Note

Adaptive Evolution of *Lactobacillus casei* under Acidic Conditions Enhances Multiple-stress Tolerance

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Acid stress is an environmental condition commonly encountered by lactic acid bacteria in the gastrointestinal tract and fermented foods. In this study, adaptive evolution of *Lactobacillus casei* was conducted in MRS broth at 37°C for 70 days, and three acid-tolerant strains were isolated. The evolved strains exhibited more than 60% increases in biomass than the parental strain when cultured at pH 4.3 for 54 h. Then, the tolerances of the evolved strains to acid stress, bile salt, and simulated gastrointestinal juice were determined. Acid tolerance was investigated by exposing the evolved strains and the parental strain in pH 3.3 and pH 2.5 of lactic acid and HCl-adjusted MRS broth respectively. The evolved strains showed higher survival rate than the parental strain. In addition, the evolved strains exhibited higher resistance to simulated gastric juice (pH 2.5), simulated intestinal juice (pH 8.0), and bile salt (1.6%). Analysis of the intracellular pH (pH_i) showed that the evolved strains exhibited higher pH_i compared with that in parental strain. The enhanced tolerance of the evolved strains to various stresses observed in this study provides a possible procedure for the food industry to select environmental-resistant strains and may be important in the production of probiotics.

Keywords: acid stress, adaptive evolution, *Lactobacillus casei*, bile tolerance, transit tolerance

Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria for producing fermented foods and are generally recognized as safe (GRAS). They are normal inhabitants of the oral cavity and the digestive tract in humans. These bacteria are used as starters to manufacture cheeses, yoghurt, sourdough bread, silage, table olives, fermented fish and sausages, and have been proposed as natural bio-preservatives in nonfermented vegetables (De Angelis and Gobbetti, 2004). In addition, some lactic acid bacteria function as a health adjuncts probiotics. The probiotic uses of these organisms include treatment of various types of diarrhea, prevention of adhesion of pathogenic bacteria to epithelial cells, and balance of intestinal microflora by modulating the growth of bacteria present in the gastrointestinal tract (Fuller, 1989; Fuller,

1991; Isolauri, 2001). For these reasons, there has been an increasing interest in creating food products containing these bacteria as dietary probiotics. However, to provide health benefits, they must overcome various chemical and physical barriers such as acid and bile in the gastrointestinal (GI) tract and in industrial processes (Duwat *et al.*, 2000). In addition, they need to be viable, active and abundant with a concentration of at least 10⁶ cfu/g in the product, throughout the specified shelf life (Samona and Robinson, 1991; Vinderola *et al.*, 2000).

Acid tolerance may be particularly important in lactic acid bacteria whose growth and transition to stationary phase are accompanied by the production of lactic acid, which results in acidification of the media, arrest of cell growth, and possibly cell death (Rallu *et al.*, 1996; Serrazanetti *et al.*, 2009). Some

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interesting findings showed that acid tolerance made these organisms less susceptible to lethal acid stress by prior exposure of the cells to moderately acidic conditions (Hartke *et al.*, 1996; De Angelis *et al.*, 2001). What's more, acid tolerance response appears to confer protection against other stresses such as heat and salt in addition to severe acid (Greenacre and Brocklehurst, 2006; Streit *et al.*, 2008; Jung *et al.*, 2009). Therefore, in this paper, we employed adaptive evolution to generate acid-resistant *Lactobacillus casei* by serially exposing mid-exponential phase strains to acidic conditions and characterized the changes of the evolved mutants in multiple-stress tolerance.

Materials and Methods

Bacteria and growth conditions *Lactobacillus casei* CICC 23185 was purchased from China Center of Industrial Culture Collection (CICC, Beijing, China). Cultures were grown statically in MRS broth (Oxoid, Hampshire, England) at 37°C. Stock cultures were prepared by growing the strain for 12–14 h and pipetting 0.7 mL into a sterile 1.5 mL vial containing 0.3 mL glycerol. Each vial was frozen immediately and was stored at –80°C until use. Each experiment started with a stock freezer vial for inoculation.

Adaptive evolution Adaptive evolution was performed according to Zhang *et al.* (2012) with some modifications. In brief, cells were grown in MRS broth adjusted to pH 5.5 with lactic acid (25%, v/v) prior to use. When the culture grown to mid-exponential phase (12 h), 2 mL of the culture was transferred into fresh MRS medium (100 mL, pH 5.5). After another 12 h of cultivation, the culture (2 mL) was transferred into new MRS medium (100 mL, pH 5.5). The transfer procedure was repeated for 10 days, and then the culture was transferred to fresh MRS broth at pH 5.0. Then, the culture was sequentially transferred to MRS at pH 4.6, and 4.3 (adjusted by 25% lactic acid) at the 25th day and 45th day, respectively (Fig. 1). The whole process of evolution was carried out for 70 days.

Acid challenge and acid tolerance analyses One milliliter of parental strain and the evolved stains grown in MRS at an initial pH of 6.2 (adjusted with lactic acid) were harvested in mid-exponential growth phase (OD₆₀₀ 2.0). To determine their acid resistance, cells were centrifuged, washed with distilled water and resuspended in modified MRS (adjust to pH 3.3 with 25% lactic acid or pH 2.5 with 6 M HCl). Aliquots of cell suspension (1 mL) were incubated at 37°C and samples were withdrawn at different times to determine viability.

Cell numbers were estimated with spot plating, where 10 µL of serially diluted samples were spotted in triplicate onto MRS agar plates and incubated at 37°C for 48 h. The number of colonies on the spot containing 5–100 colonies was counted, and CFU/mL was calculate from the average. All experiments were performed in triplicate.

Preparation of simulated human GI tract The simulated gastric juice was prepared by supplementing phosphate buffered

Fig. 1. Schematic representation of the adaptive evolution procedure

Cells were firstly grown in MRS broth at pH 5.5, and the culture at mid-exponential phase was serially transferred into fresh medium (pH 5.5). Then, continuous repeating this transfer procedure at pH 5.5 for 10 days, and the culture was sequentially transferred to MRS at pH 5.0, 4.6, and 4.3 at 10, 25, and 45 days, respectively. The whole procedure was conducted for 70 days. After evolution at different pHs, the evolved strains were isolated and acid tolerance analysis was performed. Three strains which exhibited high acid resistance were used for further adaptive evolution. The heavy lines indicate the acid tolerance, and the fine and dotted lines indicated the shifts of pH during evolution.

saline (PBS) solution (pH 2.5) with pepsin (3 g/L, 10000 U/mg, P7000, Sigma, Missouri, USA) and NaCl (0.5% (w/v)). The simulated intestinal juice was prepared by supplementing PBS buffer (pH 8.0) solution with trypsin (1 g/L, 250 U/mg, T6424, Sigma, Missouri, USA). The PBS buffer was sterilized by autoclaving at 121°C for 15 min and the simulated gastric juice and intestinal juice were filter-sterilized using a 0.22 µm filter membrane.

Determination of transit tolerance Cells (1 mL) grown to mid-exponential phase were collected and resuspended in simulated gastric juice and incubated at 37°C for 3 h. Thus the tolerance was assayed by determining the total viable count in simulated gastric juice. In the analysis of transit tolerance, after 3 h of incubation at 37°C in the simulated gastric juice, the cells were washed with PBS buffer (pH 8.0) solution and transferred to simulated intestinal juice and incubated at 37°C. The small intestinal transit tolerance was evaluated by determining the total viable count after the incubation at 37°C for 8 h.

Determination of bile tolerance The cells grown to mid-exponential phase were inoculated in MRS containing 1.6% bile salt (Oxoid, Hampshire, England) and the cell growth was monitored by determining the total viable count when incubated at 37°C.

Determination of intracellular pH (pH_i) The cells were loaded with 5 (and 6-)carboxyfluorescein succinidyl ester (Beyotime, Shanghai, China), and the determination of pH_i and calibration of

pH_i were followed the procedure described previously (Breeuwer *et al.*, 1996).

Statistical analysis Student's *t*-test was employed to investigate statistical differences. Differences between samples with *p*-values (*P*) ≤ 0.05 were considered to be statistically significant.

Results and Discussion

Adaptive evolution improves the growth performance of *L. casei* during acid stress Adaptive evolution was conducted with *Lactobacillus casei* as the parental strain at lactic acid-stressed condition. In this manner, three parallel evolved strains (indexed as Lbz-1, Lbz-2, Lbz-3) were generated after evolution for 70 days. Growth performance of the cells was measured in MRS broth at pH 4.3 at Day 0 (parental strain at the beginning of evolution), Day 10, Day 25, Day 45 and Day 70 (evolved strain at the endpoint) to track the growth shifts in each strains (Fig. 2). The biomass at 54 h increased over evolution and the evolved strains at Day 70 exhibited 1.6-fold higher biomasses when compared with the parental strain. Therefore, adaptive evolution resulted in the growth improvement with increased biomass. These increases indicate that the evolved strains display higher acid tolerance to low pH stress, which is essential for survival. This result was in agreement with that reported by Zhang *et al* (2012). Acid-tolerant *L. casei* lb-2 was isolated through adaptive evolution, and the evolved strain lb-2 also displayed higher biomass during acidic condition compared with the parental strain.

In addition, the genetic stability of the evolved strains were also investigated. The evolved strains were cultured at pH 6.2 for 50 generations, and the growth performance and the acid tolerance were monitored at evolutionary pH (pH 4.3) and acid-stressed environment (pH 3.3). Results presented in Table 1 confirmed that after 50 generations cultured in MRS at pH 6.2, no dramatic difference in growth performance and acid resistant was observed among different generations of Lbz-1. Similar results were also obtained in evolved strains Lbz-2 and Lbz-3 (data not shown). In this case, it can be considered that the acid resistance of the evolved strain maintained after serial passage.

Tolerance to acid-stressed conditions The evolved strains (Day 70) were challenged with lactic acid stress (pH 3.3) and hydrochloric acid stress (pH 2.5) for various times (Fig. 3). When subjected to lactic acid shock, survival of the parental strain sharply decreased. After shock for 8 h, only 0.2% of the parental cells survived compared with nearly 10% for the evolved strains (Fig. 3A). Tolerance to hydrochloric acid was investigated as well and the evolved strains showed a higher survival compared with the parental strain. All the evolved strains except Lbz-3 had more than 20% survival during the first hour of exposure, and more than 2-fold difference in the number of survivors was observed after 3 h (Fig. 3B).

Bile salt tolerance Bile salt disorganize the structure of the cell

Fig. 2. Growth changes during evolution
The cells were cultured in MRS with initial pH of 4.3 at 37°C for 54 h, and the growth was determined by measuring the OD₆₀₀. A: Lbz-1, B: Lbz-2, C: Lbz-3. ○parental strain, ● day 15, ▲ day 30, ■ day 50. Each point represents the mean value of three independent experiments.

membrane, and it is toxic for living cells (Succi *et al.*, 2005). Therefore, bile tolerance is considered as an important characteristic of *Lactobacillus* which enables it to survive, grow, and exert its action in gastrointestinal tract. In this study, we investigated the effect of bile salt on the growth of parental strain and the evolved strains. As shown in Fig. 3C, all of the four strains could grow in the medium with bile salt. However, the adaptive evolved strains displayed higher resistance to bile salt, since higher cell number in the evolved strains was observed (Fig. 3C).

Table 1. Growth performance and acid tolerance of the evolved strain Lbz-1 after serial passage for 50 generations at pH 6.2.

Generation	0	15	35	50
OD ₆₀₀ ¹	3.13 ± 0.09	3.28 ± 0.11	2.97 ± 0.08	3.19 ± 0.13
Survival (%) ²	34.79 ± 7.65	42.56 ± 5.18	30.77 ± 4.62	38.37 ± 5.93

¹Cells were grown at pH 4.3 for 54 h.

²Cells grown in mid-exponential growth phase were challenged with pH 3.3 of lactic acid stress for 4 h.

Fig. 3. Tolerance of cells to environmental stresses The evolved strains (Day 50) and the parental strain were challenged with lactic acid stress (A), hydrochloric acid stress (B) and bile salt (C). A : lactic acid as acidic stressor and the MRS broth was acidified with lactic acid to pH 3.3. B: hydrochloric acid as acidic stressor and the MRS broth was acidified with hydrochloric acid to pH 2.5. C: bile salt (1.6%) was added in MRS medium. ○ parental strain, ● Lbz-1, ▲ Lbz-2, ▼ Lbz-3. Error bars indicate standard deviations (n = 3).

The enhanced survival of the evolved strains demonstrated that adaptive evolution in acidic conditions improved cells resistant not only to low pH but also to other stresses. Chen and Xu (2014) isolated the ethanol-resistant *Saccharomyces cerevisiae* during Chinese rice wine fermentation via adaptive evolution by using ethanol as the selective pressure, and mutant exhibited markedly increased tolerance to ethanol, osmotic and temperature stresses. Adaptive evolution induced cross-protection and enhanced tolerance to other adverse environmental stresses. Recently, some interesting findings indicated that many stress response systems are interconnected, and there have been some reports concerning acid tolerance response in bacteria to resist other stresses. Greenacre and Brocklehurst (2006) reported that the acetic acid-adapted *Salmonella typhimurium* survived better in both NaCl and KCl stresses than their non-adapted controls did. The same is true for the HCl-induced acid resistance response of *Salmonella typhimurium* when encounter heat and salt stresses (Leyer and Johnson, 1993).

Changes in intracellular pH (pH_i) during acid stress In order to further verify the acid tolerance of both the parental strain and evolved populations, we determined the pH_i in both cells. Under normal condition (pH 6.2 adjusted with lactic acid), no obvious difference was observed in pH_i among parental and evolved strains (pH_i from 6.95 to 7.08, data not shown). During acid stress, the evolved populations seemed to have the capability of maintaining a higher pH_i compared to that of the parental strain in both acids (Fig. 4). The ability to maintain pH_i homeostasis during severe acid stress is essential for the survival of microorganisms. At a pH_o 3.3 of lactic acid, the pH_i of evolved population Lbz-3 was 5.75 in contrast to a pH_i of 5.34 in parental strain (Fig. 4A). Likewise, at a challenge pH_o of 2.5 in hydrochloric acid for 2 h, evolved population Lbz-1 maintained a pH_i of 5.57, in contrast to a pH_i of 5.31 in parental strain (Fig. 4B). These suggested that the evolved populations may effectively prevent the rapid decline of pH_i with severe acid challenge. In addition, some other mechanisms may also be involved in acid tolerance.

Transit tolerance The effect of simulated gastric juice and intestinal transit on the viability of the evolved strains is presented in Fig. 5. The survival of all the four strains decreased in simulated gastric and intestinal juices. After challenged in simulated gastric juice for 3 h, only 16.9% of the parental strain survived, while 72.4% of the evolved strain Lbz-1 survived. During the 8 h

Fig. 4. The pH_i of parental strain and evolved populations upon acid challenge. Mid-exponential growth phase cells were withdrawn and challenged at pH 3.3 or pH 2.5 adjusted by lactic acid (A) or hydrochloric acid (B) for 2 h. Error bars indicate standard deviations (n = 3). Statistically significant differences (p < 0.05) were determined by Student's test and are indicated with asterisks.

Fig. 5. Tolerance of cells to simulated human GI tract. T1: tolerance to simulated gastric juice (pH 2.5); T2: tolerance to simulated intestinal juice (pH 8.0). ○ parental strain, ● Lbz-1, ▲ Lbz-2, ▼ Lbz-3. Error bars indicate standard deviations (n = 3).

incubation in simulated intestinal juice, the evolved strains showed more than 2-fold higher survival compared with the parental strain did. Furthermore, the survival differences enlarged when the incubation time prolonged. Our results showed that the evolved strains had higher resistance to the simulated GI juice.

In conclusion, as a potential probiotic microorganism, *L. casei* has been largely applied in fermented dairy foods due to its technological properties and its health-promoting effects for humans. However, before playing a beneficial role to the host, it has to survive and retain activity during the food processing, storage, and particularly in the gastrointestinal tract. Therefore, the multiple-stress tolerance is considered as an important standard to select potential probiotic. This study demonstrates that the adaptive evolution under acidic condition enhances the resistance of cells against multiple environmental stresses. Results presented in this paper provided a possible strategy to screen potential probiotic and improve the functionality of probiotic.

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