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. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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

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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 lacticoccal proliferation when desired or to prevent spoilage in products that need no lacticoccal 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
(reasons. Scientific interest is fuelled by the fact that 
*L. lactis* is a mesophilic Gram-positive microorgan-
ism 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 phospho-
rylation state. Regulation can also take place by dif-
ferential degradation of a protein. Key examples of
stress proteins which are controlled at these various
levels are σ5 [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 I)
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 dis-
cussed for a number of stress conditions. Compari-
son of the different studies in this field is hampered
by differences in the analysis techniques and by dif-
ferences in challenge conditions and incubation times
that have been used to stress cells. Careful interpre-
tation 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 tem-
peratures or in an anaerobic environment.

2. Stress

The stress responses of *L. lactis* have gained inter-
est in recent years not only because of the industrial
relevance of the organism but also for basic scientific

### Table 1

<table>
<thead>
<tr>
<th>Stress</th>
<th>Conditions</th>
<th>Number of proteins with Enhanced expression</th>
<th>Reduced expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock</td>
<td>42°C, 5-25 min</td>
<td>most</td>
<td>17 (at 16°C)</td>
<td>[10]</td>
</tr>
<tr>
<td>Low temperature</td>
<td>8°C, 1-10 h</td>
<td>majority</td>
<td></td>
<td>[30]</td>
</tr>
<tr>
<td>Low pH</td>
<td>pH 5.5, 30 min</td>
<td></td>
<td>majority</td>
<td>[28]</td>
</tr>
<tr>
<td>Starvation</td>
<td>3 h galactose and arginine exhaustion in CDM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;45</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td>Salt</td>
<td>2.5% NaCl, 10-40 min</td>
<td>most</td>
<td></td>
<td>[12]</td>
</tr>
<tr>
<td>UV light</td>
<td>254 nm, 100 J/m²</td>
<td>30</td>
<td></td>
<td>[27]</td>
</tr>
</tbody>
</table>

<sup>a</sup>CMD, chemically defined medium.

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 addi-
tion, some potential applications of this knowledge
will be discussed.

#### 2. Stress

2.1. Heat shock

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

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against bacteriophages and bacteriocins. The rela-
tively 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 de-
scribe *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cre-
moris*. 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 grow at
40°C and in the presence of 4% NaCl, whereas most
subsp. *cremoris* strains do not, is used to phenotypi-
cally 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]. Indus-
trial subsp. *cremoris* strains probably have lost some
properties that are essential to survive in nature but
not or even disadvantageous for continuous subcul-
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peratures or in an anaerobic environment.

2.1. Heat shock

When an *L. lactis* culture was shifted from its
Table 2
Genes induced by environmental stress in *L. lactis*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of protein</th>
<th>Stress condition</th>
<th>Induction factor</th>
<th>Analysis method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>groESL</em></td>
<td>chaperone</td>
<td>heat shock</td>
<td>43°C, 15 min in GSA[^a^]</td>
<td>10</td>
<td>RNA</td>
</tr>
<tr>
<td><em>groES</em></td>
<td>chaperone</td>
<td>heat shock</td>
<td>42°C, 30 min in GM17[^a^]</td>
<td>12</td>
<td>protein</td>
</tr>
<tr>
<td><em>groEL</em></td>
<td>chaperone</td>
<td>heat shock</td>
<td>37°C, 60 min in GM17</td>
<td>3</td>
<td>protein</td>
</tr>
<tr>
<td><em>dnaJ</em></td>
<td>chaperone</td>
<td>heat shock</td>
<td>42°C, 10 min in WP[^a^]</td>
<td>3 to 4</td>
<td>RNA</td>
</tr>
<tr>
<td>ORF11/hrcA</td>
<td>negative regulator of heat shock genes</td>
<td>heat shock</td>
<td>43°C, 15 min in GSA</td>
<td>5</td>
<td>RNA</td>
</tr>
<tr>
<td><em>grpE</em></td>
<td>chaperone</td>
<td>heat shock</td>
<td>43°C, 15 min in GSA</td>
<td>5</td>
<td>RNA</td>
</tr>
<tr>
<td><em>clpP</em></td>
<td>protease</td>
<td>heat shock</td>
<td>43°C, 15 min in GSA</td>
<td>10</td>
<td>RNA</td>
</tr>
<tr>
<td><em>ftsH/hflB</em></td>
<td>regulator of heat shock response</td>
<td>heat shock</td>
<td>43°C, 10 min in GSA</td>
<td>+[^b^]</td>
<td>RNA</td>
</tr>
<tr>
<td><em>recA</em></td>
<td>SOS regulator</td>
<td>DNA damage</td>
<td>0.03% methylnitrosourea, or 1 μg ml⁻¹ mitomycin C aerated culture</td>
<td>5.2</td>
<td>protein</td>
</tr>
<tr>
<td><em>fpg</em></td>
<td>SOS regulator</td>
<td>DNA damage</td>
<td>0.03% methylnitrosourea, or 1 μg ml⁻¹ mitomycin C aerated culture</td>
<td>+[^c^]</td>
<td>RNA</td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>O₂ scavenging</td>
<td>oxidation</td>
<td>oxidation aerated culture</td>
<td>2</td>
<td>reporter</td>
</tr>
<tr>
<td><em>cspA</em></td>
<td>RNA stabilization</td>
<td>cold shock</td>
<td>10°C, 1 h in GM17</td>
<td>10</td>
<td>RNA</td>
</tr>
<tr>
<td><em>cspB</em></td>
<td>RNA stabilization</td>
<td>cold shock</td>
<td>10°C, 4 h in GM17</td>
<td>40</td>
<td>RNA</td>
</tr>
<tr>
<td><em>cspC</em></td>
<td>RNA stabilization</td>
<td>cold shock</td>
<td>10°C, 2 h in GM17</td>
<td>8</td>
<td>RNA</td>
</tr>
<tr>
<td><em>cspD</em></td>
<td>RNA stabilization</td>
<td>cold shock</td>
<td>10°C, 4 h in GM17</td>
<td>30</td>
<td>RNA</td>
</tr>
<tr>
<td>P170</td>
<td>acid stress resistance</td>
<td>low pH</td>
<td>growth at pH 5.2 vs. pH 7.0</td>
<td>50</td>
<td>reporter</td>
</tr>
<tr>
<td><em>gadCB</em></td>
<td>acid stress resistance</td>
<td>chloride</td>
<td>growth with 0.5 M NaCl vs. no NaCl</td>
<td>1000</td>
<td>reporter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>low pH</td>
<td>growth with 0.3 M NaCl, in non-buffered vs. buffered M17, pH 4.2 vs. pH 5.5</td>
<td>10</td>
<td>reporter</td>
</tr>
</tbody>
</table>

[^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.

The 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 (dnaKΔ1, 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 hsp86, and the gene asp23 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 dnaKLΔ 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 dnaKΔ 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 σ32. The level of σ32 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 3½ 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].
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 HindIII 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-translational 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 pls 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$_2$O$_2$. *L. lactis* has no catalase and depends solely on NADH peroxidase to keep H$_2$O$_2$ levels at subinhibitory concentrations. At sublethal H$_2$O$_2$ levels, *L. lactis* develops an adaptive response against lethal concentrations of H$_2$O$_2$ [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 ($\cdot$OH), the most reactive oxygen species, because this is formed in the H$_2$O$_2$- and Fe$^{2+}$-dependent Fenton reaction (H$_2$O$_2$+Fe$^{2+}$+H$^+$ $\rightarrow$ $\cdot$OH+H$_2$O+Fe$^{3+}$) [57]. $\cdot$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$_2$O$_2$ 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 ISS1 mutagenesis. Mutants defective in the *recAB* 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/m² 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 F₀F₁ 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 F₁β protein levels in this mutant were elevated at low pH, as is the case in the wild-type strain. Incubation at pH 5.5 triggered the synthesis of 33 proteins, among which DnaK, GroEL [28] and ClpP [18].

A second mechanism for pH homeostasis is the arginine deiminase (ADI) pathway. This pathway allows L. lactis to neutralize its environment by NH₃ production [70] and the concomitant ATP generation enables extrusion of cytoplasmic protons by the
FoF1 ATPase. The ADI pathway consists of three cytoplasmic enzymes: arginine deiminase, ornithine carbamoyltransferase and carbamate kinase, that catalyse the reaction: arginine+H2O+ADP+P1→ornithine+CO2+NH3+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 (arcAECTD) 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 antiporter 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 alkalize 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. di-acetylactis. 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 Pl70 promoter is not known.

To study regulation mechanisms that control acid resistance, acid resistant ISS1 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 H2O2 [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/m2) conferred cross protection against a treatment with heat, acid or ethanol and, to a lesser extent, against H2O2 [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 σB [20]. It would be interesting to elucidate whether such general stress pro-
teins and their regulator σB 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.

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 rlt and upstream of aemA 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 rlt. 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 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.
this way it shuts off expression of the P2 driven genes. Recently, a temperature sensitive repressor mutant (Rro12) was constructed by site directed mutagenesis on the basis of comparative molecular modelling. Promoter P2 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.

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
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 industrial processes. 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 natural ecosystems. Appl. Environ. Microbiol. 61, 788–792.


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