture conditions have been identified, most of which also confer tolerance to other stresses such as oxidation and heat [22]. Of the mutants selected for resistance to acid stress, 11 out of 16 (68%) were also more resistant to oxidative and heat stress. Another study utilizing a proteomic approach identified many genes in response to acid stress that were also associated to the general stress response (eg heat shock proteins) [44]. The SodA and AhpC proteins were also isolated as protective against acid stress, although these genes are generally regarded as oxidative stress related genes. Evidence suggests that low pH levels are conducive to the formation and accumulation of oxygen radicals requiring the induction of oxidative stress defense mechanisms [44].

Pre-exposure to sub-lethal acid concentrations (pH 5) results in the ability of *L. lactis* cells to tolerate exposure to normally lethal acid concentrations. This phenomenon is known as the acid tolerance response (ATR) and results in the modified expression of 22% and 32% of proteins when cultured on M17 and chemically defined media respectively [45]. Notably, proteins involved in sugar metabolism were regulated to yield less acidic end-products, a finding in agreement with other research [46]. Proteins involved in pH homeostasis, heat shock and oxidative stress were all induced in the acid tolerance response, as was the betaine ABC transporter (BusAA) which is involved in osmotic stress resistance [45]. The transport of peptides and their degradation is favored over the transport of individual amino acids under acid stress, presumably due to the greater amino acid yield for one molecule of ATP under conditions of ATP limitation caused by maintenance of the pH gradient [45].

Table 3 Genes of *L. lactis* involved in acid response.

Source	Main Identification Methods	Gene	Function
[44]	Proteomics	*groES*	Heat shock protein (protein refolding)
		groEL	Heat shock protein (protein refolding)
		dnaK	Heat shock protein (protein refolding)
		clpEP	Regulatory protease complex
		hsp14,17,26	Heat shock proteins
		ssp21	Salt shock protein
		sodA	Superoxide dismutase
		ahpC	Alkyl hydroperoxide reductase
		hpr (ptsH)	PEP-PTS system (sugar uptake)
		tig	Trigger factor (Hsp - protein folding)
		luxS	Quorum Sensing
[43]	Transport assays	*arc* operon	Arginine/ornithine transport and metabolism
[22]	IS*S1* insertion	*pstB,S*	Phosphate transporter
		gln operon	Glutamine transporter
		htp	Purine salvage
		relA	(p)ppGpp synthetase
		guaA	Purine biosynthesis
		deoB	Purine salvage
[42]	Transport assays	*atp* operon	H^+ ATPase

5. Carbohydrate Starvation

Carbohydrates are a source of carbon and energy for *L. lactis*. Utilization of carbohydrates leads firstly to exponential growth, followed by carbohydrate exhaustion, and finally to a viable, but non-culturable state [47]. This metabolically active viable, but non-culturable state has been shown to extend for a period up to 3.5 years for *L. lactis* subsp. *lactis* ML3 as determined by lipid membrane integrity and intracellular enzyme activity. Intact lipid membranes were found to be integral in maintaining ATP concentrations and the proton motive force. Peptide transport, amino peptidases, amino acid catabolic enzymes and lipases all retain activity [47]. Microarray analysis suggests that in the non-culturable state, genes encoding carbohydrate metabolic enzymes, cell division proteins and cell envelop degrading proteins were generally expressed at lower levels than cells harvested upon carbohydrate exhaustion [47]. It has long been known that carbohydrate starvation also induces resistance to other types of stress. Carbohydrate starved *L. lactis* subsp. *lactis* IL1403 was found the be more resistant than non-adapted cultures to 15mM hydrogen peroxide, 20% ethanol, incubation at 52^{o}C and exposure to 3.5M sodium chloride [48]. The response to carbohydrate availability is primarily mediated by the gene regulator CcpA. Mutation of *ccpA* in *L. lactis* subsp. *cremoris* MG1363 and subsequent microarray analysis revealed the modified expression of many sugar metabolism related genes, but also many stress related genes during the transition phase and the onset of carbohydrate starvation [49].

6. Ionic and Osmotic Stress

Salt is regularly used in cheese making for its flavour and preservative properties and importantly, as a controller of acid production by the starter culture. The effect of salt addition on acid production by cultures of *L. lactis* subsp *lactis* and *L. lactis* subsp *cremoris* has been compared [50]. *L. lactis* subsp *lactis* consistently produced more acid at salt concentrations of between 3% and 5% (w/v). At a salt concentration of 6% (w/v) all cultures were inhibited in acid production.

The mechanism for protection against osmotic stress is heavily reliant on the transport of glycine betaine and proline in *L. lactis* subsp *lactis* [51]. High intracellular concentrations of betaine and proline were observed under high osmotic stress conditions. A high affinity betaine transporter was identified which, interestingly, was induced by changes in pH rather than increases in growth medium osmolarity. Betaine was shown to have an inhibitory effect on proline transport and it was concluded that the proline transporter probably transported betaine when it was present. Glycine betaine transport was later characterized at the genetic level to reveal the *opuA* (osmo-protectant uptake) operon [52] which has since been renamed busA (betaine uptake system). The *busA* glycine betaine transport operon encodes an ABC-type transporter composed of *busAA,* the gene for the ATP binding protein and *busABC*, a gene fusion of the permease protein and the substrate binding protein. Research into the regulation of the *busA* operon identified BusR as a repressor responsive to changes in environment osmolarity [53]. The mechanism of BusR repression was found to be at the level of DNA binding. Binding of BusR to the *busA* promoter was directly proportional to ionic strength in vitro. High concentrations of ionic potassium glutamate were able to inhibit binding of BusR to the *busA* promoter (ionic). Non-ionic osmolytes such as sucrose and glycine betaine could not inhibit BusR binding to the *busA* promoter [54].

Fatty acid composition of the lipid membrane also occurs in response to salt stress. Salt stress induced higher levels of cyclopropane fatty acid (ΔC19:0) and lower levels of oleic acid (C18:1) in the lipid membrane [55]. ΔC19:0 can be considered as an unsaturated fatty acid, and as such, there is no net change in the ratio of unsaturated fatty acids to saturated fatty acids. It has been proposed that this change in the composition of the lipid membrane may regulate the activity of the glycine betaine transporter; however the mechanism is yet to be established [55].

General heat shock response proteins have also been implicated in adaptation to osmotic stress [56]. Two-dimensional polyacrylamide gel electrophoresis showed that almost all proteins with altered expression in salt stressed *L. lactis* subsp *cremoris* MG1363 were heat shock proteins. One protein denoted as ssp21 was the only highly upregulated protein identified in salt stressed cultures. Its expression was increased 35-fold when cultures were harvested 40 minutes after the addition of NaCl to a final concentration of 2.5%. The full function of ssp21 remains to be determined [56].

7. Antibiotic and Cell Wall Stress

L. lactis, as with other starter culture species, is sensitive to β-lactam antibiotics used routinely on farm for the control of Gram positive pathogens causing mastitis (e.g. *Staphylococcus* spp and *Streptococcus* spp). Despite tight regulation of antibiotic levels in milk by manufacturers, low level contamination results in slower growth of the starter culture and reduced acidification during cheese production [57]. In contrast, lysis of *L. lactis* by native autolysins is considered to be important for intracellular enzyme release for flavour development during cheese maturation [4].

The bacterial cell envelope forms the first line of defense against many stresses, including both antibiotic and enzymatic attack. Modification of the cell envelope, particularly the peptidoglycan saculus, is an essential part of the response to enzymatic attack. In *L. lactis* subsp *cremoris* MG1363, increased O-acetylation of peptidoglycan led to

resistance to lysozyme and native peptidoglycan hydrolase (autolysin) activity in a *spxB* over-expressing mutant [58]. Due to the inhibition of autolytic activity this mutant displayed an elongated cell shape, suggesting a severe defect in cell division. Over-expression of *spxB* (a gene regulator) induced the expression of the O-acetylase gene *oatA* 13-fold and increased O-acetylation of muropeptides from 3.1% in the wild type strain to 3.8%. The *pgdA* gene, encoding a peptidoglycan N-deacetylase was also found to be an important factor in the shift from N- to O-acetylation. The two component regulatory system CesSR was found to sense cell wall damage and induce the expression of *spxB* via a cesR binding site in the promoter region of *spxB* [59]. A *trmA* (*spxB* homologue) mutant was also found to confer high level resistance to peptidoglycan hydrolysis by lysozyme [58]. This finding was confirmed when a *trmA* mutant of *L. lactis* subsp *cremoris* MG1363 identified as an over-expresser of recombinant lysostaphin (PG hydrolytic enzyme) and was also resistant to lysozyme [60].

Bioinformatics revealed there are 21 genes within the regulon of the two-component system response regulator CesR, an ortholog to the LiaR and VanR proteins in *B. subtilis* and *Staphylococcus aureus* respectively [59]. Among these genes the highest induction by the bacteriocin Lcn972 was seen for the putatively annotated hypothetical proteins *llmg_0169* and *llmg_2164-2163,* up-regulated 300- and 21-fold respectively. A Δ*llmg_0169* mutant was found to be highly sensitive to heat stress and the Δ*llmg_2164-2163* mutant was sensitive to salt [61]. Both mutants were more sensitive to acid stress. Although the exact mechanism of function for these genes has not yet been determined, these results exemplify the complex network of interactions leading to stress cross-resistance.

Table 4 Genes of *L. lactis* involved in cell wall stress response.

Source	Main Identification Methods	Gene	Function
[59]	Microarray, Bioinformatics	*Llmg_0169* *Llmg_1155* *Llmg_2164*	Hypothetical protein SpxB - gene regulator Hypothetical protein
[61]	Directed mutagenesis	*llmg_0169* *Llmg_2164-2163*	Hypothetical protein Hypothetical protein
[58]	Clone library, Directed mutagenesis, Microarray	*spxB* *trmA* *mtlARF* *oatA* *pgdA*	Gene regulator (*spx* homolog) Gene regulator (*spx* homolog) Mannitol transport operon (and regulator) Peptidoglycan O-acetylase Peptidoglycan N-deacetylase

8. Stress Response Overlap

8.1 The Stringent Response

As shown in the preceding sections the stringent response is implicated in the *L. lactis* response to acid, oxidative and heat stresses [22]. The stringent response has been well studied, particularly in *E. coli* where it was first discovered, and has been well reviewed [62]. The stringent response is mediated by intracellular concentration of (p)ppGpp in response to stress, most often nutritional stress. Increased levels of (p)ppGpp rapidly decrease the rate of protein synthesis, inhibits DNA replication, inhibits rRNA transcription and can activate transcription of certain genes, particularly those involved in amino acid biosynthesis. Additionally, (p)ppGpp has been found to directly contact RNA polymerase at the site of NTP incorporation into the nascent RNA molecule. The concentration of (p)ppGpp is regulated by RelA and SpoT, both of which can synthesize and hydrolyse (p)ppGpp and furthermore, its activity can be modulated by DksA which is thought to stabilize the (p)ppGpp-RNA polymerase interaction [62].

8.2 Heat Shock Proteins (HSP)

The heat shock proteins (HSP) are molecular chaperonins functioning to refold denatured proteins. In most bacteria, the two major chaperone complexes are present: the DnaK-GrpE-DnaJ complex and the GroES-GroEL complex. The genes required for the synthesis of both of these complexes are regulated by the HrcA repressor. Both chaperone complexes respond to the presence of denatured protein which may be the result of heat, oxidative, acid and osmotic/ionic stress [63]. Regulatory proteases including HtrA, HflB and the Clp protease complex are closely associated to the chaperone complexes, degrading protein that can not be refolded [63]. The genetic location and functionality of the DnaK-GrpE-DnaK complex [64] and the GroES-GroEL complex [65] have been identified and confirmed in *L. lactis*.

8.3 Spx-like Proteins

The Spx-like proteins are short proteins ~130 amino acids in length and found throughout the low G+C Gram-positive bacteria. *L. lactis* possesses 7 Spx homologues, more than any other sequenced bacterium (Figure 1). The Spx homologs function as gene regulators, often in response to a stress stimulus. The most substantial work to date on a Spx protein is that of *B. subtilis,* after which most homologous proteins have been named. It was found that mutations in *spx* suppress *clpP* and *clpX* mutations and restore competence development [66]. Since it is a suppressor of *clpP* and *clpX* mutations, it was named Spx. Microarray analysis on Spx over-expressing cultures has revealed that Spx either directly or indirectly activates or represses the expression of over 200 genes in *B. subtilis* [67]. In particular Spx has been shown to positively regulate thioredoxin and thioredoxin reductase encoding genes [67] and sense redox conditions through its N-terminally located CXXC motif [68]. Spx disruption mutants are hypersensitive to the thiol oxidizing chemical diamide and superoxide generating chemical paraquat [67, 69]. Mutagenesis of either cysteine residue in the CXXC motif renders Spx unable to activate expression of the thioredoxin system and evidence suggests that only the oxidised form of Spx is able to activate gene expression [70]. Spx has also been shown to regulate genes involved in sulphur amino acid metabolism where it promotes the conversion of methionine to cysteine [71].

Spx is a member of the ArsC (arsenate reductase) family of proteins and is structurally different to other transcription regulators. It does not itself have sequence specific DNA binding activity but instead interacts with the α subunit of RNA polymerase (RNAP) and hence has been termed an "anti-alpha" protein [72]. The crystal structure for Spx bound to the α-subunit of RNA polymerase was solved [73]. Interestingly, the CXXC motif was not located at the Spx-RpoA interface, suggesting interaction with other components of the RNA polymerase complex or conformational change upon disulphide formation. Spx has the ability to repress genes by interfering with activator proteins (e.g. ComA response regulator) binding to RNAP [74]. In contrast, Spx can activate genes via interaction of the Spx-RNAP complex with upstream promoter DNA, followed by Spx-induced recruitment of σ and ββ' subunits and contact with the core promoter -35 and -10 elements [75].

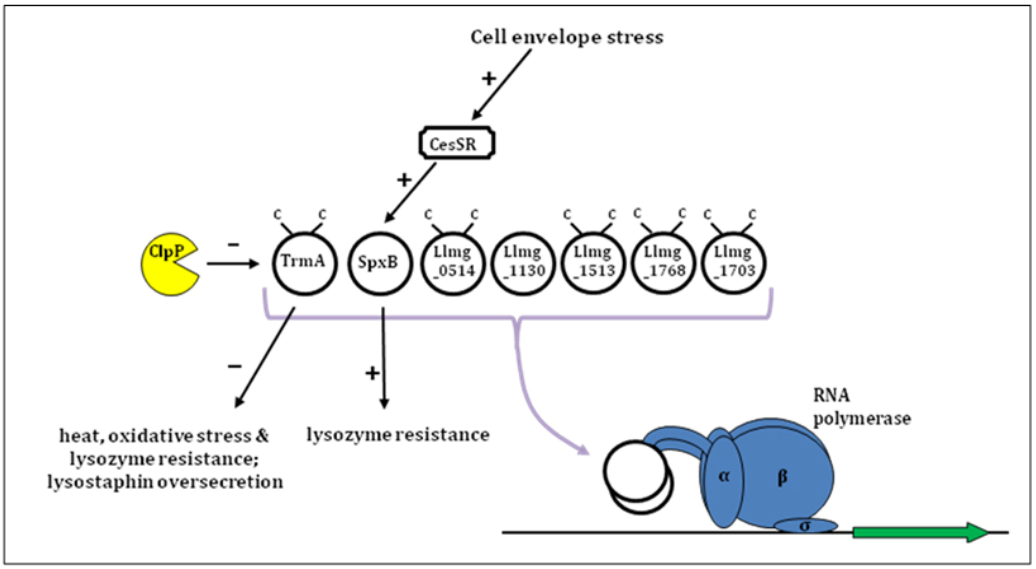

Fig. 1 Summary of proposed Spx homolog interactions and effects in *L. lactis.*

Research in *L. lactis* has shown that the *spx* homolog *trmA* (annotated as *spxA* in the *L. lactis* subsp *cremoris* MG1363 genome) mutants are resistant to oxidative [11], heat [10], and cell wall stress [60, 58]. In addition to this, elevated levels of *spxB* are stimulated by cell wall damage [59] and induce the expression of other genes to improve resistance [58]. The opposing effects of SpxB and TrmA are thought to occur via competitive binding of the RNA polymerase α-subunit (RpoA), with which they interact to direct gene expression [58]. It is thought that the other Spx homologs may also bind competitively with RpoA as shown in Figure 1; however this is yet to be reported experimentally. Mutation of other *spx* homolog genes in *L. lactis* produces both resistance and sensitive phenotypes to various stress conditions (Smith et al., unpublished). Together these results indicate that in *L. lactis*, Spx homologs play an integral role in coordinating responses to many types of stress by directing global gene expression.

9. Current Methods and Trends for Future Research

Genes involved in stress resistance and/or responding to stress have been identified using a variety of approaches including site-directed mutagenesis, random mutagenesis, microarrays, proteomics and bioinformatic methods as seen

in Tables 1-4. Each method has its own advantages and disadvantages, but all have a significant role to play in expansion of current understanding. Gene disruption or deletion provides general information about how that gene is implicated in growth under a certain conditions; however it should be pointed out that the consequential phenotypic effects may be indirect. Handling and manipulation of *L. lactis* DNA sequences has been facilitated by the development of plasmids for gene disruption or deletion such as pORI [76], pRV300 [77] and pG+host [78]. Further development of the pG+host plasmids resulted in the development of pG+host9:IS*S1* which is used for random insertional mutagenesis [79]. The NICE system plasmids have been developed for protein expression and utilize the bacteriocin nisin as an inducer of expression [80].

Microarrays, quantitative real time PCR and two-dimensional gel electrophoresis are frequently used to analyze changes in gene/protein expression which provides insight at the molecular level, particularly with respect to regulatory mechanisms. As our knowledge becomes more detailed, techniques to determine enzymatic function, protein-protein and protein-nucleic acid interaction, and structure are increasing in importance. Integrated into each of these techniques are computational methods, known collectively as bioinformatics, which improve the speed and accuracy of analysis and even experimental design. Genome sequencing has played a major role in facilitating more specific molecular research. Currently four *L. lactis* genomes have been completely sequenced including *L. lactis* subsp. *lactis* IL1403 [81] *L. lactis* subsp. *cremoris* SK11 [82] and *L. lactis* subsp. *cremoris* MG1363 [83] all of dairy origin, and most recently *L. lactis* subsp. *lactis* KF147 [84] of plant origin. Next generation DNA sequencing is emerging as a method to sequence genomes at relatively low cost and will certainly result in an increase in sequenced genomes in the coming years. Further research utilising current and novel methods will yield a better understanding of how *Lactococcus* deals with stress and potentially lead to innovations in industrial fermentation processes.

Acknowledgements. Work in our laboratory on stress resistance has been supported by University of Queensland grants to M.S.T. and a Pakistan HEC fellowship to A.H.S.

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