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Environmental stress responses in *Lactococcus lactis*

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Abstract

Bacteria can encounter a variety of physical conditions during their life. Bacterial cells are able to survive these (often adverse) conditions by the induction of specific or general protection mechanisms. The lactic acid bacterium *Lactococcus lactis* is widely used for the production of cheese. Before and during this process as well as in its natural habitats, it is subjected to several stressful conditions. Such conditions include oxidation, heating and cooling, acid, high osmolarity/dehydration and starvation. In many environments combinations of these parameters occur. Understanding the stress response behaviour of *L. lactis* is important to optimize its application in industrial fermentations and is of fundamental interest as *L. lactis* is a non-differentiating Gram-positive bacterium. The stress response mechanisms of *L. lactis* have drawn increasing attention in recent years. The presence in *L. lactis* of a number of the conserved systems (e.g. the heat shock proteins) has been confirmed. Some of the regulatory mechanisms responding to an environmental stress condition are related to those found in other Gram-positive bacteria. Other stress response systems are conserved at the protein level but are under control of mechanisms unique for *L. lactis*. In a number of cases exposure to a single type of stress provides resistance to other adverse conditions. The unravelling of the underlying regulatory systems gives insight into the development of such cross resistance. Taken together, *L. lactis* has a unique set of stress response mechanisms, most of which have been identified on the basis of homology with proteins known from other bacteria. A number of the regulatory elements may provide attractive tools for the development of food grade inducible gene expression systems. Here an overview of the growth limits of *L. lactis* and the molecular characterization of its stress resistance mechanisms is presented.

Keywords: *Lactococcus lactis*; Lactic acid bacterium; Physical stress condition; Stress resistance; Gene regulation

Contents

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1. Introduction

Lactococcus lactis has been associated with food production and preservation since ancient times. Nowadays, defined starter cultures of *L. lactis* are of great economic importance in the bulk production of cheese. This organism plays a key role in the formation of flavour and texture of cheese and in its preservation. From an industrial point of view it is important to select strains that perform well in fermentation and that resist adverse conditions occurring during the fermentation process. In addition, such strains should survive storage and handling procedures that are cheap and convenient. In some food products (e.g. fresh milk) the same *L. lactis* is regarded as a spoilage organism. The presence of *L. lactis* in food systems can be controlled by choosing specific conditions, either to promote lactococcal proliferation when desired or to prevent spoilage in products that need no lactococcal fermentation. A number of (physical) parameters can be manipulated to control lactococci without changing the safety and nutritive value of the final product. Therefore, it is important to know which conditions are favourable and which are detrimental for the life of this organism and which mechanisms are essential for its survival under such conditions.

1.1. Stress conditions

A first insight into the stress resistance of *Lactococcus* is given by its 'natural' habitats and the extremes of the conditions encountered. *L. lactis* is commonly found in milk, which is a nitrogen and carbon rich substrate, and in various plant materials, making grass a likely source of inoculation of raw milk [1]. Both on plant material and in industrial processes *L. lactis* faces a wide range of different conditions such as extremes in temperature, pH, or osmotic pressure. The organism is subject to relatively high temperatures in soil or during 'cooking' in cheddar cheese production, in which the temperature is increased to the growth limiting level of 40°C. Low temperatures occur during storage of frozen starter cultures and during cheese ripening (8–16°C). Osmotic pressure can vary from very low in rain water to high in pressed cheeses containing 0.56 M NaCl. Many of these different stress conditions will often coincide. This is, for example, the case with carbohydrate starvation and acid stress, as sugar fermentation results in high levels of lactic acid. Dried cells in lyophilized or spray dried starter cultures suffer from both osmotic and oxidative stress. Cells are exposed to both high temperatures and UV radiation in sunlight. Optimal conditions for growth are rare and require specific metabolic adaptations and can, therefore, also be seen as a stress condition [2]. An increasing number of studies show that *L. lactis*, when confronted with dangerous environmental conditions, can survive by the activation of specific protection mechanisms.

1.2. L. lactis

L. lactis strains have for long been maintained in milk and, therefore, have adapted to survival in that environment. With the establishment of the dairy industry the use of selected and defined cultures became practice. Strains have been selected for rapid acidification, proteolytic activity and resistance

against bacteriophages and bacteriocins. The relatively small set of strains that is currently used is, thus, not specifically selected for stress resistance but this may have been selected for indirectly. The various studies discussed in this review either describe *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris*. The two subspecies have different stress resistance properties but these differences have only recently been studied in more detail. Kim et al. [3] showed that the subsp. *lactis* is, in general, more robust than subsp. *cremoris* with respect to acid resistance, bile salt resistance and freezing resistance. The fact that most subsp. *lactis* strains can grow at 40°C and in the presence of 4% NaCl, whereas most subsp. *cremoris* strains do not, is used to phenotypically distinguish the two [4]. Genotypic analysis of isolates from both industrial and natural sources have revealed that subsp. *cremoris* strains isolated from nature can have a stress resistance phenotype that is similar to that of the subsp. *lactis* [5]. Industrial subsp. *cremoris* strains probably have lost some properties that are essential to survive in nature but not or even disadvantageous for continuous subculturing in a dairy environment.

The aim of this review is to describe the current insight into the environmental stress response of *L. lactis*, and some other lactic acid bacteria. In addition, some potential applications of this knowledge will be discussed.

2. Stress

The stress responses of *L. lactis* have gained interest in recent years not only because of the industrial relevance of the organism but also for basic scientific reasons. Scientific interest is fuelled by the fact that *L. lactis* is a mesophilic Gram-positive microorganism with a relatively small genome (2.5 Mbp) [6] that is unable to differentiate (by sporulation) in response to stress conditions. Stress-induced gene expression can be very complex as regulation can take place at the level of transcription, translation or mRNA stability. In addition, regulation by post-translational modifications is possible. For example, the activity of a gene product may be influenced by its phosphorylation state. Regulation can also take place by differential degradation of a protein. Key examples of stress proteins which are controlled at these various levels are σ^S [7] and CspA [8,9] in *Escherichia coli*.

The influence of stress conditions on *L. lactis* has been studied globally by analyzing their effects on growth and total protein synthesis (see Table 1) and by genetic analyses of known stress-related genes (summarized in Table 2). In the next subsections both global effects and genetic studies will be discussed for a number of stress conditions. Comparison of the different studies in this field is hampered by differences in the analysis techniques and by differences in challenge conditions and incubation times that have been used to stress cells. Careful interpretation of studies that aimed at subjecting cells to a single stress condition in a single experiment is needed as the conditions used may have posed a combination of stresses to the cells. For example, a single stress may be deleterious under standard growth conditions but may not be so at lower temperatures or in an anaerobic environment.

2.1. Heat shock

When an *L. lactis* culture was shifted from its

Table 1
Global response to environmental stress in *L. lactis*

Stress	Conditions	Number of proteins with:		Reference
		Enhanced expression	Reduced expression	
Heat shock	42°C, 5–25 min	12–17	most	[10]
Low temperature	8°C, 1–10 h	12	17 (at 16°C)	[30]
Low pH	pH 5.5, 30 min	33	majority	[28]
Starvation	3 h galactose and arginine exhaustion in CDM[a]	14	>45	[83]
Salt	2.5% NaCl, 10–40 min	12	most	[12]
UV light	254 nm, 100 J/m^2	14	30	[27]

[a]CDM, chemically defined medium.

Table 2
Genes induced by environmental stress in *L. lactis*

Gene	Function of protein	Stress condition		Induction factor	Analysis method	Reference
groESL	chaperone	heat shock	43°C, 15 min in GSA[a]	10	RNA	[14]
groES	chaperone	heat shock	42°C, 30 min in GM17[a]	12	protein	[29]
		low pH	pH 5.5, 30 min lactic acid	3.8	protein	[29]
		UV light	254 nm, 100 J/m^2	2.3	protein	[29]
groEL	chaperone	heat shock	37°C, 60 min in GM17	3	protein	[23]
		heat shock	42°C, 30 min in GM17	4.9	protein	[29]
		low pH	pH 5.5, 30 min lactic acid	2.4	protein	[29]
dnaJ	chaperone	heat shock	42°C, 10 min in WP[a]	3 to 4	RNA	[15]
		heat shock	43°C, 15 min in GSA	10	RNA	[14]
ORF1/*hrcA*	negative regulator of heat shock genes	heat shock	43°C, 15 min in GSA	5	RNA	[14]
grpE	chaperone	heat shock	43°C, 10 min in GSA	5	RNA	[14]
		heat shock	37°C, 60 min in GM17	3.7	protein	[23]
dnaK	chaperone	heat shock	43°C, 15 min in GSA	100	RNA	[14]
		heat shock	37°C, 60 min in GM17	3.4	protein	[23]
		low pH	pH 5.5, 30 min lactic acid	2.1	protein	[28]
clpP	protease	heat shock	43°C, 15 min in GSA	10	RNA	[18]
		low pH	pH 5.1, 10 min HCl	3	RNA	[18]
		salt	2.5% NaCl, 10 min	4	protein	[12]
ftsH/hflB	regulator of heat shock response	heat shock	43°C, 10 min in GSA	+[b]	RNA	[14]
		heat shock	37°C, 60 min in GM17	5.2	protein	[23]
		low pH	pH 5.1, 15 min	5 to 6	RNA	[86]
recA	SOS regulator	DNA damage	0.01% methylmethanesulfonate, or 1 μg ml^{-1} mitomycin C	3 to 5	RNA	[57]
	DNA repair	oxidation	aerated culture	+[c]	RNA	[57]
fpg	SOS regulator	DNA damage	0.01% methylmethanesulfonate, or 1 μg ml^{-1} mitomycin C	3 to 5	RNA	[57]
	DNA repair	oxidation	aerated culture	+[c]	RNA	[57]
sodA	O_2^- scavenging	oxidation	aerated culture	2	reporter[d]	[51]
cspA	RNA stabilization	cold shock	10°C, 1 h in GM17	10	RNA	[41]
cspB	RNA stabilization	cold shock	10°C, 4 h in GM17	40	RNA	[41]
cspC	RNA stabilization	cold shock	10°C, 2 h in GM17	8	RNA	[41]
cspD	RNA stabilization	cold shock	10°C, 4 h in GM17	30	RNA	[41]
P170		low pH	growth at pH 5.2 vs. pH 7.0	50	reporter	[81]
		low temperature	growth at 15°C vs. 30°C	7.5	reporter	[81]
gadCB	acid stress resistance	chloride	growth with 0.5 M NaCl vs. no NaCl	1000	reporter	[49]
		low pH	growth with 0.3 M NaCl, in non-buffered vs. buffered M17, pH 4.2 vs. pH 5.5	10	reporter	[49]

[a]GSA, glucose SA medium [102]; GM17, glucose M17 broth; WP, whey permeate.
[b]Uninduced expression level below detection limit.
[c]+, not quantified.
[d]Determined from a *lacZ* transcriptional fusion.

optimal growth temperature of 30°C to 42°C cells stopped growing [10]. Growth resumed after a shift-down to 30°C with the growth rate reaching the preshock level after 1 h at 30°C. Cells were hardly capable to recover from exposure to 50°C for 30 min. In this case growth only resumed after 48 h. The number of viable cells was slightly reduced after a 30 min heat shock at 42°C but was more than 1000-fold reduced by incubation at 50°C. However, pretreatment at 42°C for 10 min resulted in survival

of more than 10% upon heat shock at 50°C [10]. Increased expression of 12 to 17 proteins was observed upon exposure of *L. lactis* to 42°C [10–12]. This phenomenon has been observed in organisms as diverse as plants, animals and bacteria. Detailed immunological and genetic analyses have confirmed the presence of the conserved heat shock proteins (HSPs) DnaK, DnaJ, GroEL, GroES and GrpE in *L. lactis*. These HSPs are protein chaperones that function in folding and maturation of new or denatured proteins [13]. The organization of the genes encoding these proteins in *L. lactis* is partially different from other bacteria. Whereas in most cases *dnaK* and *dnaJ* form one operon, lactococcal *dnaJ* is transcribed independently from *dnaK* [14,15]. *dnak* is part of a gene cluster that is also found in other Gram-positive bacteria and consists of ORF1(*hrcA*)-*grpE*-*dnaK* [16], followed by a putative transcription terminator and ORF4 that is not induced at high temperature [14]. A *dnaK* mutant (*dnaK*Δ1, lacking 174 out of 607 amino acids from the C-terminus of the protein) shows a clear temperature sensitivity phenotype [17]. It grows at a reduced rate at 30°C, whereas it is unable to grow at 33°C or higher temperatures. The mutant strain is still able to acquire thermotolerance by preincubation at 40°C, although less efficiently than the wild-type.

Arnau et al. [14] integrated data from several groups by analyzing the expression of seven genes at the RNA level in response to a standardized heat shock in a single strain. They showed that after 15 min at 42°C *dnaK*-specific mRNA levels in *L. lactis* MG1363 had increased 100-fold and that it represented the single *dnaK* transcript. *hrcA*- and *grpE*-specific mRNAs were of similar size and the levels of both were 5-fold higher after 10 min of heat shock, indicating that these genes are transcribed together. On the basis of the nucleotide sequence, a promoter could be identified upstream of *hrcA* (see below) but not upstream of *dnaK* [16]. The discrepancy between this observation and the observed lengths of the mRNAs may be explained by efficient processing of a single messenger covering *hrcA*-*grpE*-*dnaK* [14] and differential stability of the processing products. Indeed, larger mRNAs could be detected both with *grpE* and *dnaK* probes. A *groEL* probe hybridized with a 2.2 kb transcript that was 10-fold induced after 15 min. This transcript may also include *groES*. The mRNA levels of *dnaJ*, *dnaK*, ORF1 and *grpE* had decreased significantly 20 min after the onset of the heat shock. Only the level of *groEL* transcript was lower at this time than its normal level at 30°C. The expression of heat shock genes upon induction in rich medium was generally lower and faster than in a defined medium [14]. The temporal induction of the HSPs after heat shock was monitored by pulse labelling of stressed cells and separation by 2D-PAGE [12]. DnaK production is induced 35-fold in the first 10 min of heat shock and production declines thereafter, whereas GroEL and GroES are produced at levels 45- and 35-fold over unstressed levels, respectively, in the first 10 min and synthesis continues at a high level in the following 30 min. Five other unidentified proteins were induced more than 10-fold during the first 10 min of heat shock. A second group of nine unidentified HSPs was induced to lower levels: their expression levels continued to increase during the first 30 min of heat shock at 43°C to levels ranging from 2- to 8-fold higher [12]. One of these has been identified as the protease ClpP [18]. ClpP is, most likely, involved in the degradation of misfolded proteins, as a *clpP* mutant has a reduced capacity for the degradation of puromycyl-containing polypeptides. The mutant strain expressed higher levels of the heat shock chaperones when grown at 30°C, which is an indication for the accumulation of unfolded proteins due to reduced proteolytic activity. The *clpP* mutant is unable to form colonies at 37°C. *clpP* mRNA levels increase up to 10-fold within 5 min after a shift to 43°C. A number of putative genes induced at 43°C were found using a probe made by RNA subtractive hybridization. These included transposase genes and putative *L. lactis* homologs of the cell division gene *ftsZ*, the heat shock gene *hsp*86, and the gene *asp*23 encoding an alkaline shock protein, as well as a gene of which the product showed homology to transcriptional repressors of the *deoR*-like family [19].

No central regulator for the heat shock response has been identified so far. Most attention has been focussed on the regulation of the expression of the chaperone operons. The genes *hrcA*, *dnaJ* and *groESL* are preceded by vegetative promoters and highly conserved repetitive sequence elements, consisting of two 9 bp inverted repeats separated by a

9 bp loop. These CIRCE (controlling inverted repeat of chaperone expression) elements are present upstream of heat shock genes in 27 different bacterial species [20]. They have a regulatory role as negatively acting *cis*-elements, as deletion of the CIRCE upstream of the *L. lactis dnaJ* promoter resulted in temperature-independent expression of a *dnaJ*::*amyS* fusion [15]. From studies in *B. subtilis* it is known that HrcA is a negative regulator of *dnaK* and *groE* and that HrcA can bind to CIRCE elements [20]. Experiments in *B. subtilis* support a regulatory mechanism in which the levels of active HrcA are modulated by the chaperone GroEL. GroEL is, thus, a negative regulator for chaperone expression and functions as the molecular thermometer [21]. Heat shock and ethanol lead to increased levels of non-native proteins that titrate the levels of GroEL, thereby reducing the capacity of GroEL to activate the repressor protein HrcA [22]. DnaK of *B. subtilis* plays no role in the modulation of chaperone expression, unlike *E. coli* DnaK. However, recent experiments show that in *L. lactis* the levels of the heat shock mRNAs *hrcA*, *dnaK*, and *dnaJ* and the levels of the heat shock proteins GroEL, GroES, Hsp84, Hsp85 and Hsp100 are higher in the *dnaK*Δ1 mutant than in the wild-type strain [17]. Levels of *hflB* mRNA (encoding the HflB protease, see below) were not elevated in this mutant. These data suggest that DnaK may be the central sensor for denatured proteins in *L. lactis* and can modulate chaperone expression via HrcA. Additional factors are likely to act on HrcA to explain the remaining capacity of the *dnaK*Δ1 mutant to develop thermotolerance by preadaptation and to elicit a low level heat shock response. Alternatively, these properties may also be ascribed to the N-terminal part of the DnaK protein that is still expressed in this mutant strain.

Another regulator of heat shock genes in *L. lactis* may be RecA. A culture of a *recA* mutant shifted to 37°C showed a lag of 8 h before growth resumed at a low rate [23]. Two- to 3-fold reduced levels of DnaK, GroEL and GrpE were observed in the *recA* strain and induction of these proteins by heat shock was delayed. In *E. coli*, the expression of most heat shock genes is controlled by the transcription factor σ^{32}. The level of σ^{32} is negatively regulated by HflB, which is also an HSP. An analogue of HflB (also called FtsH) has been identified in *L. lactis* [24] and its expression is induced at high temperature [23]. An *L. lactis hflB* mutant was unable to grow at elevated temperature (38°C) [24]. HflB levels were 3-fold higher in the *recA* strain at both 30°C and 37°C. The reduced levels of the HSPs in the *recA* mutant may be caused by the higher level of HflB in this strain. In a model based on these observations, RecA is proposed to regulate the heat shock response via HflB that, in turn, may govern the stability of an unknown positive factor which regulates HSP expression, as is the case in *E. coli* [23,25]. However, at present there are no indications of the existence of such a positive factor, either or not a sigma factor, in *L. lactis*. Therefore, it is still unclear what other control circuits regulate HSP expression in *L. lactis* and what the exact roles of RecA and HflB are. Some genes that may be involved in the control of heat shock resistance were identified by screening for heat resistant mutants of a *recA* strain [26]. Among these were *deoB*, *guaA*, *hpt*, *pnpA*, *pstB*, *tktA*, and *trmA*. Mutation of *deoB*, *guaA*, *hpt*, and *tktA* also conferred heat resistance in a wild-type background. These four genes play a role in nucleotide synthesis or uptake. The expression of the HSPs is also induced under other stress conditions such as low pH, salt, and UV radiation (Table 2) [12,27–29], but not by phage infection [10]. Induction of HSPs by UV light (see below), together with the data discussed above, point to the relevance of *recA* in HSP expression.

2.2. Low temperature

Whereas growth at high temperatures is deleterious to a cell, growth at low temperatures merely slows down biological processes. The doubling time of a lactococcal culture increased from 48 min at 30°C to $3\frac{1}{2}$ h, 57 h and 7 days at 16°C, 8°C, and 4°C, respectively [30]. No lag period was observed after a temperature downshift. The survival of *L. lactis* increased at low temperature. Survival of bacteria first grown at 30°C to stationary phase and subsequently incubated at 4°C for 28 days was 30%, whereas 0.03% of the culture survived when held at 30°C throughout the experiment [30]. Similarly, survival from a freezing-thawing cycle was better (95%) after preincubation at 8°C for 48 h than without such an adaptive treatment (75%) [31]. Pre-

incubation at 16°C or 4°C did not increase freezing-thawing survival significantly. Survival from freezing-thawing cycles was lower when cells were incubated in a 0.85% NaCl solution instead of culture medium [32]. Log phase cells are more resistant to freeze-thaw than cells in the stationary growth phase [32]. *L. lactis* subspecies *lactis* strains were shown to acquire freezing resistance by preadaptation at 10°C whereas strains of the subspecies *cremoris* are equally freezing sensitive with or without preadaptation [3]. Surprisingly, preincubation at 8°C for 48 h improved survival of a 30 min challenge at 52°C 60-fold compared to a non-adapted culture [31]. Incubation at low temperature resulted in the induction of a specific set of 12 proteins in *L. lactis* [30]. The level of induction depended on the incubation time and temperature. The maximum observed overexpression of one of the proteins was 10-fold after 1 h at 8°C when compared with 30°C.

After a cold shock treatment, *E. coli* and *B. subtilis* are known to overexpress several proteins (reviewed by Jones [33], Graumann [34,35] and Yamanaka [36]). Research on cold shock responses was focused on a family of small (7 kDa) highly conserved cold shock proteins (CSPs), three of which were identified in *B. subtilis* and nine in *E. coli* [34]. All three *B. subtilis* CSPs are cold induced whereas this is only true for four of the nine CSPs of *E. coli*. Some of the other *E. coli* CSPs are induced upon starvation (CspD) or are expressed constitutively (CspC and CspE). Expression of one of the cold-induced proteins, CspA from *E. coli*, was shown to be induced 200-fold at low temperature by a combination of increased transcription, stability and translation efficiency of *cspA* mRNA [8,9]. CspA and its relatives are thought to have a role as RNA chaperones. CspA was shown to bind RNA and ssDNA with a broad sequence specificity and increases the susceptibility of RNA to RNases [37]. Therefore, CspA was suggested to prevent the formation of secondary structures in RNA molecules at low temperatures and in that way to stimulate translation efficiency. The first *csp* deletion mutant described, *cspB* of *B. subtilis*, showed a defective cold shock response and a reduced induction at low temperature of 15 proteins, suggesting a regulatory function for *cspB* in the cold shock response [38]. Deletion of *B. subtilis cspC* or *cspD* did not lead to a detectable phenotype and was shown to be compensated by the enhanced expression of the remaining *csp* genes [39]. Double mutants showed a severe reduction in cellular growth (both at 37°C and at 15°C), stationary phase survival and a deregulated protein synthesis. A minimum of one *csp* gene is essential for viability of *B. subtilis*.

Recently, members of the CSP family were identified in *L. lactis* [32,40,41]. A set of primers designed on the basis of conserved regions in the known CSPs was used to amplify an internal fragment of a lactococcal *csp* gene by PCR. This cloned PCR fragment hybridized with four chromosomal *Hin*dIII restriction fragments [41]. Three of these were cloned and revealed the presence of five *csp* genes, named *cspA*, *cspB*, *cspC*, *cspD* and *cspE*. *cspA* and *cspB* were present on one fragment and were separated by 360 bp. *cspC* and *cspD* were also closely linked. The linked *csp* genes are transcribed in the same direction. A similar organization was found for *E. coli cspB* and *cspG*, which are divergently transcribed from the same genomic region as *cspF* and *cspH*, respectively [36]. The deduced amino acid sequences of the five *L. lactis* genes show 45 to 65% identity to *E. coli* CspA and *B. subtilis* CspB. The lactococcal CSPs have 52 to 85% identical amino acid residues. Expression of *cspA* is about 10-fold higher after 1 h of incubation at 10°C whereas *cspB* expression is 40-fold induced after 4 h at 10°C, as was shown by Northern hybridization. Expression of *cspC* is 8-fold higher after 2 h at low temperature and *cspD* expression reaches an optimum of 30-fold induction 4 h after a shift to low temperature (10°C). *cspE* expression appeared to be temperature independent [41]. All transcripts were about 300 nucleotides (nt) in length, indicating that all genes, including *cspB* and *cspC*, are monocistronic. The start points of the *csp* mRNAs have been mapped. The nucleotide sequences of the approximately 87 nt non-translated leaders of the messengers of *cspA*, *cspB*, *cspC* and *cspD* are highly conserved. The leader of the non-cold-induced *cspE* shows much more differences. This may point to a regulatory function of the leader, as is the case for the 5′-end untranslated region of the *E. coli cspA* messenger [42]. The similarity in the primary structure and the pIs of CspA and CspC compared to those of CspB and CspD as well as their similarity in levels and time spans of expression

in response to cold shock suggest that CspA and CspC may have a similar role in the cell. Possibly they are involved in the induction of *cspB* and *cspD*, their respective neighbours on the chromosome [41]. On the basis of amino acid sequence similarity and structural conservation it is likely that the *L. lactis* CSPs function as RNA chaperones in a way similar to *E. coli* CspA. PCR studies using degenerate primers suggest the presence of *csp* homologs in *Lactobacillus helveticus*, *Pediococcus pentosaceus* and *Streptococcus thermophilus* [32]. From another lactic acid bacterium, *Lactobacillus plantarum*, the two *csp* genes *cspL* and *cspP* have been cloned and sequenced [43]. The 66 amino acid proteins encoded by these genes differ by only eight amino acids and show about 66% identity to *E. coli* CspA. Two distinct transcripts of 330 and 760 nt were detected with a *cspL*-specific probe, whereas a single 330 nt RNA was detected with a *cspP*-specific probe. A 3- to 5-fold increase of all three transcripts over the basal level at 37°C was observed after 1 h at 10°C. Messenger RNA levels declined to preshock levels within 1 h after return to 37°C.

2.3. Osmotic stress

Bacterial cell envelopes are permeable to water. Therefore, an increase in the osmolarity of the growth medium would result in rapid efflux of water from the cytoplasm. To retain water in the cell and, thus, to maintain turgor pressure, bacteria have systems to accumulate specific solutes that do not interfere with cell physiology [44]. Such compatible solutes are either taken up from the environment or newly synthesized in the cytoplasm; the uptake mechanism is found in most lactic acid bacteria. One of the compatible solutes mostly used by bacteria is glycine betaine (betaine). Growth of *L. lactis* was shown to be inhibited at high salt concentrations: 2.5% NaCl (a level close to that in some cheese types) reduces the growth rate to 25 to 50% of the unstressed rate [12]. Growth under these conditions was stimulated considerably by the presence of betaine in the medium [45]. Cells grown in CDM in the presence of 0.5 M KCl accumulated high levels of proline and, to a lesser extent, aspartate. In the same medium in the presence of betaine, cells accumulated aspartate, glutamate and betaine but no proline. *L. lactis* has a high affinity uptake system for betaine that is constitutively expressed. In addition, a low affinity proline uptake system was also active but only so in a chemically defined medium (CDM) of high osmolarity and not in rich media or in the absence of KCl. Proline transport was inhibited by betaine and exchange of proline for betaine was also observed, suggesting that the proline transport system may also transport betaine. This would explain the observed absence of proline in the cytoplasm when betaine is present in the medium. Uptake by both transport systems is energy dependent but, most likely, not driven by the proton motive force [45]. Information on the regulation or the genetic determinants of these systems is not available. Growth of *L. plantarum* at high osmotic pressure is stimulated by betaine, which is the preferred osmolyte of this organism [46]. Levels of alanine, glutamate, proline, and glycine increased 3-, 6-, 35-, and 48-fold, respectively, upon growth in CDM with 0.8 M KCl compared to low osmolarity medium, with glutamate and proline being the most abundantly accumulated amino acids. The four amino acids accumulated to lower levels when betaine was present in the high osmolarity medium. Betaine transport was increased 3-fold in cells cultured in high osmolarity medium, suggesting enhanced expression of the transport system. Uptake rates also increased when the osmolarity of the assay buffer was raised, which points towards activation of the transport protein. Efflux of betaine upon osmotic downshock depended on the post-shock osmolarity of the medium. Separate transport systems were postulated for the uptake and efflux of betaine [47].

Analysis of protein production by 2D-PAGE revealed that total protein synthesis dropped to about 50% of the preshift level in cells stressed with 2.5% NaCl [12]. The synthesis of at least 12 proteins is induced, their level of induction varying from 2- to 9-fold. The expression of one protein, labelled Ssp21, gradually increased 35-fold during 40 min after stress induction. Most of the salt-induced proteins identified were also induced by heat shock. The heat shock proteins DnaK, GroEL and GroES were induced 5- to 9-fold within 10 min at 2.5% NaCl, resembling their induction by heat shock, although at a lower level. ClpP was induced 4-fold after 10 min at 2.5% NaCl [12].

In a search for environmentally regulated genes in *L. lactis* an NaCl inducible promoter structure was identified that was independent from the medium osmolarity but required chloride for induction [48]. The two genes transcribed from this promoter function in low pH survival. One of these, *gadC*, may code for a glutamate-γ-aminobutyrate antiporter but the involvement of the genes in osmoregulation, if any, is still unclear [49].

An *L. lactis hflB* mutant is unable to grow in M17 medium in the presence of 4% NaCl and grows very slowly in 1% NaCl whereas growth of the wild-type is only reduced at the high NaCl concentration [24]. Interestingly, the pattern of membrane-associated proteins in the *hflB* mutant is different from that of the wild-type, suggesting improper assembly of membrane proteins, some of which may be essential for salt tolerance. *hflB* expression is not induced at high osmolarity [24].

2.4. Oxidative stress and DNA damage

L. lactis is an obligatory fermenting bacterium. It can tolerate oxygen as its growth is not affected by aeration [50,51]. Cells are able to use oxygen in the presence of a carbon source by a closely coupled NADH oxidase/NADH peroxidase system. This is an alternative way to regenerate NAD^+, next to the conversion of pyruvate to lactate or ethanol. Pyruvate is then available for conversion to acetate, yielding extra ATP. Therefore, aerobic fermentation produces different (amounts of) end products as compared to anaerobic fermentation [50,52]. The activity of NADH oxidase/NADH peroxidase is about 5-fold higher in galactose grown aerated cells than in non-aerated cells. This enzyme activity may generate the highly toxic oxygen intermediate superoxide (O_2^-). Two-fold higher levels of superoxide dismutase (which removes O_2^-) were found in aerated cultures [51,52]. The toxicity of O_2^- is illustrated by the markedly reduced growth rate of a superoxide dismutase (*sodA*) mutant during aerobic growth [51]. The remaining systems can still cope with O_2^- to a certain extent. Alternative reducing capacity may be provided by glutathione of which a relatively high level is present in *L. lactis* [53]. A number of *L. lactis* strains were reported to accumulate glutathione [54]. Accumulation is medium dependent [54] and relies on transport from the environment [55].

Both NADH oxidase and superoxide dismutase produce the toxic compound H_2O_2. *L. lactis* has no catalase and depends solely on NADH peroxidase to keep H_2O_2 levels at subinhibitory concentrations. At sublethal H_2O_2 levels, *L. lactis* develops an adaptive response against lethal concentrations of H_2O_2 [56]. The *L. lactis recA* gene clearly plays a role in oxidative stress. Expression of *recA* was induced in aerated cultures and a *recA* mutant is highly sensitive to aeration, as evidenced by a lower growth rate and reduced viability during stationary phase [57]. The doubling time of an aerated *recA* culture is restored to that of a non-aerated culture by the presence of catalase or the Fe^{2+} chelator ferrozine in the medium, while catalase also improves survival. This observation points to the involvement of hydroxyl radical (°OH), the most reactive oxygen species, because this is formed in the H_2O_2- and Fe^{2+}-dependent Fenton reaction ($H_2O_2+Fe^{2+}+H^+ \rightarrow °OH+H_2O+Fe^{3+}$) [57]. °OH may be the cause of the observed higher rate of DNA damage in aerated cells as compared to standing *L. lactis* cultures [23]. In *E. coli*, O_2^- caused an increase in the intracellular pool of free iron, which promoted the rate at which H_2O_2 caused DNA damage [58].

RecA is the key protein in the SOS response to DNA damage and in homologous recombination in *E. coli*. In analogy, recombination in *L. lactis recA* was more than 10^4-fold lower than in the wild-type and the mutant was sensitive to DNA damage induced by UV light, mitomycin C or methyl methane sulphonate [23]. The latter compounds also induce an increase in the level of *recA* mRNA [57], but an increase in the RecA protein level does not occur upon introduction of DNA damage [59]. *recA* in *L. lactis* is cotranscribed with a gene encoding the DNA repair enzyme formamidopyrimidine DNA glycosylase (*fpg*) [57]. Like its *E. coli* counterpart, Fpg from *L. lactis* has DNA glycosylase activity and can nick DNA at abasic sites. Furthermore, it suppresses an *E. coli* mutator phenotype [60].

A number of genes that are in some way involved in the DNA damage response were found by IS*S1* mutagenesis. Mutants defective in the *rexAB* genes were identified by their lack of exonuclease activity;

these are more UV sensitive than wild-type cells and are recombination deficient [61]. This points to a role for *rexAB* (encoding the lactococcal *recBCD*-like exonuclease) in resisting DNA damage. Other UV and mitomycin C sensitive mutants contained lesions in genes (identified on the basis of homology) involved in DNA metabolism (*hexB*, *polA*, and *deoB*), cell envelope formation (*gerC* and *dltD*) and various metabolic pathways [62]. *hexB*, *polA*, and *deoB* mutants were sensitive to both low and high doses of UV (10 and 50 J/m^2 at 260 nm, respectively), whereas the other mutants only showed sensitivity to high doses. The strain lacking the putative mismatch repair system HexB permitted recombination between DNA molecules with mismatches and the occurrence of spontaneous mutations at a higher frequency than the wild-type. *polA* encodes the bacterial DNA polymerase I. Consequently the *L. lactis polA* strain could not support replication of a theta replicating plasmid. DeoB is part of a salvage pathway for purines and pyrimidines. The UV sensitivity of the *deoB* mutant was relieved by the presence of nucleotides in the culture medium. Although the function of the other genes in UV resistance remains to be elucidated, this set of mutants strongly suggests that UV resistance, apart from DNA repair, is mediated by other mechanisms.

The lactococcal *lacX* and *lacN* genes were found to complement an *E. coli recA* strain sensitive for some mutagens. LacX and LacN may be a sensor and response regulator of a two component regulatory system with a function in the SOS response, but their function in *L. lactis* is unknown [63].

2.5. Low pH

Lactic acid bacteria produce lactic acid during sugar fermentation. This implies that they are frequently confronted with acid stress. It is important to note that lactic acid is a weak organic acid that is not charged at low pH and can easily pass the cell membrane in the protonated form. At cytoplasmic pH, it dissociates and, thus, poses a stronger stress to cells at a given extracellular pH than for example hydrochloric acid [64]. The intracellular pH of *L. lactis* cells in suspension was slightly reduced (from 7.0 to 6.0) when the extracellular pH was reduced with HCl (from 6.75 to 5.0) [65,66] but decreased linearly (from 7.0 to 5.25) with the extracellular pH when that was adjusted with lactic acid [65]. When measured in growing cultures, the intracellular pH decreased with the extracellular pH. In this way a constant ΔpH of 0.7 units was maintained [65]. *L. lactis* can resist pH 4.5 (with HCl) in minimal medium, but its viability rapidly decreases by incubation at pH 4.0 [59]. Kim et al. [3] reported that *L. lactis* subsp. *lactis* strains can survive a pH as low as 2.5 (in M17 with HCl) and that the subsp. *cremoris* can resist a pH of 3.0. Cells can survive a low pH when adapted to a sublethal pH (5.5) for only 15 min, both in a defined medium adjusted with HCl [59] or in M17 medium with lactic acid [28]. In another study [3], three exponentially growing and pre-adapted *L. lactis* subsp. *lactis* strains were shown to resist exposure to a lethal pH (2.5) whereas a large percentage of cells of three subsp. *cremoris* strains did not survive a lethal pH of 3.0. Stationary phase cells of both subspecies survived a lethal pH equally well. Acid adaptation was shown to be chloramphenicol sensitive in *L. lactis* subsp. *cremoris* MG1363 [59] but chloramphenicol independent in *L. lactis* subsp. *lactis* IL1403 [28]. Therefore, the latter strain seems to be acid resistant without the need for de novo protein synthesis. Incubation at pH 5.5 for 30 min triggered the synthesis of 33 proteins, among which DnaK, GroEL [28] and ClpP [18].

A number of mechanisms have been shown to confer acid resistance. The primary mechanism for control of intracellular pH is the F_0F_1 ATPase that translocates protons to the environment at the expense of ATP. Both the expression level and the activity of this protein complex are increased at low pH [67,68]. An acid sensitive *L. lactis* mutant was isolated that is unable to maintain a neutral intracellular pH in an acidic environment [69]. The acid sensitivity of this mutant was shown to be caused by a mutation in the ATPase structural gene resulting in a reduced enzyme activity. The mRNA levels and $F_1\beta$ protein levels in this mutant were elevated at low pH, as is the case in the wild-type strain.

A second mechanism for pH homeostasis is the arginine deiminase (ADI) pathway. This pathway allows *L. lactis* to neutralize its environment by NH_3 production [70] and the concomitant ATP generation enables extrusion of cytoplasmic protons by the

F_0F_1 ATPase. The ADI pathway consists of three cytoplasmic enzymes: arginine deiminase, ornithine carbamoyltranferase and carbamate kinase, that catalyse the reaction: arginine+H_2O+ADP+$P_i \rightarrow$ ornithine+CO_2+NH_3+ATP. These enzymes are active at low pH (2 to 3) in several *Streptococcus* species [71]. Arginine and ornithine are exchanged without the need for metabolic energy by a membrane located antiporter. The activity of the pathway is induced 3- to 5-fold in the presence of arginine [72]. The presence of the ADI pathway is a unique phenotypic property of *L. lactis* subsp. *lactis* [4], as it is only rarely observed in the subsp. *cremoris*. A relationship between the presence of the ADI pathway and higher salt and temperature tolerance in the subsp. *lactis* is likely as ADI^+ transductants of the subsp. *cremoris* showed increased stress tolerance [73]. Recently, genetic analysis of the ADI pathway in *Lactobacillus sake* revealed the presence of five genes (*arcABCTD*) encoding the four components of the pathway and a putative transaminase gene (*arcT*) [74]. Transcription of this pathway is induced by arginine and repressed by glucose.

L. lactis expresses a glutamate-dependent acid resistance mechanism in the presence of chloride [48,49]. The system is encoded by an operon consisting of two genes, *gadC* and *gadB*, which specify a putative glutamate-γ-aminobutyrate antiporter and a glutamate decarboxylase, respectively. The combined action of these two proteins may confer acid resistance by the removal of a proton from the cytoplasm and the export of γ-aminobutyrate which is more basic than the imported glutamate. Alternatively, it may also result in the formation of a proton motive force as was shown for a *Lactobacillus* sp. that could generate ATP in the presence of glutamate [75]. Expression of *L. lactis gadCB* in cultures that were allowed to acidify further during growth, by the omission of buffer from the medium, was higher than in buffered medium and *gadCB* expression may also depend on glutamate [49]. Similar mechanisms, based on the combination of amino acid antiport and amino acid decarboxylation have been described for lactobacilli. An aspartate-alanine antiporter that could stimulate ATP formation was described in *Lactobacillus* subsp. M3 [76]. *Lactobacillus buchneri* is able to generate a proton motive force by histidine decarboxylation and electrogenic histidine-histamine antiport [77]. Interestingly, a histidine decarboxylase mutant of *Lactobacillus* 30a was unable to alkalinize its environment in the presence of histidine [78], suggesting that these systems may support acid survival by restoring the pH to levels which permit growth.

Another acid stress response mechanism was recently described in *L. lactis* subsp. *lactis* biovar. *diacetylactis*. In this organism the transcription of *citP*, encoding the citrate-lactate antiporter CitP, is induced at low pH [79]. The strain could grow to a high cell density when inoculated in growth medium at pH 4.5 containing both glucose and citrate, whereas proliferation was very poor in the presence of only glucose or citrate, or in the absence of CitP. This may be explained on the basis of studies on citrolactic fermentation in *Leuconostoc mesenteroides* describing the alkalinization of the growth medium in the presence of citrate and the generation of energy by the electrogenic precursor/product antiport of citrate and lactate [80]. In conclusion, co-metabolism of glucose and citrate provides selective advantage at acidic pH to cells expressing CitP.

In a study using random transposon insertion, clones were selected that expressed the transposon encoded *lacZ* reporter at a higher level in response to low pH [81]. One of these, PA170, showed 50-fold higher *lacZ* expression in a chemostat maintained at pH 5.2 than at pH 7.0. Expression was only observed in the stationary phase and was also induced 7-fold at 15°C compared to 30°C. Genetic analysis of this promoter revealed that a 50 bp region is sufficient for transcription initiation in response to all three inducing conditions [82]. The function of the gene transcribed from the P170 promoter is not known.

To study regulation mechanisms that control acid resistance, acid resistant IS*S1* insertion mutants in *L. lactis* were selected [59]. One of the genes identified in this way shows homology with *ahrC*, a regulator of arginine synthesis and catabolism in *B. subtilis*, and may be involved in control of the arginine deiminase pathway. A number of the other acid resistant mutants contained insertions in genes that have roles in nucleotide metabolism and may act in the biosynthesis of (p)ppGpp, the stringent response regulator. This result points to the possible involvement of the stringent response in pH regulation in *L. lactis* [59].

2.6. Starvation

Exhaustion of an essential nutrient limits growth of a culture which then enters the stationary growth phase. It is generally accepted that stationary phase is the most common state of bacterial cells in nature. Glycolytic and arginine deiminase pathway activities decline rapidly upon entry of *L. lactis* in the stationary phase [83]. These activities could be restored by the addition of sugar. Resumption of glycolytic activity was slower after a longer period in stationary phase and was chloramphenicol sensitive. In contrast, arginine deiminase pathway activity raised to the prestarvation rate independent from the duration of starvation and required no protein synthesis. Total cell protein is degraded during exponential growth and in the first 30–90 min of stationary phase, but proteins were stably maintained afterwards [83]. Cells react to starvation by increasing the level of 14 proteins, despite the lack of metabolic energy. Major changes were also observed in the protein composition of the cytoplasmic membrane, but amino acid transport capacity was hardly affected in the stationary phase [83]. Although *L. lactis* is unable to react to starvation by, for instance, the development of competence or the formation of spores, it does develop multiple stress resistance. The first sign of such an adaptation was the observation that lactose starved cells survived longer when cultured at a lower imposed growth rate before starvation [84]. *L. lactis* appeared to be highly resistant, already at the onset of the stationary phase, to incubation at 52°C or pH 4.0, or to the presence of 20% ethanol, 3.5 M NaCl or 15 mM H_2O_2 [85]. Analysis of acid resistance of cells during growth revealed that it is acquired during the late exponential phase. The latter is not surprising as growth of *L. lactis* always results in acid formation.

Genetic studies on starvation response have only just started.

2.7. Cross protection and global regulation

Subjection to a mild stress makes cells resistant to a lethal challenge with the same stress condition, as has been discussed above for a number of stress conditions. Moreover, preadaptation to one stress condition can render cells resistant to other stress imposing conditions. This is of relevance for cells in environments where they can be exposed to combinations of stress conditions. The best example of such a multiple stress resistance is that acquired in the stationary phase. Also, exposure to UV light (100 J/m^2) conferred cross protection against a treatment with heat, acid or ethanol and, to a lesser extent, against H_2O_2 [27]. Another aspect of the same phenomenon was seen by total protein analysis in 2D gels. UV challenge induced 14 proteins in *L. lactis* of which four were also induced by other stress conditions. Similarly, nine of the 33 proteins that were induced at low pH were also induced by heat shock [28]. This is suggestive of an overlap between the control circuits regulating responses to these stresses. Other evidence for the existence of such interaction is the involvement of *recA* in both oxidative stress, and heat shock and the acid inducibility of *hflB* [86], which is also implicated in heat shock response. Interestingly, in a selection procedure for thermoresistant mutants of the thermosensitive *L. lactis recA* strain, a number of the disrupted genes appeared to be the same as those found in selection for acid resistant mutants [59,29]. One of these (*deoB*) was also found by selection for UV resistant mutants [62]. These observations suggest a link between the heat shock response and acid resistance in *L. lactis*. A number of the genes found in these mutagenesis studies are involved in metabolic pathways for guanine and phosphate. As these are related to (p)ppGpp synthesis, the stringent response may be involved. However, there is no evidence for direct control of heat shock genes or regulators and acid resistance genes by the stringent response.

Another open question as to the control of stress responses in *L. lactis* is the involvement of sigma factors. Despite several attempts to identify alternative sigma factors, thus far only the vegetative sigma factor has been described [87,88]. Control mechanisms in *L. lactis* are likely to resemble those in the Gram-positive bacterium *B. subtilis* more than those of the Gram-negative *E. coli*. Indeed, remarkable similarities in the control of heat shock genes, including the CIRCE element and *hrcA*, have been found between *L. lactis* and *B. subtilis* (see above). Another group of *B. subtilis* genes involved in general stress resistance is controlled by σ^B [20]. It would be interesting to elucidate whether such general stress pro-

teins and their regulator σ^B are present in *L. lactis*. The striking properties of *recA* in relation to heat shock response and oxidative stress suggest that control of stress responses in *L. lactis* is markedly different from other bacteria, as has been noted for the control of other cellular functions [89].

3. Development of expression systems for *L. lactis*

Another reason to study stress response mechanisms of *L. lactis* is related to its biotechnological potential. As *L. lactis* has the GRAS status (generally regarded as safe), it is an interesting tool for the production of new fermented food products or for in situ modification of foods. Strains with new traits, different from the ones currently known may be desirable. These can be obtained by the introduction of genes from non-lactococcal sources, thereby opening the way for specific new applications. Genetically *L. lactis* is the best characterized species of the lactic acid bacteria. Sophisticated techniques for the genetic modification of *L. lactis* have been developed in the last 15 years [90,91]. These include cloning vectors, integration systems, expression signals, and selection markers entirely based on lactococcal DNA.

The gene expression systems developed thus far direct, with a few exceptions, constitutive gene expression. However, for the expression of lethal gene products, inducible gene expression systems are indispensable. Such systems are also preferred in many other cases in order, for instance, to obtain high yields of non-lethal proteins. The induction of certain activities during an (industrial) process requires expression signals that allow tight control. The inducing signals should be food compatible and should, thus, be either a safe food additive or a change in an environmental condition that occurs naturally or during the fermentation process, or can be easily incorporated in the process. In other words, regulatory systems that respond to changes in the environment that normally control stress adaptation mechanisms hold promise for use in food grade inducible gene expression systems.

3.1. Stress inducible gene expression systems

The expression of a number of lactococcal operons is regulated (reviewed in [89]). Only a few of these depend on stress conditions. The regulatory elements involved that have been used for the controlled expression of other genes will be discussed here.

Regulation signals of lactococcal heat shock genes have been used for heterologous gene expression. However, the levels of induction from these promoters upon heat shock were rather low [92,93]. A fusion of the *dnaJ* promoter with *usp45*::*amyS* showed a 4-fold higher level of secreted *Bacillus stearothermophilus* α-amylase activity 30 min after a shift from 30°C to 42°C [15].

P_{gad}, the promoter of the lactococcal *gadCB* operon [49], can be induced more than 1000-fold by growth in the presence of 0.5 M NaCl. In addition, expression is induced at low pH. A positive regulator encoded by *gadR* is indispensable for the chloride-dependent expression of *gadCB*. The P_{gad} promoter was amplified together with *gadR* and this expression cassette was inserted upstream of the lysis cassette *lytPR* of the lactococcal bacteriophage r1t and upstream of *acmA* of *L. lactis*, the gene specifying the cell wall hydrolase. Induction of *L. lactis* carrying these fusions with NaCl resulted in overexpression of the cell wall degrading enzymes and in cell lysis, evidenced by the release of cytoplasmic enzyme activity [94]. The basal expression level of the fusions was very low and did not harm the cells.

Another way of obtaining a stress inducible promoter is by mutagenesis of a regulated promoter and/or its repressor that normally respond to a different stimulus. This approach was chosen for the exploitation of the genetic switch element of the temperate lactococcal bacteriophage r1t. This element is normally activated by DNA damage caused by UV radiation or by chemicals like mitomycin C and results in a change in the life cycle of the phage from the lysogenic state to the to lytic phase. The switch element consists of two divergently transcribed genes, *rro*, encoding the phage repressor, and *tec*. Expression of the genes (including *tec*) of the lytic growth phase downstream of promoter P_2 is repressed by Rro. When *E. coli lacZ* was placed on a plasmid downstream of P_2, β-galactosidase expression was induced about 70-fold after addition of 1 μg ml^{-1} mitomycin C [95]. Rro was shown to bind specifically to an operator sequence present twice in the promoter region and once in *tec*. In

this way it shuts off expression of the P_2 driven genes. Recently, a temperature sensitive repressor mutant (Rro12) was constructed by site directed mutagenesis on the basis of comparative molecular modelling. Promoter P_2 driven expression of *lacZ* is efficiently repressed by Rro12 at 24°C and is 600-fold higher at 40°C [96].

4. Concluding remarks

The increasing number of studies on stress in *L. lactis* allows an overview of its stress response behaviour and an opportunity for comparison with other organisms. Various stress conditions and the cellular responses to these conditions are summarized in Fig. 1. Best studied is the response to heat shock. In particular, the insight into the mechanisms that control the expression of the chaperone proteins in response to various conditions is increasing. In contrast, almost nothing is known of the regulatory mechanisms controlling the responses to other types of stress, including the induction of non-chaperone heat shock proteins. The number of induced proteins observed in 2D-PAGE analyses indicates that much more genes and proteins are involved in stress responses than currently known and indicated in Fig. 1.

Obviously, growth studies confirm that *L. lactis* is a mesophile. It is, however, able to survive more harsh conditions, in particular when pretreated with a mild stress condition. It is questionable whether the laboratory conditions and culture media are representative for survival in a natural situation, either in a milk (derived) environment or on plant material. The results of, for example, an acid resistance test differ considerably depending on the use for the low pH challenge of either a buffer, a defined medium, or a complex medium (our unpublished observations). Natural situations may confront *L. lactis* not only with physical stress but also with toxic compounds. Interestingly, *L. lactis* appears to harbour specialized multidrug transporters for the removal of certain classes of (putative toxic) drugs or metabolites from its cytoplasm, as reviewed elsewhere [97].

It is no surprise that the systems employed by *L. lactis* for protection against various environmental stress conditions are similar to those found in other bacterial species and higher organisms. Several of such mechanisms are not present in more closely related Gram-positive species but were found in more distant Gram-negative bacteria. Whereas the stress resistance systems found thus far are well conserved at the protein level, the underlying molecular mechanisms responsible for the timing and fine tuning of the expression of these proteins in response to environmental changes are in most cases rather different and unique for *L. lactis*. A striking example is the regulation of the chaperone protein expression, which depends on HrcA and DnaK and in which also RecA is involved. The latter was thus far seen as a key protein in homologous recombination and DNA repair.

The mechanisms for sensing environmental conditions and the coupled cellular signalling routes are still undefined. Regulation could be organized either globally or be stress specific. There are no signs thus far for the presence of multiple sigma factors such as has been described for *E. coli* and, in particular, for *B. subtilis*. Only the gene for the vegetative sigma factor, *rpoD*, has been found [87,88] and no deviating promoter sequences for certain classes of stress genes have been reported. Many sensors in other species that monitor environmental conditions belong to the class of two component regulatory systems. These usually comprise a membrane located histidine protein kinase that senses a specific signal and transfers this by phosphorylation to a response regulator. This protein, when phosphorylated, can activate cellular processes, often at the level of transcription. The two component regulatory systems in lactic acid bacteria characterized thus far regulate the expression of bacteriocins by sensing the concentration of bacteriocin or a bacteriocin-like peptide in their environment (reviewed in [98]). However, recent data show that genes for other homologs of histidine protein kinases and response regulators are present on the chromosome of *L. lactis*. The cellular processes controlled by these systems and the conditions they respond to are still unknown [99].

The present knowledge on the growth limits of *L. lactis* supports the insights gained from centuries of practical use of the organism in dairying. This forms a basis for the systematic evaluation of combinations of different strategies to obtain optimal food preservation. The available data can be combined with the

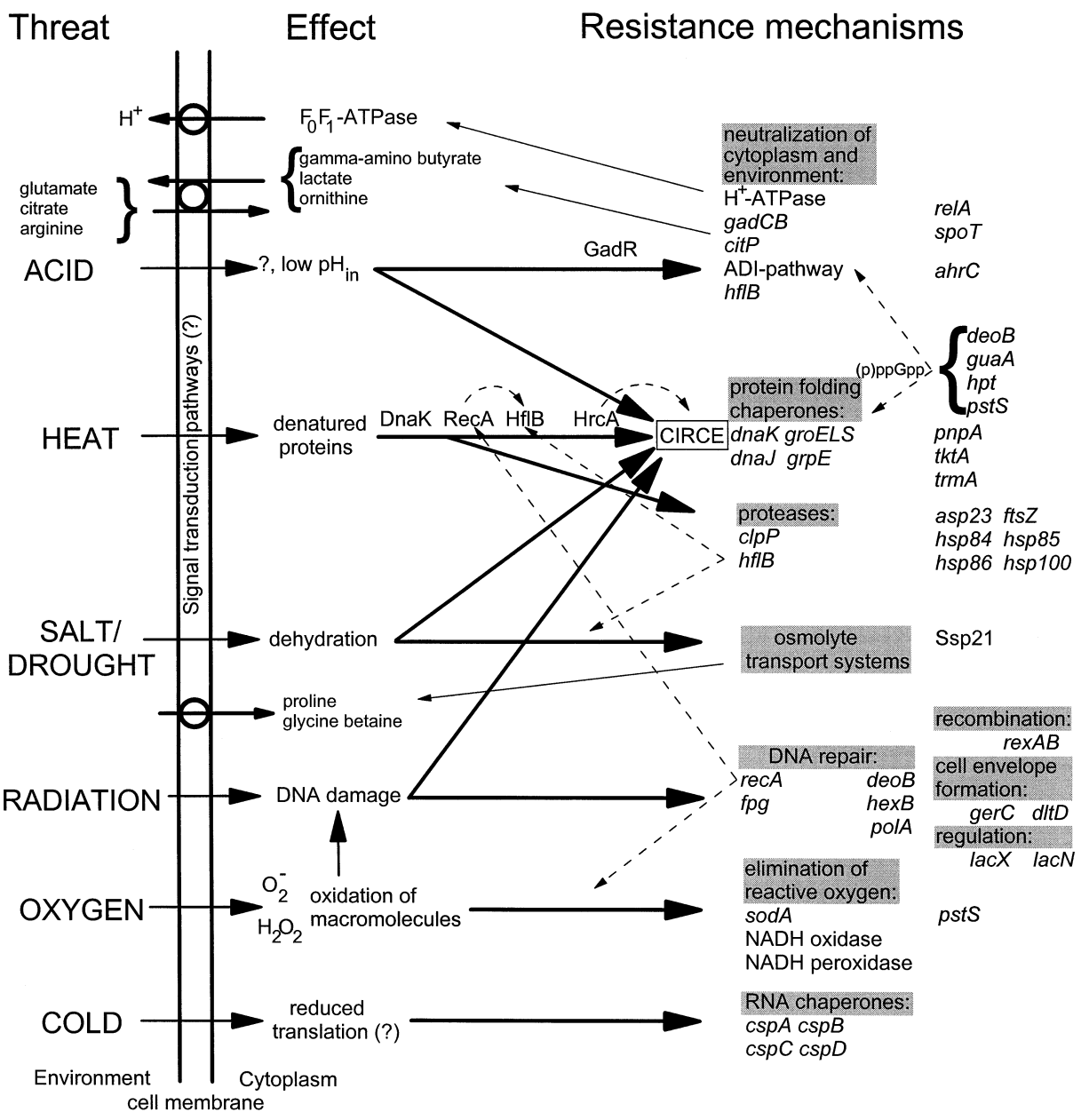

Fig. 1. Schematic view of the known stress resistance mechanisms operative in *L. lactis*. The fat arrows point to the induced genes/proteins specific for that condition. Thin arrows point to membrane located stress resistance mechanisms. Broken arrows and protein names above the fat arrows indicate regulatory functions. At the right proteins that were identified as spots in 2D-PAGE or genes that gave stress resistant or sensitive phenotypes when mutated are listed. Their exact roles are to be elucidated.

evaluation of alternative conservation techniques like high pressure treatment [100] and sonication [101]. The stress resistance properties of *L. lactis* discovered now could form the basis for the design of alternative procedures to optimize the conditions for the efficient and low cost maintenance, storage, transport and application of strains that are used in industrial fermentations. The understanding of the

underlying molecular mechanisms may provide targets for specific manipulation to promote or prevent lactococcal growth. For example starter cultures that are stored frozen will have a higher viability when incubated at 10°C prior to freezing. An alternative storage procedure is to dry starter cultures. Survival of the osmotic stress imposed by this treatment will be promoted by preculturing the starter in the presence of osmolytes. A number of studies [70–72,79,80] have made clear that the ability to ferment citrate and/or arginine has two advantages: it provides cells with an alternative source of energy and gives protection against low pH. This notion can be used for the selection of strains and for the design of culture media. Stress-induced proteins are clear molecular markers for the fitness of starter cultures. These proteins indicate that a culture has been subjected to a stress and, therefore, that the cells may not optimally perform in a following large fermentation. On the other hand, these markers could also be used as positive indicators for a culture that is fully adapted to resist an upcoming stress condition. Mutants affected in a stress resistance mechanism, e.g. acid resistance, may proliferate only partially under certain conditions. These may be useful to control fermentation, for example to limit acidification of a culture. Otherwise, mutants with improved stress resistance properties, for instance due to a break in a negative control circuit, may survive adverse conditions that occur in industrial processes.

Of particular interest for future work will be the processes operating in cells that enter the stationary growth phase in a low pH environment, as this is a condition commonly encountered by and unique for lactic acid bacteria.

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