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MINI-REVIEW

Progress in engineering acid stress resistance of lactic acid bacteria

Chongde Wu · Jun Huang · Rongqing Zhou

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Abstract Lactic acid bacteria (LAB) are widely used for the production of a variety of fermented foods, and are considered as probiotic due to their health-promoting effect. However, LAB encounter various environmental stresses both in industrial fermentation and application, among which acid stress is one of the most important survival challenges. Improving the acid stress resistance may contribute to the application and function of probiotic action to the host. Recently, the advent of genomics, functional genomics and high-throughput technologies have allowed for the understanding of acid tolerance mechanisms at a systems level, and many method to improve acid tolerance have been developed. This review describes the current progress in engineering acid stress resistance of LAB. Special emphasis is placed on engineering cellular microenvironment (engineering amino acid metabolism, introduction of exogenous biosynthetic capacity, and overproduction of stress response proteins) and maintaining cell membrane functionality. Moreover, strategies to improve acid tolerance and the related physiological mechanisms are also discussed.

Keywords Acid stress · Lactic acid bacteria · Stress response · Amino acid metabolism · Cell membrane

C. Wu (✉) · J. Huang · R. Zhou (✉)
College of Light Industry, Textile and Food Engineering, Sichuan University, Chengdu 610065, China
e-mail: cdwu@scu.edu.cn
e-mail: zhourqing@scu.edu.cn

C. Wu · J. Huang · R. Zhou
Key Laboratory of Leather Chemistry and Engineering, Ministry of Education, Sichuan University, Chengdu 610065, China

Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of low-GC, nonsporulating Gram-positive bacteria, which ferment a range of carbon sources primarily to lactic acid (Gaspar et al. 2013). In food industry, LAB are mainly used for food and beverage fermentation, production of add-in ingredients, bacteriocins, exopolysaccharides (Zhu et al. 2009; Table 1). In addition, LAB are also used to produce bulk and fine chemicals including organic acids, polyols, and vitamins (Gaspar et al. 2013; Table 1). However, as cell factories, LAB encounter various stress conditions during the industrial production and in the gastrointestinal tract. Among various environmental stresses, acid stress is one of the most important survival challenges, and acid tolerance is one of the criterias to select potential probiotics (Parvez et al. 2006). During fermentation, the growth of LAB is accompanied by lactic acid production leading to acidification of the media, arrest of cell growth, and possibly cell death due to the entrance of undissociated form of lactic acid into the cytoplasm by simple diffusion (Serrazanetti et al. 2009). Intracellular lactic acid dissociates, changes the intracellular pH, and disrupts the cytoplasm anion pool, which affects the integrity of purine bases and results in denaturing of essential enzymes inside the cells (Warnecke and Gill 2005). Thus, improving the acid stress resistance is important for the industrial application of LAB.

In response to acid stress, LAB have evolved stress-sensing systems and employed numerous mechanisms to withstand harsh conditions and sudden environmental changes, including the maintenance of intracellular pH homeostasis, cell membrane functionality, and upregulation of stress response proteins (Lebeer et al. 2008; Wu et al. 2012b; O'Sullivan and Condon 1997; Fig. 1). In addition, acid tolerance response (ATR) appears to confer protection against environmental

Table 1 Application of lactic acid bacteria

Application	Products	Strains	References
Dairy industry	Cheese	*Lactococcus lactis*; *Leuconostoc* spp.	(Zhu et al. 2009)
	Yoghurt	*Streptococcus thermophilus*; *Lactobacillus delbrueckii*	(Zhu et al. 2009)
Food fermentation	Wine	*Oenococcus oeni* PSU-1; *Lactobacillus acetolerans*	(Mills et al. 2005; Zheng et al. 2013)
	Pickled vegetables	*L. lactis*; *Leuconostoc mesenteroides*; *Lactobacillus plantarum*	(Xiong et al. 2012)
	Soy sauce	*Lactobacillus salivarius*; *Tetragenococcus halophilus*; *Lactobacillus fermentum*; *S. thermophiles*	(Yan et al. 2013; Tanaka et al. 2012)
	Vinegar	*Lactobacillus panis*; *Lactobacillus pontis*	(Xu et al. 2011)
Commodity chemicals	Lactic acid	*Lactobacillus rhamnosus*; *Lactobacillus brevis*	(Abdel-Rahman et al. 2013; Cui et al. 2011)
	2,3-Butanediol	*L. lactis*	(Gaspar et al. 2011)
	1,3-Propanediol	*Lactobacillus reuteri*	(Vaidyanathan et al. 2011)
	Ethanol	*L. plantarum*	(Liu et al. 2006)
	Succinic acid	*L. plantarum*	(Tsuji et al. 2013)
	Butanol	*L. brevis*	(Berezina et al. 2010)
Food ingredients	Alanine	*L. lactis*	(Ye et al. 2010)
	γ-Aminobutyric acid	*L. brevis*	(Cho et al. 2011)
	Diacetyl	*L. lactis*	(Guo et al. 2012)
	Acetadehyde	*L. lactis*	(Bongers et al. 2005)
	Bacteriocins	*L. lactis*	(Beshkova and Frengova 2012)
Nutraceuticals	Xylitol	*L. lactis*	(Nyyssölä et al. 2005; Monedero et al. 2010)
	Mannitol	*L. lactis*; *L. mesenteroides*	(Song and Vieille 2009)
	Sorbitol	*Lactobacillus casei*; *L. plantarum*	(De Boeck et al. 2010; Ladero et al. 2007)
	Riboflavin	*L. lactis*	(Burgess et al. 2004)
	Folate	*L. lactis*	(Sybesma et al. 2003)
	Vitamin B12	*L. reuteri*	(Santos et al. 2008)
	Isoprenoids	*L. lactis*	(Song et al. 2012)
	Phenylpropanoids	*L. lactis*	(Martínez-Cuesta et al. 2005)
Polysaccharides	Exopolysaccharide	*L. lactis*	(Patel et al. 2012)
	Hyaluronic acid	*L. lactis*	(Prasad et al. 2012)

stresses by prior exposure of cells to moderately acidic conditions (De Angelis et al. 2001). Meanwhile, omics methods combined with molecular techniques have contributed to the understanding and validation of the molecular mechanisms involved in acid tolerance, and many feasible strategies (engineering general stress response proteins, maintaining cell membrane functionality, and regulating amino acid metabolism) have been proposed to improve the acid stress resistance of LAB (Wu et al. 2012a, 2013b; Trip et al. 2012). Thus, it is necessary to timely summarize the progress to further stimulate the research interest in this field. In this review, we provide an overview of the recent progress in engineering acid stress resistance of LAB, with emphasis on engineering intracellular microenvironment (engineering amino acid metabolism, introduction of exogenous biosynthetic capacity, and overproduction of stress response proteins) and maintaining cell membrane functionality based on physiological and omics analysis.

Engineering intracellular microenvironment of LAB

Regulation of intracellular amino acid metabolism

Regulation of intracellular amino acid metabolism is a common mechanism utilized by LAB upon environmental stresses. Arginine deiminase (ADI) system is a widely reported regulative system in LAB during acid stress. This system converts arginine and subsequently leads to the production of NH_3, CO_2, and ATP. The generation of ATP enables extrusion of cytoplasmic protons by H^+-ATPase (Burne and Marquis 2000). Previous research demonstrated acid stress induced the accumulation of arginine in *Lactobacillus casei*, and *Streptococcus faecium* could degrade arginine at extremely low initial pH of 2.5 and raise the pH to nearly 8.0 with 80 mM NH_3 accumulation (Wu et al. 2012a). Moreover, the addition of arginine improved the survival of *L. casei* during

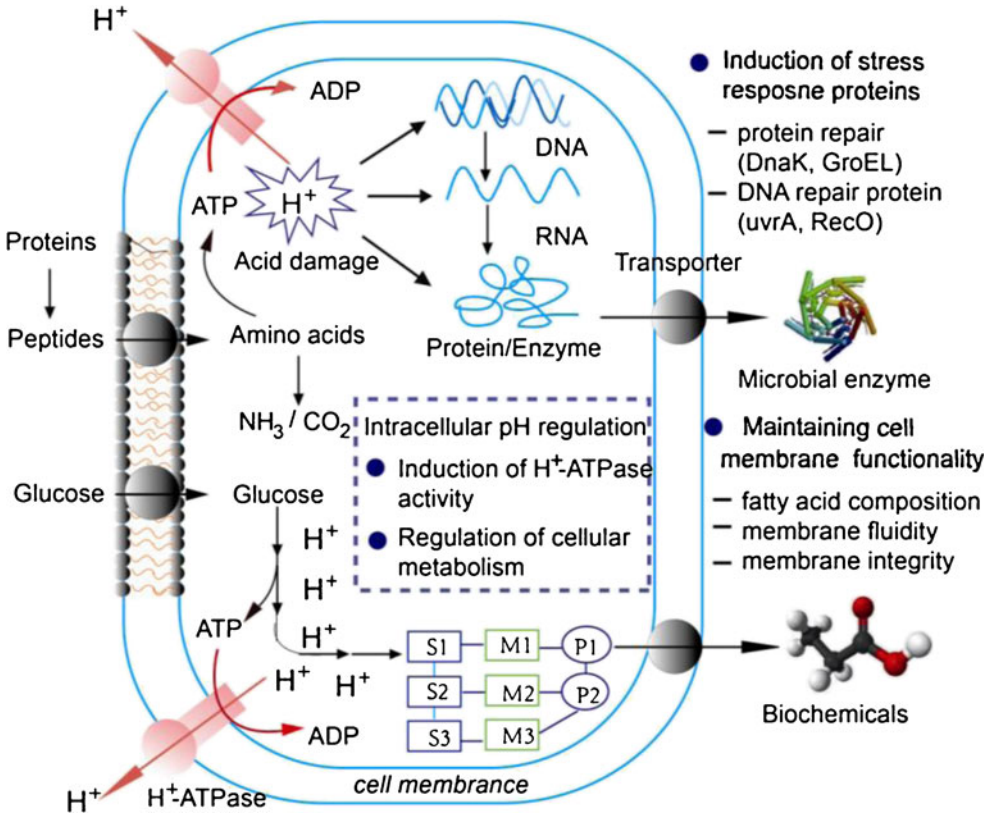

Fig. 1 Responses of lactic acid bacteria as a cell factory to acid stress. *S* substrate, *M* intermediate, *P* product

acid stress by increasing the activity of H^+-ATPase and intracellular ATP levels (Zhang et al. 2012b). An example with *Streptococcus suis* showed that knockout of *arcABC* encoding genes involved in the ADI system resulted in decreased ammonia production and decreased cell growth during acidic conditions (Fulde et al. 2011).

Intracellular accumulation of aspartate is another response induced by LAB during acid stress, and the biomass and survival at low pH were significantly improved in the presence of aspartate (Wu et al. 2013a). In addition, an aspartate-dependent acid survival system was also characterized in *Yersinia pseudotuberculosis*. The expression of aspartase (AspA), which catalyzed the deamination of aspartate to form fumarate and ammonia, increased acid survival of *Y. pseudotuberculosis* (Hu et al. 2010).

Regulation of branched-chain amino acids (BCAA) leucine, isoleucine, and valine was also an ATR in LAB. During acid stress, the enzymes (IlvA, IlvC2, IlvD, and IlvE) involved in BCAA metabolism were overproduced, and deamination of BCAA was postulated as a mechanism to maintain the internal pH of the cells (Sánchez et al. 2007; Ganesan and Weimer 2004). Knockout of the *ilvE* gene led to decreased F_0F_1-ATPase activity and acid tolerance in *Streptococcus mutans* (Santiago et al. 2012). In addition, decarboxylation was also reported to protect cells against acid damage by generation of ATP and consumption of a single proton (Higuchi et al. 1997). Trip et al. (2012) heterologously expressed the histidine decarboxylation pathway in *L. lactis*, and this pathway enabled cells to survive at low pH in the presence of histidine.

Introduction of exogenous biosynthetic pathway

Nowadays, a vast genetic toolbox for the regulation of LAB gene expression levels is available, allowing the manipulation of acid tolerance through metabolic engineering (Table 2). Previous researches showed that glutathione can protect LAB against a variety of environmental stresses (acid, oxygen, cold and salt stresses; Kim et al. 2012; Zhang et al. 2010a, b, 2012a; Li et al. 2003). To further investigate the protective roles of glutathione during stressed conditions, two genes *gshA* and *gshB*, encoding γ-glutamylcysteine synthetase and glutathione synthetase, respectively, from *Escherichia coli*, were expressed in *L. lactis* NZ9000. As expected, the recombinant strain exhibited higher resistance to acid stress compared to the control strain (Zhang et al. 2007; Fu et al. 2006).

Previous study showed that acid stress led to substantial accumulation of trehalose in *Propionibacterium freudenreichii* during acid stress (Cardoso et al. 2004). Inspired by this observation, Carvalho et al. (2011) introduced the *P. freudenreichii* trehalose de novo biosynthetic pathway into *L. lactis* to investigate the effect of trehalose production on the tolerance of host strain to acid stress. As expected, the mutant exhibited higher tolerance to acid (pH 3.0) and cold (4 °C) shock, as well as to heat stress (45 °C; Carvalho et al. 2011).

Table 2 Improving acid stress resistance of lactic acid bacteria by metabolic engineering

Strains	Genetic modifications	Phenotype	References
Introducing exogenous biosynthetic pathway			
Lactococcus lactis	Expressed glutathione synthetase genes *gshA* and *gshB* from *Escherichia coli*	The survival increased 15-fold when challenged at pH 2.5 for 30 min	(Zhang et al. 2007)
L. lactis	Introduction of trehalose biosynthetic pathway from *Propionibacterium freudenreichii*	Higher survival to acid, cold, and heat stresses was obtained	(Carvalho et al. 2011)
Bifidobacterium breve	Introduction of betaine-uptake system from *Listeria monocytogenes*	Increased tolerance to gastric juice and osmolarity was achieved	(Sheehan et al. 2007)
Expression of general stress response protein			
L. lactis	Heterologous expression of *E. coli dnaK*	The maximum biomass increased 1.44-fold in the presence of 0.5 % lactic acid	(Abdullah-Al-Mahin et al. 2010)
L. lactis	Heterologous expression of *shsp* gene from *Streptococcus thermophilus*	The host cells displayed significantly higher survival under acid, heat, ethanol, bile salt and H_2O_2 stresses	(Tian et al. 2012)
L. lactis	Heterologous expression of *RecO* gene from *Lactobacillus casei*	Significantly higher survival during acid, salt, H_2O_2 stresses was achieved	(Wu et al. 2013b)

Overproduction of stress response proteins by genetic modification

With the development of genome sequencing and other high-throughput technologies, it has enabled us to engineer the robustness of industrial microbes at a global or systems biology levels (Zhu et al. 2012). At present, several systems biology approaches (e.g., genomics, transcriptomics, proteomics, metabolomics), combined with the molecular techniques have been employed to further understand the physiological mechanisms of LAB, and based on these, strategies to improve the physiological functions and engineer stress tolerance of LAB were proposed (Fig. 2). For example, Broadbent et al. (2010) investigated the acid stress response of *L. casei* ATCC334 during acid stress by transcriptional analysis, and the results showed that the two genes involved in malolactic fermentation (*mleS*, malolactic enzyme; *mleP*, malate/lactate antiporter) and eight genes cluster for histidine biosynthesis (LSEI_1426–1434) were significantly upregulated. To further validate the microarray data, 30 mM malate or 30 mM histidine were supplemented into the acid challenge medium, and the presence of either malate or histidine in the medium at pH 2.5 resulted in a more than 100-fold increase in cell survival after 60-min incubation, and greater than 10^7-fold improvement after 2 h (Broadbent et al. 2010).

Generally, bacteria maintain protein homeostasis under normal or stressed conditions using various mechanisms including the action of a group of regulatory proteins. Likely, LAB upregulated the expression of general stress response proteins in response to environmental stress (Wu et al. 2011, 2012a), among which molecular chaperones and DNA repair proteins were widely investigated (Table 3). Heterologous expression of *dnaK* from *E. coli* in *L. lactis* NZ9000 resulted in improved tolerance to lactic acid, NaCl, and ethanol stresses (Abdullah-Al-Mahin et al. 2010). Tian et al. (2012) expressed a small shock protein (*shsp*) gene from *Streptococcus thermophilus* in *L. lactis*, and the recombinant strain displayed significantly higher survival rate under acid, heat, ethanol, bile salt, and H_2O_2 stresses. In addition, comparative proteomic analysis with *L. casei* parental strain and its acid-

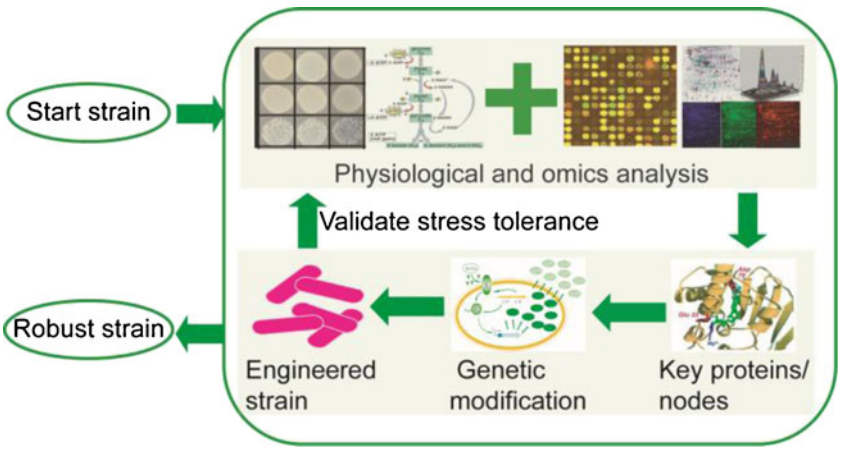

Fig. 2 Schematic representation of the approach to identify and validate stress-related genes via the omics-based technologies

Table 3 Protectants used for improving acid stress resistance of lactic acid bacteria

Protectant	Strains	Engineering strategy	Phenotype	References
Arginine	*Lactobacillus casei* Zhang	Exogenous addition of arginine to the media	2.1-fold increase in survival at pH 3.3 was obtained	(Wu et al. 2012a)
Aspartic acid	*L. casei* Zhang	Exogenous addition of aspartate to the media	The growth and survival during acid stress increased in the presence of aspartate	(Wu et al. 2013a)
Histidine	*L. casei* ATCC334	Exogenous addition of histidine to the media	100-fold increase in survival was obtained during acid stress	(Broadbent et al. 2010)
Glutathione	*Lactococcus lactis* SK11, *Leuconostoc mesenteroides*	Exogenous addition glutathione to the medium	Significantly higher survival of cells was observed	(Zhang et al. 2007; Kim et al. 2012)
Glucose	*Lactobacillus rhamnosus* GG	Addition of 1–19.4 mM glucose in simulated gastric juice	Significantly higher survival was obtained during gastric exposure	(Corcoran et al. 2005)
Tween 80	*L. rhamnosus* GG	Incorporation of 1 g/l tween 80 in the growth media	1,000-fold higher survival was achieved in simulated gastric juice	(Corcoran et al. 2007)
Gum acacia	*Lactococcus paracasei* NFBC338	10 % (w/v) gum acacia was supplemented into the media	100-fold increase in survival when exposed to porcine gastric juice	(Desmond et al. 2002)
Citrate	*L. lactis*	Exogenous addition of 13.2 mM sodium citrate to the media	Higher cell growth and glucose consumption were obtained at low pH	(Claudia 2008)

resistant mutant demonstrated that higher expression of DNA repair proteins (e.g., MutL, MutS2, UvrC, RecO) were observed in the mutant. Engineering the overproduction of DNA repair protein RecO in *L. lactis* NZ9000 was carried out, and the recombinant strain exhibited higher tolerance to lactic acid, salt, and H_2O_2 stresses (Wu et al. 2013b).

The whole-genome sequencing has yielded increasing numbers of completed genomes of LAB, many of which are publicly available on the Internet. This allows us to characterize their gene expression profiles and to identify the genes during environmental stresses. In addition, it provides an effective platform for us to engineer LAB with improved robustness. However, it should be noted that the strains obtained by genetic engineering may be hampered by legal issues and the general public opinion during industrial application. Therefore, further efforts should be made to ensure the acceptability of recombinant LAB in industrial manufacture, especially in food industry.

Engineering cell membrane functionality

As the first barrier of the cell, cell membrane separates cells from their environments and is a primary target for damage during environmental stresses. Changes in the cell membrane can protect the cell from environmental damage by modifying the physicochemical properties of membrane (Mykytczuk et al. 2007). Upon acid stress, *L. casei* increased the fluidity of cell membrane and increased the proportions of monounsaturated fatty acids, as well as mean chain length (Wu et al. 2012b). Therefore, engineering the production of unsaturated fatty acids could be a potential method to increase the acid tolerance of LAB. FabM, a novel enzyme, responsible for the production of monounsaturated fatty acids, was identified in *S. mutans*, and the FabM-defective mutant was extremely sensitive to acid stress compared with the wild type (Fozo and Quivey 2004). However, the acid-sensitive phenotype was relieved by growth in the presence of monounsaturated fatty acids or through genetic complementation (Fozo and Quivey 2004). In addition, production of cyclopropane fatty acids (CFA) was also a general stress response to acid stress (Broadbent et al. 2010; Wu et al. 2012b). Previous work with *E. coli* demonstrated that the CFA-defective mutant exhibited decreased resistance to acid stress, and the acid tolerance was restored by incorporation of a functional *cfa* gene (Chang and Cronan 1999). Conversely, recent work with *L. lactis* subsp. *cremoris* wild-type strain, the *cfa* mutant, and the complemented strain showed that the cyclopropanation of unsaturated fatty acids was not essential for survival under acidic conditions (To et al. 2011). Thus, further investigation concerning the detailed protective mechanisms of CFA during acid stress is necessary.

Adaptive evolution

Adaptive evolution, as a convenient approach to study many microbial phenomena, such as the emergence of new pathogens and the acquisition of environmental resistance factors, can address fundamental question on adaptation to selection pressures and evolution, and it has also become a widely used tool for biotechnological applications, improving yields and reducing costs in industrial settings (Fig. 3; Portnoy et al. 2011; Fong et al. 2005; Wang et al. 2011). Recently, adaptive evolution has been used with great success to gain insight into the genetic basis and dynamics of adaptation (Teusink et al. 2009). An example with *L. casei*, Zhang et al. (2012b) isolated acid-resistant mutant lb-2 by adaptive evolution for 70 days,

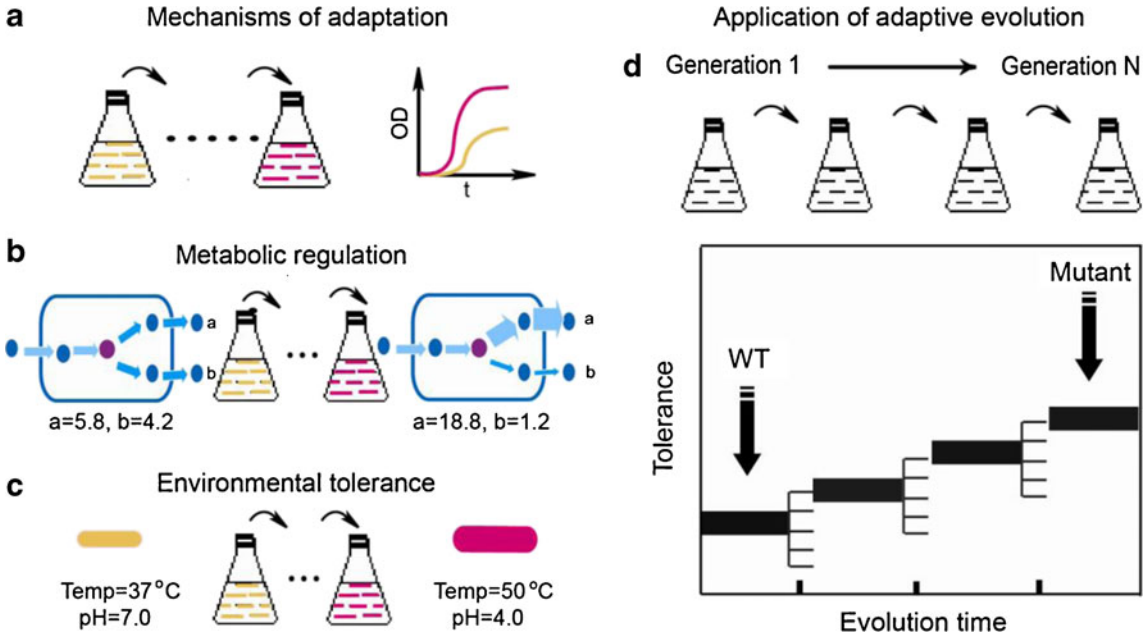

Fig. 3 Application of adaptive evolution. **a** Investigation of the mechanisms to environmental adaptation. **b** Engineering cellular metabolism for enhanced biosynthetic capacity of desired product (*a*) and decreased amount of by-product (*b*). **c** Improved robustness to environmental stresses. **d** Schematic representation of the procedure for adaptive evolution

and the evolved mutant exhibited higher biomass and survival during acid stress. Analysis of the intracellular microenvironments showed that the acid tolerance mutant displayed higher intracellular pH and NH_4^+ concentration in acidic conditions. In addition, at least 40.0 % and 23.9 % higher contents of intracellular arginine and aspartate were observed in the mutant compared with that in the parental strain (Zhang et al. 2012b). Moreover, proteomic analysis showed that higher expressions of many proteins including chaperonin (groEL), DNA repair protein (RecO) were observed in the evolved strain, and the overproduction of RecO in *L. lactis* led to increased tolerance to acid and NaCl stresses (Wu et al. 2012a, 2013b). These results suggest that adaptive evolution might be a useful method to engineer robust LAB.

Pre-adaptation and cross-protection

In response to environmental stress, LAB employ sophisticated mechanisms to combat stress. In many cases, similar responses were induced during different stressful conditions (heat, acid, oxygen, and cold), and as such, these mechanisms of resistance were interconnected. In this respect, preadaptation (pretreatment of a strain to a sublethal level can improve its resistance toward a potential severe stress) and cross-protection (one kind of stress tolerance confers protection against other stresses). Notably, Broadbent et al. (Broadbent et al. 2010) enhanced the survival of *L. casei* ATCC 334 to severe acidic conditions (pH 2.0) by prior exposure of the cells at pH 4.5 for 20 min. Moreover, a dramatic increase in survival to a severe acid stress (pH 3.9) was obtained by pre-exposing the *L. lactis* subsp. *lactis* cells for 30 min to a mildly acid shock at pH 5.5 (Hartke et al. 1996). Cross-protection was also reported as an effective approach to increase the acid stress resistance of LAB. For example, *L. plantarum* pre-exposed to sublethal heat treatment displayed enhanced growth at pH 5.0 (De Angelis et al. 2004). Generally, pre-exposure to mild acidic condition induced an ATR, which protected cells against multiple-environmental stresses. Pre-exposure of *L. lactis* subsp. *cremoris* to sublethal acid treatment displayed enhanced tolerance to acid, heat, NaCl, H_2O_2, and ethanol stresses (O'Sullivan and Condon 1997). In conclusion, pre-adaptation and cross-protection led to significant improvement of LAB to acid stress. However, the exact molecular mechanisms involved in pre-adaptation and cross-protection are not fully understood, and further exploration is also needed.

Exogenous addition of protectants

Exogenous addition of protectants is a relatively straightforward way to protect cells against acid stress or improve acid tolerance of LAB. Recently, numerous protectants have been developed to protect LAB against acid stress including amino acids, fatty acids, and saccharides (Table 3). For example, the addition of arginine increased the survival of *L. casei* Zhang at low pH (Zhang et al. 2012b). Physiological analysis showed that the exogenous arginine improved the viability of cells during acid stress by increasing the H^+-ATPase activity and intracellular ATP level (Zhang et al. 2012b). Generally,

regulation of ADI system is a widely reported ATR in a variety of bacteria including LAB, *E. coli*, *S. mutans*, *Listeria monocytogenes*, and *Bacillus* spp. (Zhao and Houry 2010; Senouci-Rezkallah et al. 2011; Matsui and Cvitkovitch 2010; Ryan et al. 2009). In another study with *L. casei*, aspartate was supplemented into the MRS media, and this led to the increment of the biomass and survival during acid stress (Wu et al. 2013a). The subsequent analysis of the intracellular microenvironment revealed that higher concentrations of intermediates involved in glycolysis and tricarboxylic acid cycle were observed, and *L. casei* shifted the metabolic pathway by increasing the flux from aspartate to arginine (ADI system) and decreasing the flux from aspartate to asparagine (Wu et al. 2012a, 2013a). Yet another example revealed that the addition of Tween-80 to the growth medium of *L. rhamnosus* resulted in 1,000-fold higher survival during exposure to gastric juice (Corcoran et al. 2007). Analysis of the fatty acids composition of *L. rhamnosus* revealed a 55-fold higher oleic acid content and a significantly higher unsaturated/saturated fatty acids ratio in the membrane of cells in the presence of Tween-80 (Corcoran et al. 2007). These results suggest that exogenous addition of protectants could be a feasible strategy to improve acid stress resistance of LAB.

Concluding remarks

Acid stress is a common environmental challenge to LAB, and improving the acid stress resistance is crucial to the application of LAB as probiotic. The advent of genome sequencing has increase our understanding of the molecular biology of LAB, and based on this, many post-genomic approaches (such as omics method) have accelerated the identification of genes/proteins involved in stress response and tolerance. This knowledge contributes to the design of rational approaches to engineering LAB with increased robustness. Moreover, specific fermentation conditions may be employed on the basis of the understanding of characterization of LAB to increase the stress tolerance. In conclusion, these systems biology approaches combined with the molecular techniques have provided us more opportunities to engineering LAB with improved robustness and industrial functionality.

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