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Two-component signal transduction systems as key players in stress responses of lactic acid bacteria

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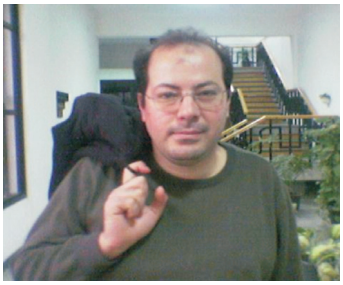
ABSTRACT

Lactic acid bacteria (LAB) continue as an important group of gram-positive bacteria that have been extensively exploited in food industries and various biotechnological applications. Some LAB species are, however, opportunistic pathogens and were reported to be associated with overwhelming number of human infections. During the use of LAB in industry or over the course of human infection, these bacteria are exposed to environmental stress. While LAB display adaptive mechanisms to cope with adverse conditions, the regulation of these mechanisms remains to be elucidated. Recent completion of genome sequencing of various LAB strains combined with the development of advanced molecular techniques have enabled the identification of a number of putative two-component signal transduction systems, also known as two-component regulatory systems (2CRS), in LAB. Examining the effect of deleting genes specifying putative 2CRS proteins in these organisms has revealed the involvement of 2CRS in the responses of LAB to different stresses. There are lines of evidence indicating that certain 2CRS may mediate a general stress response in Enterococcus faecalis *and* Streptococcus pyogenes. *This review highlights the influence of 2CRS on the physiology of LAB during optimal growth and survival/growth on exposure to environmental stress.*

Keywords: *lactic acid bacteria, stress responses, two-component signal transduction systems*



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(*photo by Mohamed Samir Darwish*)

Overview of lactic acid bacteria

Lactic acid bacteria (LAB) are a group of phylogentically-related gram-positive organisms which are able to ferment carbohydrates producing lactic acid. They share general generic and physiological features including: (1) low (<55%) G + C content; (2) fastidious nutritional requirements; (3) inability to synthesise porphyrin groups which makes them unable to produce catalases and cytochromes and thus adopt fermentative metabolic pathway rather than a respiratory one; (4) nonsporeforming; (5) nonaerobic, but aerotolerant; and (6) acid-tolerant[1]. However, different LAB show variations on these general characteristics and in other biological traits including morphology (cococci/bacilli), mode of sugar fermentation (homofermentative/heterofermentative), optimal growth temperature (mesophilic/thermophilic), *etc*... These variations are, in some cases, to the extent that makes one wonder on the feasibility of combing these bacteria in a single group (see Axelsson[1] for interesting examples). They are really the characteristics of being G^+ and fermenting sugars, with lactic acid as a major end product, that practically define different bacteria as LAB. It is currently believed that these bacteria involve the following genera: *Streptococcus* (*S.*), *Lactococcus* (*Lc.*), *Enterococcus* (*E.*), *Lactobacillus* (*Lb.*), *Pediococcus* (*P.*), *Leuconostoc* (*Ln.*), *Aerococcus* (*A.*), *Carnobacterium* (*C.*), *Oenococcus* (*O.*), *Tetragenococcus* (*T.*), *Vagococcus* (*V.*) and *Weisella* (*W.*). Other genera including *Gemella*, *Erysipelothrix*, *Alloiococcus*, *Dolosigranulum*, *Globicatella* and *Lactosphaera* were also reported as LAB but this is still associated with controversy.

Bacterial species and strains within the previous genera are wide spread in the nature, being resident of humans, animals, plants and

foods. While some of these bacteria are very beneficial for human health and have been exploited for the preparation of fermented foods, others are opportunistic pathogens. As with other bacteria, both beneficial and negative influences of LAB had been early appreciated before characterising bacteria on scientific bases. It is estimated that the preparation of naturally fermented foods dates back to 6000 BC during the civilisations of the Fertile Crescent in the Middle East, very early before man even recognised the presence of bacteria[2]. However, two scientific achievements have initiated our current detailed knowledge and massive industrial exploitation of LAB. These were the discovery of lactic acid fermentation by Louis Pasteur in 1857 and the development of LAB purification and preservation methods by the beginning of the twentieth century. Since then, LAB have continued to be central in the preparation of fermented foods and other biotechnological applications. They are considered to be the second to the yeast, *Saccharomyces cerevisia* in terms of their practical and economic value to global food fermentation industries (Jeffery Broadbent, personal communication). It is estimated that 13 million tons of cheese are annually prepared[3], for which diverse LAB are used for milk fermentation and enhancing cheese flavour, texture and preservative qualities[4]. Of the above LAB, species of six genera are important for industrial food and beverage fermentations including *Lactococcus* sp. (milk fermentations), *Lactobacillus* sp. (milk, meat, vegetable, cereal fermentations), *Leuconostoc* sp. (milk, vegetable fermentations), *Pediococcus* sp. (vegetable, meat fermentations), *Oenococcus oeni* (wine fermentations) and *Streptococcus thermophilus* (milk fermentations)[4]. Not only are these organisms employed in fermentation industries, but their usefulness also extends to other important biotechnological applications including the production of antimicrobial substances, *e.g.* nisin by *Lc. lactis*, enzymes by *Lb. brevis*, biopolymers by *Ln.* sp., ethanol and lactic acid by *Lb. casei*, *Lb. brevis* and *Lb. lactis*.

The regular consumption of food fermented by LAB has been presumed to promote good health. It was interestingly proposed by Elie Metchnikoff in 1907 that the consumption of fermented milks on a frequent basis may prolong human life. This was based on the hypothesis that regular uptake of LAB with foods may serve to provide the intestine with colonising cells of these organisms. Those LAB colonisers would help to exclude pathogenic bacteria, notably putrefactive clostridia, from the intestine or inhibit their activity by the production of lactic acid. While there are reservations on this hypothesis, it is considered as a milestone in the development of

what is now know as the "probiotic" concept. Probiotics are selected LAB and LAB-related strains, mostly of the *Lactobacillus* and *Bifidobacterium* species, which are incorporated into fermented milks. It is claimed that when probiotics successfully colonise the human gut, they serve one or more of the following benefits: inhibition of pathogens, enhancement of the immune system, lowering blood cholestrol, synthesis of vitamins and providing prophylactics against colon cancer. Recently, another interesting medical application of LAB has emerged, in which LAB are used as oral delivery vehicles for digestive enzymes and vaccines.

Despite all these useful industrial and medical applications, a number of LAB species/strains are human pathogens. Those include species of streptococci and enterococci such as *Streptococcus pneumoniae*, *Str. pyogenes*, *Str. agalactiae*, *Enterococcus faecalis*, *E. faecium* and *E. durans*. Interestingly, all LAB, like other heterotrophic bacteria, are opportunistic pathogens and beneficial LAB species were found associated with serious infections in the heart, infective endocarditis, and the urinay tract[5]. However, these infections are generally rare and only develop in immuno-compromised persons with disease risk factors such as heart defects. They also emerge when LAB species already existing in human body, rather than in ingested food, gain access to the blood stream.

Very frequently, both beneficial and pathogenic LAB experience adverse environmental conditions (stress) (Figure 1). This occurs, for instance, while LAB pass, as food ingredients or contaminants, through the stomach where they are exposed to severe low pH ranging from 1.5 to 3.0. During the preparation of fermented foods, industrial LAB may also experience diverse stresses. For example, during the making of several cheese varieties, milk is fermented by the addition of appropriate LAB "starter" cultures such as *Lc. lactis*, *S. thermophilus* that acidify milk. Then, an enzymatic preparation, known as "rennet", is added to achieve milk coagulation. The resultant curd (coagulum) is cut into pieces, progressively heated to elevated temperatures and finally scooped into moulds, in which they are pressed to eliminate moisture. Curd or cheese is also salted by the addition of dry salt (NaCl) or soaking in a brine solution. Employing these procedures, LAB are thus exposed to acid, heat and osmotic stresses. Even when LAB are prepared for commercial distribution in the form of lyophilised starter cultures, they encounter freezing and drying stresses. On the other hand, during infection by pathogenic LAB, bacterial cells

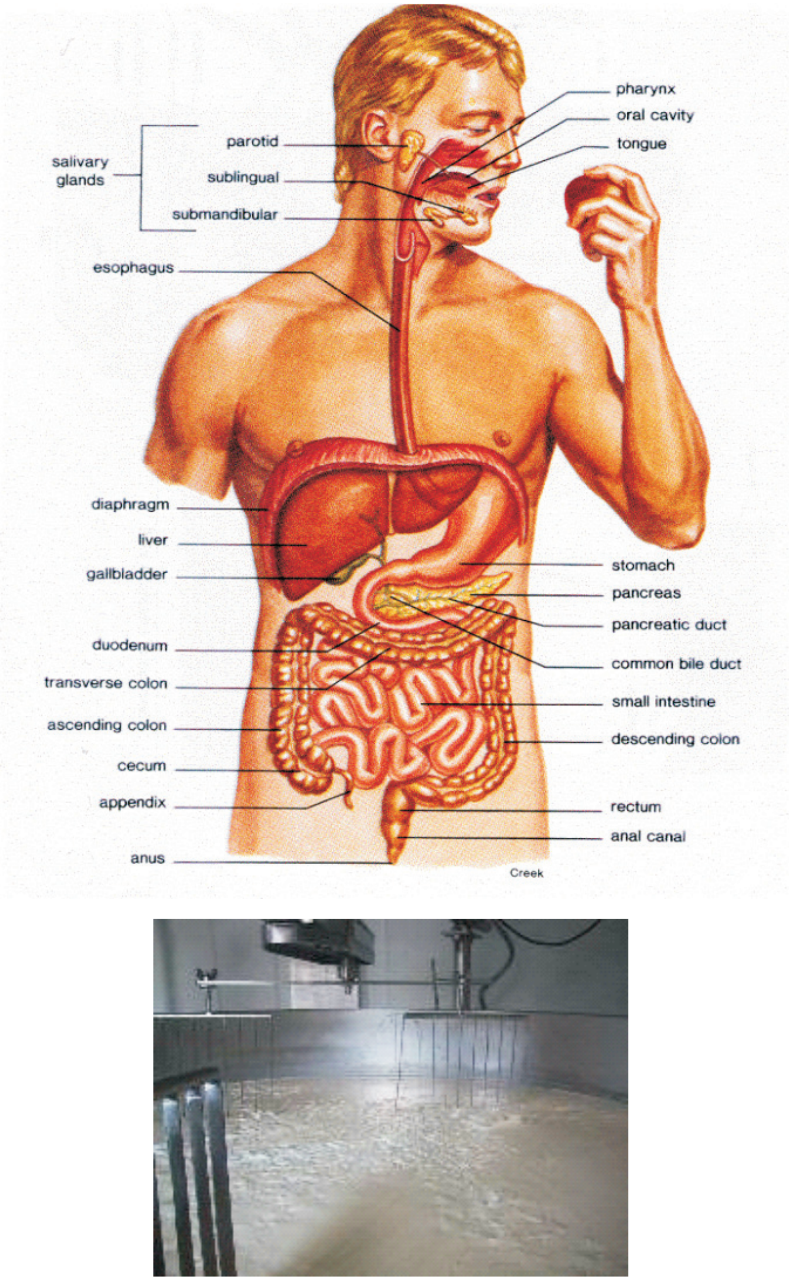

Fig. 1. *Examples of stressful situations encountered by LAB. (A) during their passage through the digestive channel, included within fermented foods, LAB are exposed to severe acidity in the stomach and free short fatty acids in the intestine; (B) LAB are exposed to acid, heat and osmotic stresses during cheese-making.*

may also be subjected to different stresses including low pH in the stomach, vagina or abscesses, high temperature associated with fever and osmotic stress provoked by the release of perspiration in the skin infections[6].

Developing adaptive strategies to cope with these environmental hardships is therefore essential for LAB to pose their functional characteristics in food fermentation or to also deal with human defence systems. It is particularly important for probiotic LAB that they tolerate stomach acidity to allow sufficient cell numbers to reach the intestine in a viable state. The study of stress-adaptive mechanisms in LAB is thus central for our basic understanding of their important industrial and medical aspects. This understanding has been exploited in vital applications including the production of LAB strains of improved performance in fermentation industries and probiotic LAB of higher potential for promoting good health. Studying stress-responsive mechanisms in pathogenic LAB may also aid the development of effective medical treatments against their infections.

Regulation of stress responses in LAB

On exposure to stress, LAB display adaptive responses whose genetic and phenotypic features are similar to those described in model bacteria, *i.e*. *Escherichia coli* and *Bacillus subtilis*. For instance, LAB are equipped with genes encoding heat shock proteins, cold shock proteins and amino acid deaminases and decarboxylases, which were shown to be expressed by model bacteria to aid their survival during heat, cold and acid stress, respectively. Like *E. coli*, LAB also show stress-habituating phenomena such as thermotolerance, cryotolerance and acid adaptation. In these, the organism increases its tolerance to severe stress following brief exposure to mild levels of the same stress. Furthermore, LAB are able to develop cross-protection responses, in which the protective effect of previous stress exposure induces endurance to other related/unrelated stresses (For excellent reviews on stress responses of LAB, see Girgis *et al*.[7] and van de Guchte *et al*.[8]). However, the regulation of stress responses is not yet well-characterised in LAB.

Given the similarities in these responses between LAB and model bacteria, it could be presumed that both would accommodate similar stress regulatory processes. A prime mechanism in *E. coli* and *B. subtilis* is the stress-induced expression of alternative sigma factors (σ) that direct RNA polymerase to transcribe stress-

responsive genes. The presence of sigma factors is not restricted to these specific organisms but they also exist in most members of the γ branch of the proteobacteria, and in several G^+ species related to *B. subtilis*. While several alternative sigma factors have been discovered in bacteria, σ^S and σ^B appear to be the most important in G^- and G^+ organisms, respectively[9]. This arises from their being involved in regulating the transcription of genes encoding proteins aiding adaptation under diverse stress conditions. This presents them as regulators of general stress responses in bacteria. Annotations of available LAB genome sequences did not show the presence of either σ^S or σ^B orthologs (Jeffery Broadbent, *personal communication*). However, some LAB strains contain gene homologues of other alternative σ-factors in *B. subtilits*, the model organism for G^+ bacteria. Those involve orthologs of SigX and SigH in *Lc. lactis* IL1403[10], and *Lb. plantarum*[11] WCFS1, respectively. While SigX and SigH were reported to be involved in regulating heat stress response and sporulation, respectively, in *B. subtilis*, no similar functions have been assumed for their orthologs in LAB. An interesting finding is the presence of an alternative σ-factor designated SigV in *E. faecalis*, which shows little similarity to most other sigma factors, but was reported to be involved in cell response to ethanol, acid and osmotic stresses[12]. It is suggested that SigV exerts its regulatory effect through a two-component partnering mechanism, in which an anti-SigV protein extending through the cytoplasmic membrane binds and inactivates its cognate SigV[13]. On exposure to reactive environmental changes, SigV is released and directs RNA polymerase to transcribe stress-responsive genes. While a putative *sigV* gene was also detected in *Lc. lactis* IL1403 genome, no orthologs of this gene were found in the genome of *Lb. helveticus* CNRZ 32 (Broadbent and Steele, *personal communication*).

Overall, it appears that most LAB species lack alternative sigma factors that serve regulatory roles as in *E. coli* and *B. subtilis*. While a two-component system involving an alternative σ-factor and a cognate transmembrane molecule was found to display stress-response adjusting functions, the existence of such a system is restricted to specific LAB strains. However, this system is reminiscent of the two-component signal transduction systems that are implicated in regulating a myriad of cellular reactions including sporulation, chemotaxis, virulence and stress adaptation in G^- and G^+ model organisms[14]. There has been a growing evidence over the last decade indicating the wide spread distribution of these systems in various LAB genomes and that a number of these

systems are involved in stress responses expressed by these bacteria. The present article discusses the extent to which the two-component signal transduction systems may be involved in the physiology of LAB under optimal growth conditions and during exposure to environmental stress. Because of their regulatory roles in various bacteria, those systems are also referred to as ''two-component regulatory systems'' (2CRS), a term that will be adopted throughout this review.

Functioning and organisation in 2CRS

On exposure to adverse environmental conditions, bacterial cells modify their physiology as to survive hostile conditions, or to even pursue growth during mild stress. To elicit a successful adaptive response, cells are presumed to display a cascade like the one shown in Figure 2[15,16]. In this, stress is initially sensed by a cellular structure that detects environmental changes and elicits a signal. On successful delivery of this signal, cells induce or repress the expression of specific genes whose protein products are involved in survival or growth during stress. Stress signals may also directly influence the functionality of protein components involved in regulatory systems, *e.g.* flagellar motors mediating chemotaxis. When cells manage to adapt to stress employing the response they produced, they develop negative feedback signals that inhibit stress sensing and/or further stimulating signal flow. However, it is presumed that the synthesis of an adaptive response does not in reality result from such a simple stress circuit. Alternatively, a single kind of stress may be sensed by different sensors that provoke various signals stimulating several changes[16]. While arrays of regulatory components directly aid cell's survival/growth, others only trigger the expression of other regulatory components involved in stress adaptation (Figure 3). The outcome of such a regulatory network would be reflected in an adaptive cell response[16].

A number of systems have been described to act as stress communicators sensing stress and eliciting signals – together these two functions constitute what is known as a ''signal transduction'' process. These systems involve two-component signal transduction systems (also known as ''two-component regulatory systems'' (2CRS)), quorum-sensing systems, FNR system, catabolite repression phosphotransferase-based systems and serine-threonine kinases[17,18]. The 2CRS show interesting features that are consistent with the above stress-response flow paradigm (Figure 4). 2CRS

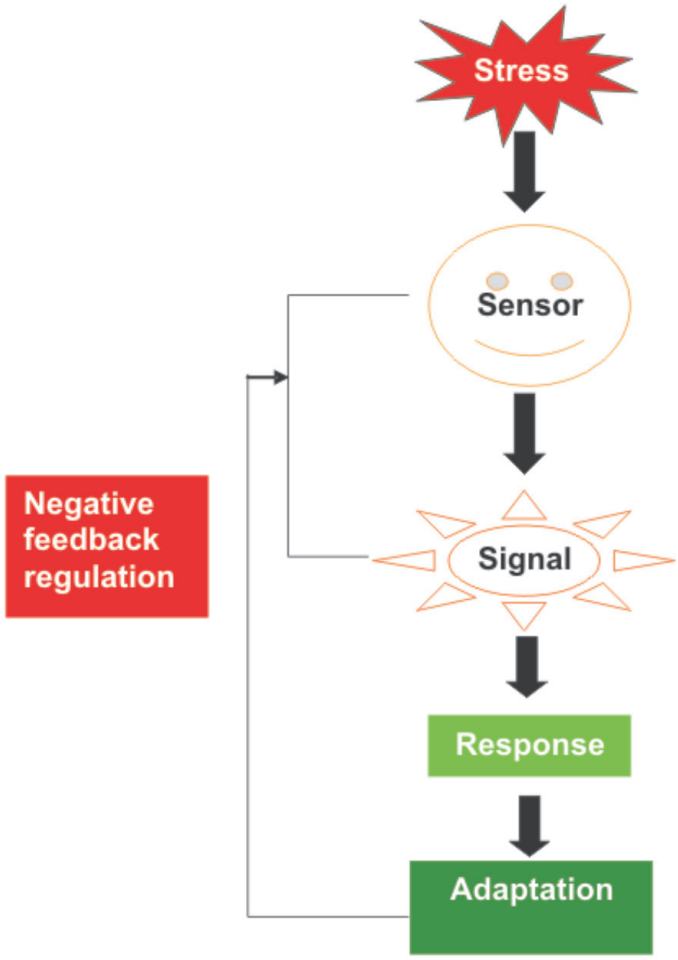

Fig. 2. *Simplified illustration of stress response cascade in bacteria (Adapted after Neidhardt[15] and El-Sharoud[16]).*

proteins are reported to be relatively abundant in eubacteria where they take important part in regulating various stress and virulence responses[20]. However, given the relative simplicity of bacterial cells and relative complexity of 2CRS, such significant contribution of 2CRS to cell physiology appears interesting[18]. A recent bioinformatics analysis of 145 prokaryotic genomes showed that simpler "one-component systems" involving single molecules, each of which contains both sensing input and response output domains, predominate 2CRS[21]. However, the relative significance of each system to cell behaviour can not solely be determined depending on such a quantitative approach. Further experimental and theoretical

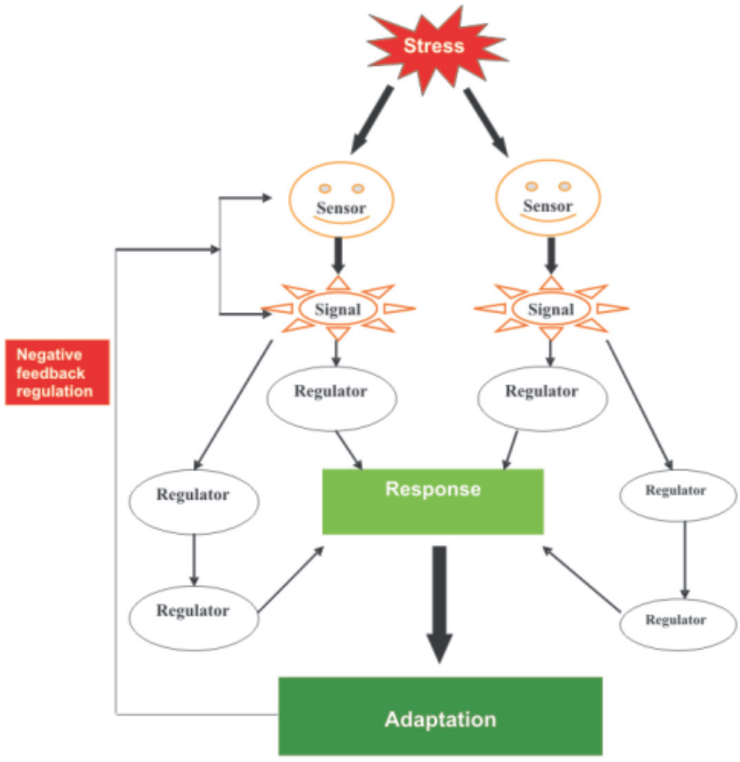

Fig. 3. *More realistic view of the development of stress response in bacteria (adapted after Neidhardt[15]).*

analysis of the significance of cellular processes regulated by these systems are required to elucidate their actual value to bacterial cells. It should be also taken into account that there is a controversy over defining individual protein molecules with sensing and output domains as ''systems''. It is argued that these domains are typical features of regulatory proteins and that the term ''system'' implies a combination of related elements functioning in a rather complex whole.

A typical 2CRS consists of two proteins: a histidine protein kinase (HPK) (sensor kinase) and a response regulator (RR) (Figure 4). Signal transduction is generally achieved through phosphorylation and dephosphorylation reactions that involve the HPK and RR components. These reactions may be simplified as follows[17] (Figures 4 and 5):

1. HPK autophosphorylates using ATP as a phosphoryl group donor.

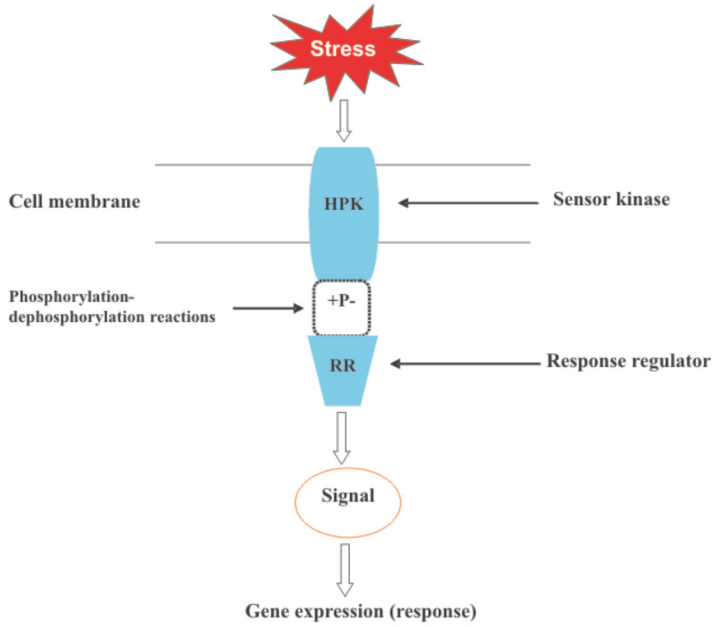

Fig. 4. *Involvement of 2CRS in sensing environmental stress and generating a response (adapted after El-Sharoud[16] and Parkinson[19]).*

2. Phosphorylated HPK activates a cognate RR through the transfer of the acquired phosphoryl group.
3. Activated RR induces or represses the transcription of responsive genes or the functionality of proteins involved in response to environmental changes.
4. RR is de-phosphorylated by phosphatase enzyme or HPK.

The HPK senses environmental stimuli which modulate its autophosphorylation or RR dephosphorylation activities leading to variations in the expression of genes or functionality of proteins involved in cell response to environmental changes. It should be, however, noted that the above reactions do not necessarily follow the above order but they may occur simultaneously. While all characterised 2CRS depend on such series of phosphorylation and dephosphorylation reactions, some systems show variations on the above mechanisms. For example, stress sensing may not be initially achieved by the HPK component in some 2CRS, but by another sensing molecule that then triggers the HPK component. This is seen in the 2CRS involved in the regulation of nitrogen and phosphate assimilation in *E. coli* (For a comprehensive review on structural and functional features of 2CRS, see Stock *et al.*[22]).

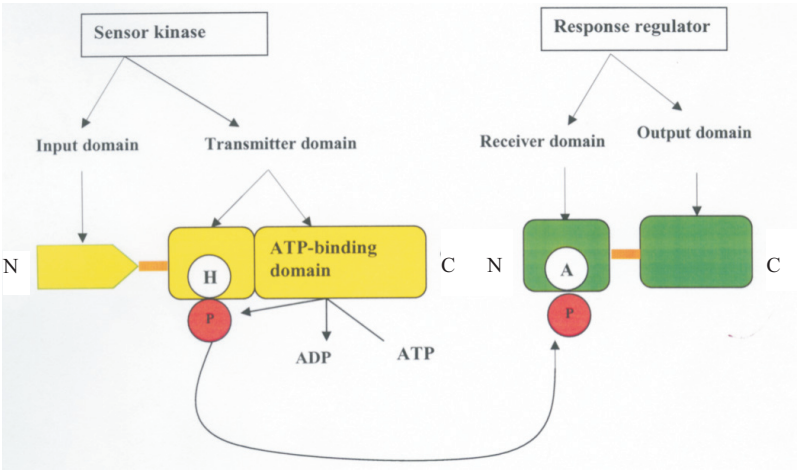

Fig. 5. *Structure and functioning in 2CRS (adapted after West and Stock[20]), see text for details. N: N-terminal end. C: C-terminal end. H: Histidine residue. A: Asparate residue. P: Phosphoryl group.*

The HPK component is comprised of an input domain (ID) and a transmitter domain (TD) (Figures 4 and 5). The ID is frequently located at the N-terminal end of the HPK and is variable in size and structure. It may exist as a periplasmic, cytoplasmic or transmembrane domain and act as a sensor of environmental stimuli. The TD contains approximately 250 amino acid residues, one of them is the histidine residue that accepts a phosphoryl group from ATP for the phosphorylation of the HPK. On the other hand, the RR component may involve 1–3 domains, of which a conserved domain of approximately 125 amino acid residues including asparate must exist. This domain is frequently located at the N-terminus of the RR and designated a ''receiver domain'' (RD) since it receives a signal of environmental changes in the form of a phosphoryl group from an activated TD. The RD contains a conserved phosphorylation site involving aspartate residues, one of which accepts a phosphoryl group from the cognate TD. Here, the phosphoryl transfer is more mediated by the RR rather than the HPK. This is indicated by the ability of the RR component to catalyse its own phosphorylation using other phosphodonors including small molecules such as acetyl phosphate and phosphoramidate[23,24]. The majority of the response regulators involve an additional domain designated an ''output domain'' (OD) at the C-terminal end. The phosphorylation of the RD stimulates the associated OD to modulate the transcription of relevant genes. Some 2CRS involve OD containing DNA-binding motifs that

enable them to directly affect transcription by physically interacting with the promoter region in targeted genes. While RRs are located in the cytoplasm, HPKs usually involve hydrophobic regions extending through the cytoplasmic membrane in addition to other extracytoplasmic and cytoplasmic motifs in the input and transmitter domains, respectively.

The specificity of a 2CRS to interact with a particular kind(s) of environmental stimuli and to modulate the expression of particular genes is governed by the presence of specific amino acid sequences at the N-terminal and C-terminal ends of the HPK and RR components, respectively. These sequences differ between different 2CRS. However, there are conserved amino acid regions at the interacting C-terminus and N-terminus of the HPK and RR components, respectively. The presence of these regions has enabled the identification of several putative 2CRS in various bacterial species including LAB (see below). Since these regions are located at the interacting ends of HPKs and RRs, they may give rise to a ''cross-talk'' phenomenon. This involves divergence or convergence of the phosphoryl transfer pathway[25]. The pathway may diverge as a single HPK phosphorylates more than one RR or converge as a single RR is phosphorylated by more than one HPK.

The organisation of the *hpk* and *rr* genes in some 2CRS mirrors the partnering relationship between their protein products. In these systems, *hpk* and *rr* are located adjacent to each other forming an operon, with *rr* frequently being located upstream of *hpk*. However, in some other 2CRS, these genes are located at distant loci.

Characterisation of 2CRS in LAB

The identification of 2CRS in LAB backs up to the 1990's with the discoveries of systems regulating the biosynthesis of bacteriocins (see below). However, the detection and characterisation of new 2CRS have been greatly facilitated by the completion of genome sequencing in several LAB strains. Annotations of genetic sequences have revealed the presence of a number of putative *hpk-rr* genetic pairs in LAB including nine pairs in *Lb. acidophilus* NCFM[26], five in *Lb. helveticus* CNRZ 32 (Jeffery Broadbent and James Steele, *personal communication*) and 17 in *E. faecalis* V583[27]. Given the extensive effort required for sequencing the whole genome of bacterial strains, simpler methods have, however, been developed. One of these is a PCR-based technique that amplifies internal fragments in putative *rr* genes. This is achieved by the use of degenerate primers targeting DNA sequences specifying highly

Table 1 *Distribution of putative 2CRS recently characterised in lactic acid bacteria*

Organism	Number of putative 2CRS	Ref.
Lb. acidophilus NCFM	9	26
Lc. lactis ssp. *cremoris* MG1363	6	30, 31
Lb. sakei 23K	5	29
Lb. helveticus CNRZ 32	5	Broadbent and Steele, personal communication
E. faecalis V583	17	27
S. pyogenes JRS4	13	6

conserved amino acid motifs in the receiver domain of RR components in previously-studied bacteria[28,29]. On identifying putative *rr* genes, the surrounding genetic regions are then sequenced and their deduced amino acid sequences are compared with those of the conserved motifs in the HPK component. The rational behind this comparison is based on the observation that *hpk* and *rr* are frequently arranged in a pair of adjacent genes, see above. The application of this procedure allowed the detection and characterisation of five putative 2CRS in *Lb. sakei*[29].

Another interesting approach to detect 2CRS has exploited the cross-talk phenomenon described above. In this approach, recombinant plasmids involving gene fragments of the examined LAB organism are introduced into cells of *E. coli* mutant strain lacking functional HPKs. Restoring the phenotypic traits regulated by 2CRS in the latter strain will thus indicate functional complementation occurring between putative HPK(s) encoded by the inserted plasmid and RR components already existing in the mutant bacterium. Carrying out this procedure followed by sequence analysis of inserted plasmids uncovered the existence of five putative *hpk* genes in *Lc. lactis* subsp. *cremoris* MG 1363[30]. Further analysis of these *hpk*s and their neighbouring sequences showed that this strain contained six putative 2CRS[31]. Table 1 shows the distribution of recently discovered putative 2CRS in industrial and pathogenic LAB. In addition to this list, Deville *et al.*[28] have identified 4 putative *rr* genes in *Lb. delbrueckii* ssp. *bulgaricus* CNRZ 777.

Effect of 2CRS on the growth of LAB

Unlike most G^- bacteria whose 2CRS are not required for growth under optimal conditions, the YycFG 2CRS is essential for the growth of G^+ organisms with low G+C content including LAB

species[32]. Homologues of *yycG* encoding the HPK component in this system were detected in genomes of industrial, probiotic and pathogenic LAB such as *Lc. lactis*, *Lb. johnosonii* and *S. pneumoniae*, respectively[32]. The importance of YycFG to bacterial growth may stem from its involvement in regulating the expression of housekeeping genes implicated in cell wall biosynthesis and cell division[33,34,35].

Among recently characterised as putative 2CRS in LAB, the Rrp-31/HpK-31, LlrA-LlkinA, HK/RR11 and Err05-Ehk05 systems were also shown to be important for normal growth of *Lb. sakei*[29], *Lc.* subsp. *cremoris*[31], *S. mutans*[36] and *E. faecalis* JH2-2[37], respectively. However, the cellular processes affected by these 2CRS remain to be elucidated. Although one report[37] shows that the Err05-Ehk05 system affects the transcription of the *sagA* gene, this gene was reported to be essentially implicated in cell responses during environmental stress rather than under optimum growth conditions[38].

Interestingly, the extent to which the above 2CRS may affect cell growth varies with different systems. For example, the YycFG system is very essential for growth as a mutant strain of *E. faecalis* V583 deleted in an ortholog of *yycF* failed to develop visible colonies on agar media[27]. Whereas, inactivation of the *rrp-31* gene of the Rrp-31/HpK-31 system in *Lb. sakei* did not prevent growth but caused earlier growth cessation after reaching one-fifth the maximum cell numbers that the parent organism produced by the end of the exponential phase[29]. Deletion of the *rr11* or *hk11* in the HK/RR11 reduced the growth rate of *S. mutans*, but both the parent and mutant strains yielded almost the same maximum numbers of cells[36].

Effect of 2CRS on stress responses in LAB

Since the 1990's, 2CRS have been described to be implicated in the regulation of the transcription of genes involved in the biosynthesis of class 1 and class 2 bacteriocins[39,40] by LAB. Bacteriocins are antimicrobial peptides/proteins produced by bacterial species, including LAB, to inhibit other closely-related organisms. An important example is nisin whose gene transcription is regulated by a 2CRS designated NisRK[41]. Nisin is produced by strains of *Lc. lactis* ssp. *lactis* and inhibits G^+ bacteria including strains of the same species. Within this context, the production of nisin, and thus the expression of NisRK, may be regarded as a strategy adopted by LAB to defend their existence in sub-optimal environments. Given

this and also the regulatory roles of 2CRS in stress responses of model bacteria[14], 2CRS may be expected to serve similar regulatory functions during the exposure of LAB to environmental stress. Investigating this possibility has been an essential aspect in most studies characterising 2CRS in LAB.

(a) Acid stress

Low pH (acid stress) is typically the kind of stress that is frequently encountered by LAB during the preparation of fermented foods and their passage in the stomach. To cope with this, LAB display adaptive systems including amino acid decarboxylation-antiporter reactions, arginine deaminase pathway, proton-translocating ATPases and citrate transport system[7]. 2CRS were found to affect the survival of LAB under acidic conditions. For example, the insertional inactivation of the *lba1524hpk* gene specifying a putative HPK component of the LBA1524HPK-LBA1525RR 2CRS in *Lb. acidophilus* NCFM was found to reduce cell survival in pH 3.5[39]. Similarly, a mutant strain of *E. faecalis* OG1RF deleted in the *etaR* gene encoding the RR component in the EtaRS 2CRS was reported to be more sensitive to pH 3.4 than the parent organism[42]. The deletion of *llkinA* or *llrA* of the LlrA/LlkinA 2CRS in *Lc. lactis* ssp. *cremoris* MG1363 also resulted in significant reductions in acid tolerance at pH 4.0[31]. These effects are consistent with the observation that the deduced protein sequences of LBA1524HPK, EtaR, LlkinA and LlrA are similar to those of their counterparts in the LisRK system, which was reported to be involved in the acid tolerance phenotype in the strain LO28-M9 of *Listeria monocytogenes*[43]. While this suggests that similar 2CRS may serve similar roles in related organisms, it should be borne in mind that this is not a general role. For instance, the HPK and RR components in the Rrp-31/Hpk-31 2CRS in *Lb. sakei* show similarities to their counterparts in the VanR/VanS system[29]. While the RR component (VanR) in the latter system is necessary for the resistance of *E. faecium* to the antibiotic vancomycin[44], the inactivation of the *rrp-31* gene in *Lb. sakei* increased its resistance to the same antibiotic[29].

Interestingly, inactivation of the *llkinB* gene of the LlkinB/LlrB 2CRS system slightly increased the survival of *Lc. lactis* ssp. *cremoris* in pH 4.0[31]. The same effect was also reported when its cognate *llrB* was deleted. It might be suggested that the LlkinB/LlrB system served to inactivate the transcription of genes involved in cell response to acid stress.

2CRS have also been shown to affect the growth of LAB under mildly acidic conditions, pH 5.5–6.0. Deletion of the *rr* genes in the Rrp-1/Hpk-1 and Rrp-48/Hpk-48 systems in *Lb. sakei*[29] and in the Err10-Ehk10 system of *E. faecalis*[37] was associated with a reduction in the growth of these organisms in moderately low pH. Inactivation of *hk11* or *rr11* also decreased the growth rate of *S. mutans* in pH 5.5[38]. However, while the deletion of *covS* encoding putative HPK in the CovR/S 2CRS prevented the growth of *S. pyogenes* in pH 6.0, this was not shown when its cognate *covR* was inactivated or when both *covS* and *covR* were deleted in the same mutant strain[6]. Based on several lines of evidence, it was suggested that CovS inactivates CovR under mildly stress conditions. Since CovR serves as a negative regulator of the transcription of stress-responsive genes, its inactivation by gene deletion or by CovS will presumably allow the expression of those genes. Whereas, deletion of *covS* aids CovR to maintain its repressive effect, which gives rise to cell vulnerability to stress. However, it has to be emphasised that the inhibitory influence of CovS towards CovR was only displayed during exposure to mild stress. This is indicated by the observation that CovR was still able to repress stress-responsive genes under optimal growth conditions despite the inactivation of CovS.

There were preliminary attempts to probe the regulatory processes through which 2CRS may exert the above effects on the survival/growth of LAB during acid stress. By examining the mRNA expression of approximately 97.4% of annotated genes in *Lb. acidophilus* NCFM, Azcarate-Peril *et al.*[39] found that the deletion of the *lba1224hpk* gene had pleiotropic effects on the expression of 80 genes in this organism. These included genes specifying oligopeptide-transporters (Opps) of the ABC transporter family. It was suggested that Opps may serve as transporters of signalling peptides that stimulate cell response to environmental changes, a frequently reported mechanism in LAB. Within this context, the deletion of *lba1224hpk* with its concomitant effect on the expression of *opps* may negatively affect bacterial survival to acid stress. Similarly, Morel-Deville *et al.*[29] have speculated that transcriptional regulatory links might exist between the *rrp-1/hpk-1* gene pair and its adjacent sequences encoding membrane transporters in *Lb. sakei*, and this may give rise to reduced growth in low pH on the deletion of *rrp-1*. A simpler explanation was, however, suggested for acid sensitivity caused by *llrA* deletion in *Lc.* ssp. *cremoris*. It was reported that gene deletion affected the expression of arginine deaminase (AD) which aids adaptation during acid stress by the production of ammonia[31].

Despite the positive effects of a number of 2CRS on the growth/survival of LAB during acid stress, these systems appear not to affect ''acid adaptation'' in these bacteria. This was seen in *Lb. acidophilus* and *S. mutans* where mutant strains of these organisms deleted in *hpk* or *rr* of 2CRS involved in cell response to acid stress (LBA1524HPK-LBA1524 and HK/RR11) were still able to adapt to low pH[39,36]. Acid tolerance in these mutant strains was increased following their exposure to mildly acidic conditions, pH 5.5. This is an interesting observation given the suggestion that 2CRS may take part in an advanced ''learning'' behaviour in bacteria[18,45]. In this, the induction of the synthesis of the HPK and/or RR components on exposure to environmental changes would fasten or intensify bacterial responses to similar future conditions. That is to say that bacteria ''learn'' from the past. The ''learning'' behaviour was proposed as a feature of 2CRS displaying environmental-induction of their own components (autoamplification). It was also described to be different from the ''adaptation'' response. However, the theoretical distinction between both phenomena is not very clear and one ''autoamplifying'' 2CRS in *Salmonella* Typhimurium, *i.e.* PhoPQ (Socini *et al.* 1995) was reported to be involved in acid adaptation[46]. If autoamplification is necessary for a 2CRS to be involved in a learning/adaptation behaviour, it will then remain to investigate whether the components of the LBA1524HPK-LBA1524 and HK/RR11 systems are not autoregulated.

(b) Heat stress

LAB are frequently prone to elevated temperatures during the processing of fermented foods, see above. Pathogenic LAB also encounter higher temperatures during the course of human infections associated with fever. While there are thermophilic and thermoduric (heat tolerant) species within LAB, *e.g. Lb.* ssp. *bulgaricus* and *Enterococcus.* sp., respectively, extended exposure to non-optimal high temperature represents environmental stress that may lead to damage in structural proteins and inactivation of enzymes. As mentioned above, LAB express a range of regulatory proteins, heat shock proteins, that act as chaperones preventing or repairing protein misfolding.

The effect of 2CRS on regulating the response of LAB during heat stress conditions was studied. It was found that the inactivation of the RR genes *err04*, *err08* or *err18* was associated with reduced growth of *E. faecalis* JH2-2 at 48°C[37]. Furthermore, the

deletion of *covS* in the CovR/S system prevented colony formation of *S. pyogenes* at 40°C[6]. As with acid stress, see above, deletion of *covR* or double mutation in *covS* and *covR* did not affect tolerance to heat and it was suggested that CovS also served to inactivate CovR under mild heat stress conditions.

Interestingly, the deletion of *err10* in *E. faecalis* JH2-2 or its homologue *etaR* in the OG1RF strain increased the tolerance of both organisms during growth at 50°C[37,42]. This was associated with the induction of the heat shock proteins DnaK and GroEL in the mutant strains.

(c) Oxidative stress

LAB are generally considered as aerotolerant anaerobes whose growth may be retarded by extensive exposure to oxygen. Although oxygen, on its own, is not toxic to LAB, it enters into metabolic reactions leading to the production of cytotoxic partially reduced oxygen species (ROS) including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). ROS cause damage to central cellular molecules including DNA, lipids and proteins. Vigorous agitation of milk inoculated with fermenting LAB may thus generate serious stressful conditions affecting the viability of these bacteria. Interestingly, some species of *Lactobacillus* may produce H_2O_2 inhibiting other LAB when they are mixed together during the preparation of fermented foods[47]. However, the production of ROS by human phagocytic cells is a useful mechanism that is employed for defence against pathogenic bacteria including species of LAB.

Few 2CRS were found to be involved in the response of LAB to oxidative stress. Of these is the Rrp-31/Hpk-31 system in *Lb. sakei*[29]. This bacterium produces catalase which hydrolyses H_2O_2 and thus aids tolerance under aerobic conditions. While aeration did not affect the growth of a parent strain of *Lb. sakei*, the deletion of *rrp-31* caused earlier growth cessation under aerated conditions, compared to growth under non-aerated conditions. The maximum cell numbers reached by the *rrp-31*-deficient mutant under aerated conditions was one-quarter that of the same strain under non-aerated conditions. The same RR gene was also found to aid cell survival during more severe oxidative stress exerted by exposure to H_2O_2. Similarly, the inactivation of the *rrF* gene in *Lc. lactis* ssp. *cremoris* caused sensitivity to H_2O_2 [31].

(d) Osmotic stress

With the exception of *E. faecalis*, *Tetragenococcus* (formerly known as *Pediococcus halophilus*), and specific strains such as *Lb. acidophilus* IFO 3532, LAB are not generally osmotolerant organisms. However, during their use in the manufacture of fermented foods, LAB may be subjected to hyperosomotic conditions commonly produced by the presence of NaCl added for inhibiting spoilage organisms and enhancing food flavour. Like in other bacterial species, the cytoplasmic accumulation of "compatible solutes" is employed by osmotolerant LAB strains to cope with higher concentrations of NaCl[48,49]. Compatible solutes are molecules, *e.g.* glycine betaine that help to balance the osmotic pressure of the cytoplasm with that of the hyperosmotic environment, without interference with cellular metabolism. This enables cells pursuing active metabolism and alleviating the negative impact of hyperosmotic conditions. However, unlike other bacteria, LAB can only uptake compatible solutes rather than synthesising them[8].

The effect of 2CRS on the response of the relatively osmotolerant LAB *E. faecalis* to osmotic stress was studied. It was found that a mutant strain deleted in the *err10* gene, encoding a putative RR component, grew at a slightly slower rate in 6.5% NaCl compared to the parent *E. faecalis* JH2-2 organism[37]. Inactivation of *covS* in *S. pyogenes* prevented colony formation in the presence of 0.65 M (1.8%) NaCl[6]. Osmotic stress is not uncommon for this pathogenic LAB, given the release of perspiration with its salt content during skin infection.

Are 2CRS general stress regulators in LAB?

The above findings indicate an important role of 2CRS in the response of LAB to environmental stress. However, it should be noted that the inactivation of putative 2CRS in LAB is not typically associated with decreased growth/survival during stress since a number of mutant LAB strains deleted in different 2CRS behaved very similarly to their parent counterparts, see Morel-Deville *et al.*[29] and O'Connell-Motherway *et al.*[31]. Interestingly, the inactivation of RR or HPK components in certain 2CRS conferred tolerance to stress, see above.

While some 2CRS appear to be involved in cell response to a single type of stress, others show multiple implications in different stresses, *e.g.* CovR/S and Ehk10-Err10. The observation that the latter systems affected cell responses to unrelated stresses raises the

possibility that they could mediate a general stress response in LAB[6].

The involvement of the Ehk10-Err10 system in cell behaviour during exposure to various stresses including heat, acid and bile salts is even striking and indicates a general regulatory role of this 2CRS in *E. faecalis*. While the deletion of the *err10* gene caused sensitivity to low pH, it aided growth at an elevated temperature and in the presence of 0.07% bile salts[37]. Previous work with *E. faecalis* demonstrated that the organism showed a ''cross protection'' relationship between bile salts and heat stresses[50]. That is when cells were exposed to sub-lethal levels of bile salts they acquired increased tolerance to heat and *vice versa*. This was associated with the common expression of 18 stress proteins, including DnaK and GroEL on exposure to either stress kind. Consistently, the enhanced growth of *err10*-deleted *E. faecalis* mutant strain during heat stress or in the presence of bile salts was associated with increased expression of the latter two proteins[37]. These observations suggest that the Ehk10-Err10 2CRS was involved in the regulation of the cross protection phenomenon, which could further indicate its involvement in a general stress response, if any, in *E. faecalis*. Interestingly, the organism did not show a cross protection relationship between acid stress and heat stress or bile salts[50]. Exposure to bile salts was found to even increase vulnerability to acidic conditions. This is consistent was the contrasting effects that the deletion of *err10* provoked in cell response to low pH and heat/bile salts.

While it is tempting for some authors, in light of the above findings, to suggest that 2CRS may functionally replace σ^S or σ^B in LAB, I feel that holding such a comparison is not appropriate. While 2CRS, in their prototypical mode, serve a dual function of sensing stress and eliciting relevant cell response by affecting gene transcription, alternative sigma factors do not sense stress, but only mediate gene transcription. It is interesting to note that the transcription of *rpoS*, encoding σ^S in G^- bacteria, was found to be regulated by 2CRS in *Pseudomonas fluorescence*[51] and *E. coli*[52]. Alternative sigma factors appear, in these cases, as components within the hierarchical and modular organisation of the 2CRS.

Another aspect that is really appealing to stress-response microbiologists is to presume the existence of unified regulatory mechanism(s) within bacterial groups. This was found in a number of related G^- and G^+ bacteria where, σ^S and σ^B, act as master stress-response regulators, respectively. As shown above, 2CRS may serve this role in LAB, but an important aspect to consider is the

phenotypic heterogeneity displayed by members within this group. Such heterogeneity could be generated by operating different regulatory functions. This raises the possibility that different stress-regulatory mechanisms may exist within LAB.

Methodological aspects

Despite the identification of several 2CRS in LAB (Table 1), it is expected that more systems might be discovered with modifications in the characterisation procedures[28]. As seen above, the identification of most 2CRS in LAB was the result of either comparing newly identified genome sequences in LAB with those encoding RRs and HPKs in other bacteria, or targeting and amplifying putative 2CRS genome sequences. The latter procedure also depends on conserved 2CRS genome sequences previously reported in other organisms, see above. Within this context and given the biological differences between LAB and other bacterial species, *e.g. E. coli* and *B. subtilis*, it may be suggested that other yet-undiscovered 2CRS might exist in LAB. A possible way to uncover those systems is to probe membrane-associated proteins and examine their involvement in phosphorylation–dephosphorylation reactions.

While current evidence suggests a significant role of 2CRS in the response of LAB to environmental stress, the cellular processes within which these systems are involved remain to be identified. It appears from the literature that probing these processes only using transcripteomic tools will not be sufficient and might be misleading in some cases. For instance, the inactivation of the *lba1524hpk* gene was found to reduce the survival of *Lb. acidophilus* under acid stress[39], see above. To examine cellular functions affected by the deletion of this gene, mRNA expression of approximately 97.4% of annotated genes in this organism was assayed. Unexpectedly, there was an increase in the mRNA level of the deleted *hpk* gene and its cognate *rr* gene. While the authors attributed acid sensitivity in the *hpk*-deleted mutant strain to changes in the expression of genes implicated in proteolytic pathways, it was surprising that there were also increases in the mRNA expression levels of oligopeptide-transporter genes. Thus, proteomic approaches combined with genomic and transcripteomic analyses have to be adopted if a comprehensive view of the effect of 2CRS on cell physiology is sought.

Conclusions and future prospects

2CRS appear to be variably implicated in stress responses of LAB. While this will aid better understanding of the regulation of stress responses in these bacteria, further work is needed to elucidate the cellular mechanisms through which 2CRS may exert their regulatory effects. Future studies may also involve developing procedures for characterising 2CRS unique to LAB, which might not be detected using current methodologies that depend on conserved HPK and RR regions previously reported with other G^- and G^+ microorganisms. Although the influence of 2CRS on the physiology of LAB was examined under various adverse conditions, this effect has also to be investigated under other industrial conditions, including cold storage temperatures and lyophilisation.

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