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A short peptide designed from late-embryogenesis abundant protein enhances acid tolerance in *Escherichia coli*

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Abbreviations: CFU, colony-forming units; DMSO, dimethyl sulfoxide; IPTG, isopropylthio-D-galactoside; LB, Luria-Bertani; LEA, late embryogenesis abundant; OD_{600}, optical density at 600 nm; PBS, phosphate-buffered saline; GDAR, glutamic acid-dependent acid resistance.

Abstract

Unsuitable pH is a major limiting factor for all organisms, and a low pH can lead to organism death. Late embryogenesis abundant (LEA) peptides confer tolerance to abiotic stresses including salinity, drought, high and low temperature, and ultraviolet radiation same as the LEA proteins from which they originate. In this study, LEA peptides derived from group 3 LEA proteins of *Polypedilum vanderplanki* were used to enhance low pH tolerance. Recombinant *Escherichia coli* BL21 (DE3) cells expressing the five designed LEA peptides were grown at pH 4, 3, and 2. The transformants showed higher growth capacity at low pH as compared to control cells. These results indicate that LEA peptide could prevent *E. coli* cell death under low pH conditions.

Introduction

Abiotic or environmental stresses such as high soil salinity, extreme temperature, water deficiency, and unsuitable pH are major limiting factors for the growth and productivity of all living organisms. In most cases organisms have a well-defined range of pH that is tolerated, outside of which death due to osmoregulatory or respiratory failure can occur [1]. pH is a critical environmental factor that determines bacterial community composition in both soil [2] and water [3, 4]. Acidic soils limit crop production worldwide; approximately 50% of the world's potentially arable soils are acidic, which is associated with toxic levels of aluminum (Al) and manganese (Mn) and suboptimal levels of phosphorous (P) [5].

Various biochemical and physiological strategies have been developed to help organisms better adapt to and tolerate biotic and abiotic stresses [6] including genetic manipulation [7] to enhance the accumulation of low-molecular-weight osmolytes and thus increase tolerance to water or salt stress [8]. Transcription of peptide- or protein-coding genes is activated as a stress response [8, 9]. Several studies have investigated the mechanisms of acid tolerance in plants [10] and microorganisms [11]. Late embryogenesis abundant (LEA) proteins were first identified in higher plants such as cotton and wheat [12, 13] and play important roles in the response to environmental stresses [14, 15], including protecting cellular structures from the effects of water loss and desiccation [16, 17] and proteins from stress-induced damage [18, 19]; ion sequestration or scavenging [20]; and refolding of denatured proteins [21]. They can also act as chaperones to resist cellular damage [22].

LEA proteins have been described not only throughout the plant kingdom but also in other organisms ranging from invertebrates to prokaryotes [23, 24]. The survival of aquatic larvae of *Polypedilum vanderplanki* in a near-dehydrated state under drought conditions in semi-

arid areas is attributed to LEA proteins [16]. Although many studies have investigated the function of LEA proteins in conferring tolerance to environmental stresses such as salinity, drought, temperature, and radiation, there have been no reports on their contribution to low pH tolerance, despite the importance of pH for organism survival. Elucidating the role that these proteins play in acid tolerance can have applications in crop production, acid mine drainage bioremediation, biofuel production, and biofertilization.

The acid resistance systems in *Escherichia coli* have been extensively studied, one of which is the glutamic acid dependent resistance (GDAR) system, which is the most effective mechanism of acid resistance; through this system, one molecule of H^+ is consumed via decarboxylation of glutamate to γ-aminobutyric acid by glutamic acid decarboxylase (GadA/GadB) [25]. Both GadA and GadB are chemically identical isoforms of glutamate decarboxylase encoded by the *gadA* and *gadB* genes [26].

Our previous study showed that LEA peptide derived from LEA protein enhanced cell viability under heat, cold, salt stresses [27] and ultraviolet (UV) radiation [28]. In the present study, we investigated the contribution of mutated LEA peptides constructed in our earlier study [29] to acid tolerance in transformed *Escherichia coli* BL21 (DE3) in order to clarify their mechanism of action and their relationship to other well-known mechanisms of acid tolerance in bacteria, especially GDAR system, including their potential role as chaperones that protect cellular proteins.

Materials and methods

Peptide design and plasmid construction

Five different LEA peptides (LEA I, II, E, K, and S) were designed based on the 13-mer peptide MDAKDGTKEKAGE corresponding to LEA I in our previous study [29]. LEA II was generated by replacing the threonine at position 7 with leucine, while substitution of glycine at positions 6 and 12 in LEA II with glutamic acid, lysine, and serine yielded LEA E, K, and S, respectively. Details on plasmid construction for expression of the peptides in *E. coli* BL21 (DE3) can be found in our previous report [30]. Details of LEA peptide amino acid sequences are shown in Table 1.

Cell culture

E. coli BL21 (DE3) were transformed with pRSF plasmid containing the different types of LEA peptides (I, II, E, K, and S) or an empty plasmid (negative control) by the heat-shock method. Transformants were plated on a Luria-Bertani (LB) agar plate supplemented with 50 µg/ml kanamycin and incubated overnight at 37°C and recombinants were subcultured at 37°C for 14 h in LB broth containing 50 µg/ml kanamycin. The cultures were diluted 100-fold with fresh LB medium and incubated at 37°C for 2–3 h until the optical density at 600 nm (OD_{600}) was 0.5 (mid-log phase). Peptide expression was induced by adding isopropylthio-D-galactoside (IPTG) to a final concentration of 0 and 0.1 mM, and the cultures were incubated at 37°C (120 rpm) for 4 h.

Tolerance and growth capacity of E. coli under low pH stress

After 4 h, *E. coli* cells were harvested by centrifugation for 10 min at 4000 rpm. The supernatant was discarded, and the cell pellet was resuspended in phosphate-buffered saline (PBS; 10-fold dilution). A 2-ml volume of each resuspended pellet was inoculated into 50 ml LB medium with different pH (4 and 7 as controls), followed by incubation at 37°C and 160 rpm for 2 h. The cultures were serially diluted and 100 µl of the last two dilutions were used to inoculate LB agar plates supplemented with kanamycin 50 µg/ml. The plates were incubated at 37°C for 14 h and the number of colony-forming units (CFU) was recorded, with the mean value for each culture calculated to identify the most effective LEA peptide for further study. M9 minimal medium with different pH (4 and 7) was also used instead of LB medium, where 50 ml of M9 medium supplemented with 50 µg/ml kanamycin was inoculated with 2 ml of each resuspended pellet followed by incubation at 37°C and 160 rpm for 2 h.

Maximum acid tolerance capacity

The effect of the selected LEA on the growth capacity of *E. coli* BL21 (DE3) was investigated at different pH values (2, 3, and 4) in addition to pH 7 (control). Other factors including the type of LEA peptide (LEA K), IPTG concentration (0.1 mM), and culture medium (LB) were fixed. The number of CFU at each pH was recorded and the mean number was calculated.

Effect of IPTG concentration on acid tolerance

IPTG at different concentrations (0.0, 0.01, 0.1, 0.5, and 1 mM) was added to the cultures at mid-log phase (OD_{600} = 0.5). Other factors including the type of LEA peptide (LEA K), pH (4), and medium (LB) were fixed. The number of CFU at each pH was recorded and the mean number was calculated.

Cell viability assay

Cell viability was evaluated with a colorimetric assay using 96-well clear flat-bottomed plates (Nunclon; Thermo Fisher Scientific, Waltham, MA, USA), water-soluble tetrazolium (WST) reagent, and dimethyl sulfoxide (DMSO) (Microbial Viability Assay Kit-WST; Dojindo Laboratories, Kumamoto, Japan). Each plate included blanks, controls, or acid-treated recombinant *E. coli* expressing LEA K induced with different concentrations of IPTG (0, 0.01, 0.1, 0.5, and 1 mM). The cells were cultured in LB broth at pH 4 for 2 h, with samples prepared in triplicate. The cell suspension (180 µl) was added to the plate; 20 µl WST (9:1 WST reagent:DMSO) was then added, followed by incubation for 1 h. The absorbance at 450 nm was measured on a microplate reader (PerkinElmer, Waltham, MA, USA), with filters at a maximum wavelength of 460 nm. Cytotoxicity was determined as percent cell viability (relative to the WST-DMSO reduction) compared to the control.

Total RNA extraction

Total RNA was extracted from five samples which included LEA K that was induced with different concentrations of IPTG (0.01, 0.1, 0.5, 1 mM) and LEA K without induction utilized the control (0 mM IPTG) grown for 2 h in 50 ml LB broth (pH 4) using RNAprotect Bacteria Reagent (Qiagen, USA) for stabilization of cultures, then purification of RNA was performed using RNeasy Mini column (Qiagen, USA) following the manufacturer's instructions. After extraction, the purified RNA was quantified spectrophotometrically using Nano-drop (Thermo Scientific, USA), and analyzed on 1% agarose gel.

Quantification of LEA K expression levels after induction with different concentrations of IPTG at pH4 using real-time PCR

The total RNA extracted from the samples that were induced with different concentrations of IPTG (0, 0.01, 0.1, 0.5, 1 mM) were used as a template in performing the One Step Reaction, where both reverse transcription and real-time PCR were performed in one step using One Step TB Green PrimeScript PLUS RT-PCR Kit (Takara, Japan). The reaction was performed in 20 µl scale, containing 10 µl (2x One Step TB Green RT-PCR Buffer 4), 1.2 µl (Takara Ex Taq HS Mix), 0.4 µl (PrimeScript PLUS RTase Mix), 0.8 µl of 10 µM of each primer, 0.4 µl (ROX Reference Dye 50x), 2 µl of template RNA, and 4.4 µl RNase free dH2O. The reaction was performed using the Step One real-time PCR system (Applied Biosystems). The sequence of specific primers designed for LEA K was F-LEA K, R-T7 ter and F-16s and R-16s for 16S rRNA as endogenous control are depicted in Table 2. The threshold cycle (Ct) for unknown samples were determined by using the Step One software (Applied Biosystems), and the fold-change levels were calculated based on the $2^{-\Delta\Delta Ct}$ method [31, 32].

Quantification of gad A/B gene expression using real-time PCR

The extracted total RNA from two samples (0 mM and 0.1 mM) were used as a template for the One Step Reaction as discussed before. The sequences of primers sets for F-GAD A/B, R-Gad A/B, for quantification of *gad a/b* mRNA, and the same primer set with 16srRNA was used as the endogenous control (Table 2). The $2^{-\Delta\Delta Ct}$ method was used, as mentioned above.

Statistical analysis

All data obtained were evaluated by statistical analysis with the CoStat computer program V 6.303 (2004) [33]. Tukey-HSD LSR at a significance level of 5% was used to differentiate

between the means, in case of comparing between more than two groups, otherwise significance evaluation was assessed based on the t-test.

Results and Discussion

LEA peptide has been widely studied for its role in conferring tolerance to abiotic stresses including heat [27, 34], cold [35–37], drought [38, 39], osmosis [40, 41], heavy metal [34], desiccation [42], and UV radiation [28]. In the present study, we evaluated the capacity of five 13-mer LEA peptides derived from group 3 LEA proteins of *P. vanderplanki* to confer tolerance to low pH stress. We previously reported that LEA I maintains its function under salt, heat, cold [27], and UV radiation [28] and can enhance the expression of target proteins [30].

Effect of LEA peptide expression in E. coli on acidic pH tolerance

E.coli cells expressing the five types of LEA peptides grown at pH4 for 2h, where the expression was induced with 0.1 mM IPTG, showed enhancement in growth in the rank order of LEA K > LEA S > LEA E > LEA II > LEA I (Fig. 1).

LEA K was selected for further studies. The results obtained were statistically evaluated via conducting a factorial experiment investigating the effect of different types of LEA peptides as the main factor, the absence or presence of the IPTG inducer as the second factor, and the interaction between both factors. Results showed that there was a significant difference between LEA K and LEA (E, II, I) respectively. While no significant difference was found for LEA S, there was a significant difference between the absence and presence of induction. The presence of five basic amino acids (lysine) in the structure of LEA K may play a vital role in tolerating acid, which is reflected by the higher growth capacity of *E. coli* BL21 (DE3) transformed with

LEA K compared with other tested LEA peptides at pH4. We used the empty vector pRSF-duet 1 as the negative control in this experiment, which uses a T7 promoter. By comparing the growth capacity of negative control and positive control cultures (containing LEA peptides but without IPTG induction), the positive controls showed higher growth capacities, although positive controls showed variable growth capacities which may be attributed to leaky expression from T7 promoter in pRSF vector through a phenomenon known as leakage [43], where there is a low level of recombinant protein expression from the T7 promoter in the pRSF-duet 1 plasmid even before induction.

Effect of LEA K expression in E. coli on acidic pH tolerance

LEA K expression was induced by adding 0.1 mM IPTG, and after harvesting cells were inoculated in LB broth with different pH (2, 3, 4, and 7) with and without IPTG. The CFU counts showed that cultures grown at pH 7 had the highest growth capacity, while LEA K expression enhanced the growth of *E. coli* BL21 at lower pH values (pH 4 and 3) compared to cultures without IPTG induction. No growth was observed at pH 2 regardless of IPTG induction (Fig. 2A). To determine whether the nutrient content of the medium influenced the low pH tolerance of LEA K-expressing cells, we performed experiments using M9 minimal medium at pH 4 and 7, similar results were obtained using M9 minimal medium at pH 4, but at pH 7 LEA K expression enhanced growth capacity as compared to cultures without IPTG induction (Fig. 2B), however cells grown in LB at pH 7 without induction had higher growth than those with induction suggesting that M9 medium does not support optimal *E. coli* BL21 (DE3) growth as was mentioned by others [44]. LEA K was an effective tool in preventing the cell death at low pH,

but only above the threshold level (pH 3), as it may prevent the aggregation of cellular proteins at low pH and thus prevent cell death; however, only above a threshold pH of 3.

Effect of IPTG concentration on LEA K expression and acid tolerance

LEA K expression was induced at different concentrations of IPTG (0.0, 0.01, 0.1, 0.5, and 1 mM) using LB broth at pH 4 stress. Results showed that the rank order of IPTG concentration yielding the highest CFU counts was 0.1 > 0.01 > 0.5 > 1 > 0.0 mM (Fig. 2C). There was a significant difference between all of the induced samples compared to those without induction (0 mM). Furthermore, there was no significance found between samples induced with different concentrations of IPTG. We previously observed that *E. coli* growth and viability increased in an IPTG concentration-dependent manner (0–1 mM) [27, 28]; however, here we found that tolerance to low pH was not enhanced by increasing IPTG concentration, with maximal CFU and viability observed at 0.1 mM IPTG. Higher IPTG concentrations may increase LEA K expression to toxic levels and may also be directly lethal to *E. coli* BL21 (DE3) [45]. LEA K contain five basic amino acids (lysine) with positive charges and the increases observed in the LEA K expression levels corresponded to elevation of the IPTG concentration (from 0.1 mM to 1 mM) could have affected and disrupted the cell membrane due to attachment between the positively charged amino acids and the negatively charged cell membrane (phospholipid) as mentioned by Ginsberg et al., who stated the role of the synthetic poly-basic amino acids was to damage the cell membrane leading to bacterial cell death [46].

Effect of LEA K expression on the viability of E. coli at pH 4

LEA K expression was induced with different concentrations of IPTG (0–1 mM), in LB medium at pH 4. Cell viability was evaluated with the WST assay for 2×10^4 cells/well with a 2%–5% coefficient of variation among replicate wells. Cell viability was increased as a function of IPTG concentration, with maximal growth observed at 0.1 mM IPTG (Fig. 3). Cells expressing LEA K showed increased viability at pH 4, indicating that LEA peptides and specifically, LEA K confer low pH tolerance to *E. coli*. Increasing IPTG concentration from 0.1 to 1 mM did not further enhance cell viability.

Quantification of LEA K expression levels after induction with different concentrations of IPTG at pH4 using real-time PCR

The expression level of LEA K in samples induced by different concentrations of IPTG (0–1 mM) were quantified using Real-Time PCR, 0 mM represented the control, and the endogenous control used is 16S rRNA as shown in Table 3. Where the expression levels of LEA K showed that the elevated levels were directly correlated to increases in the IPTG concentration. However, differences in the expression levels between each IPTG concentration were exiguous, which might have resulted from the short lifetime, small size, and instability of the transcribed mRNA.

Quantification of gad A/B gene expression using real-time PCR

The change in *gad A/B* gene (glutamic acid decarboxylase) expression levels were determined in both LEA K induced with 0.1 mM IPTG and LEA K without induction as control in pH 4, to determine whether the acid tolerance obtained using LEA K is related to GDAR system or not. As shown in Table 4 expression levels of *gad A/B* in comparison with endogenous control (16S rRNA) did not change in both the samples. This result indicates that the low pH tolerance

through expression of LEA K was not related with the GDAR system. These results are in agreement with those of Woo et al [47] who stated that GDAR system is not active in *E. coli* BL21 but active in *E. coli* K-12 MG1655. DNA sequence of the genes encoding the key enzymes of GDAR system (i.e. GadA/B, GadC) were not different in these strains; however, *E.coli* BL21 lacks RcsB (an essential component in GDAR regulation through forming heterodimer with GadE) and some small RNAs such as DsrA that are involved in stimulation of translation of RpoS, which is involved in activation of GadE using the GadXW circuit [48, 49].

E. coli BL21 (DE3) is widely used as a host strain for protein expression and purification [50]. Various strains of microorganisms have been developed that exhibit increased tolerance to fermentation conditions [51]; low pH is one of the most restrictive of these conditions that must be overcome for large-scale microbial bioprocessing. Moreover, acidification leads to a decrease in metabolic enzymes activity [52] and protein unfolding [53]. The results of our study provide a new method for improving tolerance to low pH stressor during fermentation, however this effect is not related to GDAR system. Given that LEA proteins can stabilize enzyme complexes and cell membrane structure [54, 55] by stimulating protein production [29], we speculate that LEA peptides can function as molecular shields or chaperones that prevent cellular protein aggregation and damage, and our hypothesis is supported by results of a previous study [56], which showed that specific periplasmic chaperones, HdeA and HdeB, play an important role in protection of periplasmic and membrane proteins from damage caused by low pH.

Conclusions

LEA peptides especially LEA K confer acid tolerance in *E.coli* BL21 (DE3) but only above the threshold level (pH3), however the tolerance was not enhanced by increasing the expression level of LEA K in correspondence to IPTG concentration elevation with maximal tolerance capacity at 0.1 mM IPTG. The mechanism of low pH tolerance via LEA K expression is unclear however we hypothesized that LEA peptides can function as molecular shields or chaperones preventing cellular proteins from damage, hence further studies should be conducted to detect the mechanism involved in the acquired tolerance.

Author contributions

KM and SI conceived and designed the project, interpreted the data, analyzed the data and wrote the paper. KM performed experiments.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Kimmel, W. G. (1983). The impact of mine drainage on the stream ecosystem. Pennsylvania coal: resources, technology and utilization. *The Pennsylvania Academy of Science Publication*, *5*(1), 424–437.
2. Fierer, N. , Morse, J. L., Berthrong, S. T., Bernhardt, E. S., & Jackson, R. B. (2007),

Environmental controls on the landscape‐scale biogeography of stream bacterial communities. *Ecology*, *88*, 2162–2173.

3. Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D., & Bertilsson, S. (2011). A Guide to the Natural History of Freshwater Lake Bacteria. *Microbiology and Molecular Biology Reviews*, *75*(1), 14–49.
4. Ren, L., Jeppesen, E., He, D., Wang, J., Liboriussen, L., Xing, P., & Wu, Q. L. (2015). pH influences the importance of niche-related and neutral processes in lacustrine bacterioplankton assembly. *Applied and Environmental Microbiology*, *81*(9), 3104–3114.
5. Kochian, L. V, Piñeros, M. A., Liu, J., & Magalhaes, J. V. (2015). Plant adaptation to acid soils: The molecular basis for crop aluminum resistance. *Annual Review of Plant Biology*, *66*(1), 571–598.
6. Orcutt, D. M. (2000). The Physiology of Plants Under Stress: Soil and Biotic Factors, *John Wiley & Sons*.
7. Larcher, W. (2003). Physiological plant ecology: ecophysiology and stress physiology of functional groups, 3rd edition, *Springer-Verlag Berlin Heidelberg*.
8. López-Maury, L., Marguerat, S., & Bähler, J. (2008). Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. *Nature Reviews Genetics*, *9*, 583–593.
9. Ramond, J. B., Welz, P. J., Le Roes-Hill, M., Tuffin, M. I., Burton, S. G., & Cowan, D. A. (2014). Selection of Clostridium spp. in biological sand filters neutralizing synthetic acid mine drainage. *FEMS Microbiology Ecology*, *87*(3), 678–690.
10. Kochian, L. V., Hoekenga, O. A., & Piñeros, M. A. (2004). How do crop plants tolerate acid soils? Mechanisms of aluminium tolerance and phosphorous efficiency. *Annual*

Review of Plant Biology, *55*(1), 459–493.

11. Lund, P., Tramonti, A., & De Biase, D. (2014). Coping with low pH: Molecular strategies in neutralophilic bacteria. *FEMS Microbiology Reviews*, *38*(6), 1091–1125.

12. Cuming, A. C. (1999), in Seed Proteins, LEA Proteins (P. R. Shewry and R. Casey ed., *Springer, Dordrecht*, pp. 753–780.

13. Galau, G. A., Hughes, D. W., & Dure, L. (1986). Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. *Plant Molecular Biology*, *7*(3), 155–170.

14. Silveira, V., Santa-Catarina, C., Balbuena, T. S., Moraes, F. M. S., Ricart, C. A. O., Sousa, M. V., Guerra, M. P., Handro, W., & Floh, E.I.S. (2008). Endogenous abscisic acid and protein contents during seed development of *Araucaria angustifolia*. *Biologia Plantarum*, *52*(1), 101–104.

15. Hincha, D. K., & Thalhammer, A. (2012). LEA proteins: IDPs with versatile functions in cellular dehydration tolerance. *Biochemical Society Transactions*, *40*(5), 1000-1003.

16. Sogame, Y., & Kikawada, T. (2017). Current findings on the molecular mechanisms underlying anhydrobiosis in *Polypedilum vanderplanki*. *Current Opinion in Insect Science*, *19*, 16–21.

17. Rodriguez-Salazar, J., Moreno, S., & Espín, G. (2017). LEA proteins are involved in cyst desiccation resistance and other abiotic stresses in *Azotobacter vinelandii*. *Cell Stress and Chaperones*, *22*(3), 397–408.

18. Grelet, J., Benamar, A., Teyssier, E., Avelange-Macherel, M.-H., Grunwald, D., & Macherel, D. (2005). Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. *Plant Physiology*, *137*(1), 157-167.

19. Hong-Bo, S., Zong-Suo, L., & Ming-An, S. (2005). LEA proteins in higher plants:

Structure, function, gene expression and regulation. *Colloids and Surfaces B: Biointerfaces*, *45*(3–4), 131–135.

20. Gao, J., & Lan, T. (2016). Functional characterization of the late embryogenesis abundant (LEA) protein gene family from *Pinus tabuliformis* (Pinaceae) in *Escherichia coli*. *Scientific Reports*, *6*(19467), 1–10.

21. Hundertmark, M., & Hincha, D. K. (2008). LEA (Late Embryogenesis Abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *BMC Genomics*, *9*(1), 1–22.

22. Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Current Opinion in Biotechnology*, *17*(2), 113–122.

23. Hand, S. C., Jones, D., Menze, M. A., & Witt, T. L. (2006). Life without water: expression of plant LEA genes by an anhydrobiotic arthropod. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *307*(1), 62–66.

24. Kikawada, T., Nakahara, Y., Kanamori, Y., Iwata, K. I., Watanabe, M., McGee, B., Tunnacliffe, A., & Okuda, T. (2006). Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. *Biochemical and Biophysical Research Communications*, *348*(1), 56–61.

25. Capitani, G., De Biase, D., Aurizi, C., Gut, H., Bossa, F., & Grütter, M. G. (2003). Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. *The EMBO journal, 22*(16), 4027–4037 .

26. Bergholz, T. M., Tarr, C. L., Christensen, L. M., Betting, D. J. and Whittam, T. S. (2007). Recent gene conversions between duplicated glutamate decarboxylase genes (gadA and gadB) in pathogenic *Escherichia coli*. *Molecular Biology and Evolution*, *24*(10), 2323–

2333.

27. Pathak, N., & Ikeno, S. (2017). *In vivo* expression of a short peptide designed from late embryogenesis abundant protein for enhancing abiotic stress tolerance in *Escherichia coli*. *Biochemical and Biophysical Research Communications*, *492*(3), 386–390.

28. Huwaidi, A., Pathak, N., Syahir, A., & Ikeno, S. (2018). *Escherichia coli* tolerance of ultraviolet radiation by *in vivo* expression of a short peptide designed from late embryogenesis abundant protein. *Biochemical and Biophysical Research Communications*, *503*(2), 910–914.

29. Pathak, N., Hamada, H., & Ikeno, S. (2018). Construction and characterization of mutated LEA peptides in *Escherichia coli* to develop an efficient protein expression system. *Journal of Molecular Recognition*, *31*(1), 1–6.

30. Ikeno, S., & Haruyama, T. (2013). Boost protein expression through co-expression of LEA-like peptide in *Escherichia coli*. *PLoS ONE*, *8*(12), e82824.

31. Winer, J., Jung, C. K. S., Shackel, I., & Williams, P. M. (1999). Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. *Anal. Biochem.*, *49*, 41–49.

32. Schmittgen, T. D., Zakrajsek, B. A., Mills, A. G., Gorn, V., Singer, M. J., & Reed, M. W. (2000). Quantitative reverse transcription–polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Analytical biochemistry*, *285*(2), 194–204.

33. Jones, D. H. (1994). Statistical Methods, 8th ed., *Iowa State University Press*.

34. Hu, T., Zhu, S., Tan, L., Qi, W., He, S., & Wang, G. (2016). Overexpression of OsLEA4 enhances drought, high salt and heavy metal stress tolerance in transgenic rice (*Oryza*

sativa L.). *Environmental and Experimental Botany*, *123*, 68–77.

35. Liu, Y., Liang, J., Sun, L., Yang, X., & Li, D. (2016). Group 3 LEA Protein, ZmLEA3, Is Involved in Protection from Low Temperature Stress. *Frontiers in Plant Science*, *7*, 1–10.

36. Zeng, X., Ling, H., Yang, J., Li, Y., & Guo, S. (2018). LEA proteins from Gastrodia elata enhance tolerance to low temperature stress in *Escherichia coli*. *Gene*, *646*, 136–142.

37. Boudet, J., Buitink, J., Hoekstra, F. A., Rogniaux, H., Larré, C., Satour, P., & Leprince, O. (2006). Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. *Plant Physiology*, *140*, 1418–1436.

38. He, S., Tan, L., Hu, Z., Chen, G., Wang, G., & Hu, T. (2012). Molecular characterization and functional analysis by heterologous expression in *E. coli* under diverse abiotic stresses for OsLEA5, the atypical hydrophobic LEA protein from Oryza sativa L. *Molecular Genetics and Genomics*, *287*(1), 39–54.

39. Battaglia, M., & Covarrubias, A. A. (2013). Late Embryogenesis Abundant (LEA) proteins in legumes. *Frontiers in Plant Science*, *4*, 1–11.

40. Tunnacliffe, A., & Wise, M. J. (2007). The continuing conundrum of the LEA proteins. *Naturwissenschaften*, *94*(10), 791–812.

41. Wang, H., Wu, Y., Yang, X., Guo, X., & Cao, X. (2017). SmLEA2, a gene for late embryogenesis abundant protein isolated from Salvia miltiorrhiza, confers tolerance to drought and salt stress in *Escherichia coli* and S. *miltiorrhiza. Protoplasma*, *254*(2), 685–696.

42. Shimizu, T., Kanamori, Y., Furuki, T., Kikawada, T., Okuda, T., Takahashi, T., Mihara, H., & Sakurai, M. (2010). Desiccation-induced structuralization and glass formation of

group 3 late embryogenesis abundant protein model peptides. *Biochemistry*, *49*(6), 1093–1104.

43. Briand, L., Marcion, G., Kriznik, A., Heydel, J. M., Artur, Y., Garrido, C., Seigneuric, R., & Neiers, F. (2016). A self-inducible heterologous protein expression system in *Escherichia coli*. *Scientific reports*, *6*, 33037.

44. Paliy, O., & Gunasekera, T. S. (2007). Growth of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources and salt contents. *Applied Microbiology and Biotechnology*, *73*(5), 1169–1172.

45. Dvorak, P., Chrast, L., Nikel, P. I., Fedr, R., Soucek, K., Sedlackova, M., Chaloupkova, R., de Lorenzo, V., Prokop, Z., & Damborsky, J. (2015). Exacerbation of substrate toxicity by IPTG in *Escherichia coli* BL21(DE3) carrying a synthetic metabolic pathway. *Microbial Cell Factories*, *14*(1), 201.

46. Ginsburg, I., Heerden, P. V. Van, & Koren, E. (2017). From amino acids polymers , antimicrobial peptides , and histones , to their possible role in the pathogenesis of septic shock : a historical perspective. *Journal of inflammation research*, *10*, 7–15.

47. Woo, J. M., Kim, J. W., Song, J. W., Blank, L. M., & Park, J. B. (2016). Activation of the glutamic acid-dependent acid resistance system in *Escherichia coli* BL21(DE3) leads to increase of the fatty acid biotransformation activity. *PLoS ONE*, *11*(9), 1–20.

48. Foster, J. W. (2004). *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nature Reviews Microbiology*, *2*(11), 898–907.

49. Lange, R., & Hengge-Aronis, R. (1994). The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes and Development*, *8*(13), 1600–1612.

50. Gilsdorf, J., Gul, N., & Smith, L. A. (2006). Expression, purification, and characterization of *Clostridium botulinum* type B light chain. *Protein Expression and Purification*, *46*(2), 256–267.

51. Lennen, R. M., & Herrgård, M. J. (2014). Combinatorial strategies for improving multiple-stress resistance in industrially relevant *Escherichia coli* strains. *Applied and Environmental Microbiology*, *80*(19), 6223–6242.

52. Hayes, E. T., Wilks, J. C., Sanfilippo, P., Yohannes, E., Tate, D. P., Jones, B. D., Radmacher, M. D., BonDurant, S. S., & Slonczewski, J. L. (2006). Oxygen limitation modulates pH regulation of catabolism and hydrogenases, multidrug transporters, and envelope composition in *Escherichia coli* K-12. *BMC Microbiology*, *6*, 1–18.

53. Goto, Y., Calciano, L.J., & Fink, A.L. (1990). Acid-induced folding of proteins. *Proceedings of the National Academy of Sciences*, *87*(2), 573–577.

54. Chourey, K., Ramani, S., & Apte, S. K. (2003). Accumulation of LEA proteins in salt (NaCl) stressed young seedlings of rice (*Oryza sativa* L.) cultivar Bura Rata and their degradation during recovery from salinity stress. *Journal of Plant Physiology*, *160*(10), 1165–1174.

55. Reddy, A. R., Chaitanya, K. V., & Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology*, *161*(11), 1189–1202.

56. Hong, W., Wu, Y. E., Fu, X., & Chang, Z. (2012). Chaperone-dependent mechanisms for acid resistance in enteric bacteria. *Trends in Microbiology*, *20*(7), 328–335.

Figure legends

Figure 1 Growth capacity of transformed *E. coli* BL21 (DE3) expressing LEA peptides (I, II, K, E, and S) in LB medium at pH 4. Expression was induced with 0.1 mM IPTG. Factorial experiment included Two Way ANOVA with completely Randomized (F-test), followed by multiple comparisons based on the Tukey-HSD at the 5% level, values followed by the same letter are not significantly different from each other; small letters represent the main factor (different types of LEA peptides) comparisons, there was a significant difference between the absence and presence of induction (second factor) data not shown.

Figure 2 (A) Growth capacity of *E. coli* BL21 (DE3) expressing LEA K in LB medium at different pH (2, 3, 4, and 7). Expression was induced with 0.1 mM IPTG. (B) Growth capacity of *E. coli* BL21 (DE3) expressing LEA K at different pH (4 and 7) in M9 minimal medium with 0.1 mM IPTG used as an inducer. For (A) and (B) $^{*}P < 0.05$ vs. control (0 mM IPTG); †not significant (*t* test). (C) Effect of IPTG concentration on acid tolerance was conferred by LEA K at pH 4 in LB medium. (One Way ANOVA Completely Randomized (F-test) was followed by multiple comparisons based on Tukey-HSD at 5% level, values followed by the same letter do not significantly differ from each other.

Figure 3 Viability of *E. coli* BL21 (DE3) expressing LEA K peptide induced with different concentrations of IPTG and cultured for 2 h in LB medium at pH 4. (One Way ANOVA Completely Randomized (F-test) followed by multiple comparisons based on Tukey-HSD at the 5% level, values followed by the same letter do not significantly differ from each other.

Table 1 Amino acid sequences of designed LEA peptides used in this study

LEA peptide	**Amino acid sequence**
LEA I	MDAKD**G**TKEKA**G**E
LEA II	MDAKDG**L**KEKAGE
LEA E	MDAKD**EL**KEKA**E**E
LEA K	MDAKD**KL**KEKA**K**E
LEA S	MDAKD**SL**KEKA**S**E

Table 2 Sequences of primer sets designed for QPCR

Primer code	Sequence
(1) F-LEA K	5′ –ATGGATGCGAAAGACAAACTG- 3′
(2) R-T7 ter	5′ – TGCTAGTTATTGCTCAGCGG- 3′
(3) F-GAD A/B	5′- CTCGTCAGAACCTAGCCA- 3′
(4) R-Gad A/B	5′ –TCGGCAACCATATTTACGCA- 3′
(5) F-16s	5′ –TGGATCAGAATGCCACGG- 3′
(6) R-16s	5′ –ACCTTGTTACGACTTCACC- 3′

Table 3 LEA K expression level after induction with different concentrations of IPTG in pH4 using real-time PCR

Sample	Ct *LEA K* Average	Ct 16S rRNA Average	ΔCt	ΔΔCt	LEA K expression fold change 2ˆ- ΔΔCt
(Control) **LEA K 0 mM IPTG**	18.62±0.18	8.01±0.08	10.61	-	2ˆ0 = 1.00
LEA K 0.01 mM IPTG	14.90±0.21	8.36±0.11	6.54	-4.07	2ˆ4.07 = 16.80
LEA K 0.1 mM IPTG	15.30±0.24	9.02±0.10	6.27	-4.34	2ˆ4.34 = 20.25
LEA K 0.5 mM IPTG	13.78±0.32	8.03±0.16	5.75	-4.86	2ˆ4.86 = 29.04
LEA K 1 mM IPTG	13.55±0.20	7.94±0.19	5.61	-5.00	2ˆ5.00 = 32.00

Table 4 *gad A/B* gene expression fold change

Sample	**Ct *gad a/b* Average**	**Ct 16S rRNA Average**	**ΔCt**	**ΔΔCt**	***gad a/b* expression fold change 2ˆ- ΔΔCt**
(Control) **LEA K 0 mM IPTG**	29.35±0.40	12.93±2.32	16.42	-	2ˆ0 = 1.00
LEA K 0.1 mM IPTG	31.66±0.31	14.47±0.05	17.17	0.75	2ˆ-0.75= 0.59

Figures

Figure 1

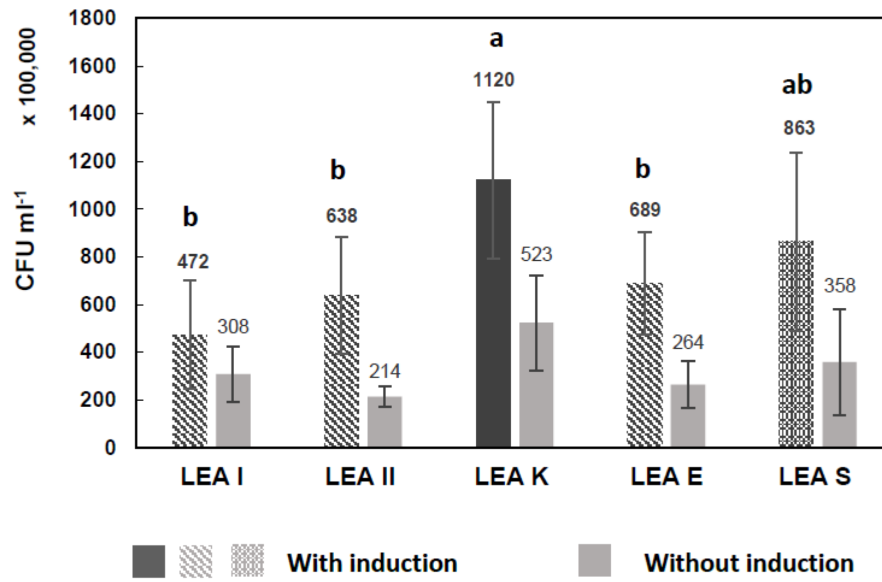

Figure 2

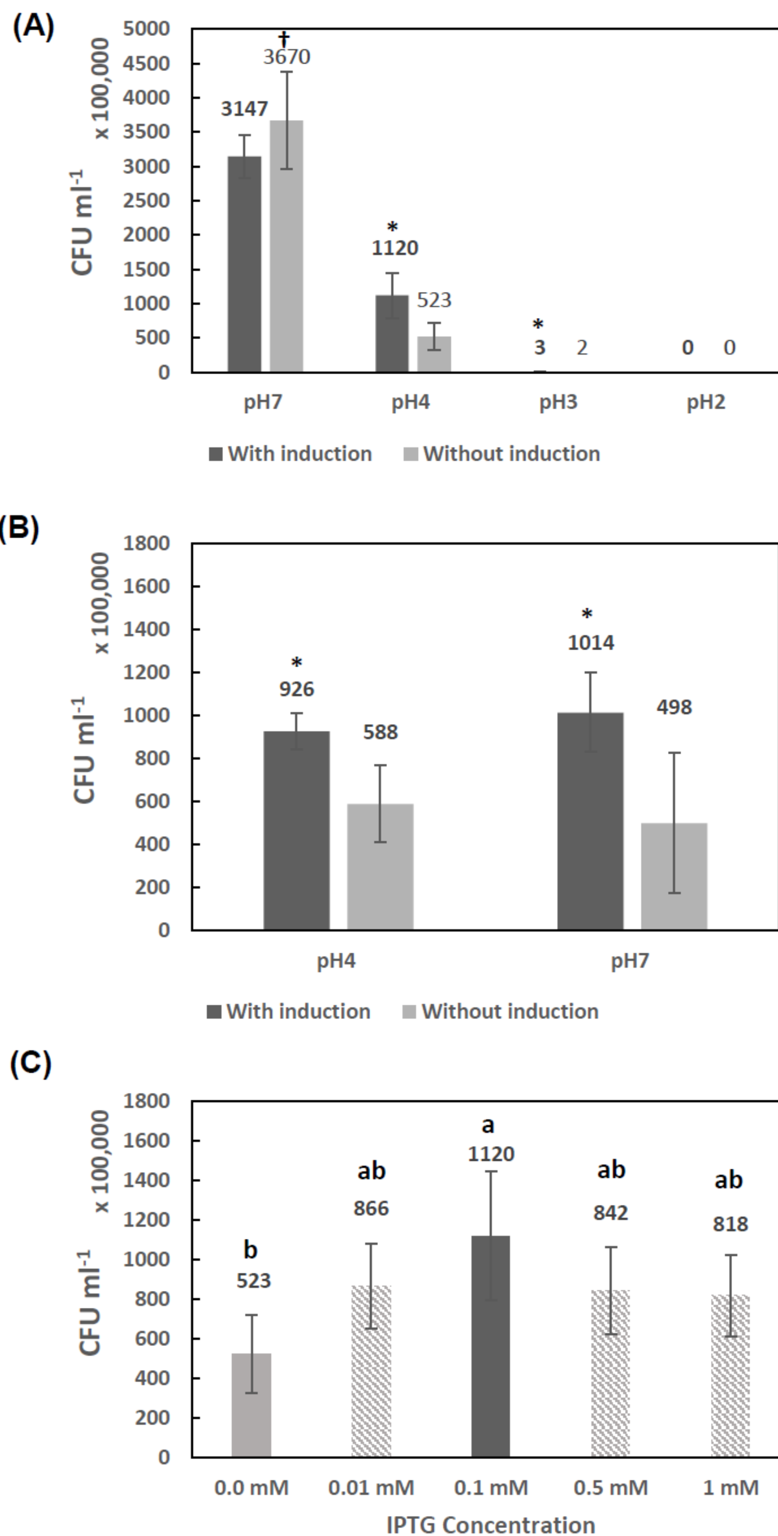

Figure 3