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ORIGINAL PAPER

Expression of genes involved in exopolysaccharide synthesis in *Lactiplantibacillus plantarum* VAL6 under environmental stresses

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Abstract

Environmental factors can alter exopolysaccharide biosynthesis in lactic acid bacteria (LAB). To further clarify this potential relationship, the mRNA expression of genes involved in exopolysaccharide synthesis such as *glmU, pgmB1, cps4E, cps4F*, *cps4J*, and *cps4H* in *Lactiplantibacillus plantarum* VAL6 under different conditions including temperature, pH, sodium chloride (NaCl), and carbon dioxide (CO_2) intensification culture was studied. The transcriptomic data revealed that the exposure of *L. plantarum* VAL6 at pH 3 increased the expression level of *cps*4H but decreased the expression levels of *pgmB1* and *cps4E*. Under pH 8, *cps4F* and *cps4E* were significantly upregulated, whereas *pgmB1* was downregulated. Similarly, the expression levels of *cps4F*, *cps4E*, and *cps4J* increased sharply under stresses at 42 or 47 °C. In the case of NaCl stress, *glmU*, *pgmB1*, *cps4J*, and *cps4H* were downregulated in exposure to NaCl at 7 and 10% concentrations while *cps4E* and *cps4F* were upregulated at 1 h of 10%-NaCl treatment and at 5 h of 4%-NaCl treatment. Remarkably, CO_2 intensification culture stimulated the expression of all tested genes. In addition, simultaneous changes in expression of *cps*4E and *cps*4F under environmental challenges may elicit the possibility of an association between the two genes. These findings indicated that the expression level of *eps* genes is responsible for changes in the yield and monosaccharide composition of exopolysaccharides under environmental stresses.

Keywords Environmental stress · Gene expression · *Lactobacillus plantarum* · qPCR · Real-time PCR

Introduction

Lactic acid bacteria (LAB) are ubiquitous appearance in food by their generally recognized as safe status (Ismail and Nampoothiri 2010). LAB are capable of producing exopolysaccharides (EPSs) which are widely used in various applications (Baruah et al. 2016). EPS production in LAB has been received a lot of attention in recent years due to the unique biological properties of these biopolymers (Silva et al. 2019). This is exemplified in *Lactiplantibacillus plantarum*, which is commonly utilized in the health and food areas owning to its ability to produce EPSs with specific functions regarding antitumor and antioxidant activities and prebiotic effects (Silva et al. 2019).

In nature as well as in industrial applications, LAB are often exposed to adverse environmental conditions (Le Marrec 2011). Indeed, LAB have various adaptive mechanisms to protect themselves from environmental stresses including changes related to the expression of genes and proteins (Mbye et al. 2020), to the cell's envelope with enhanced

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synthesis of EPSs (Huu Thanh et al. 2014). Many studies demonstrated that environmental stresses such as high temperature (Huu Thanh et al. 2014), acidic or alkaline pH, NaCl (Nguyen et al. 2021), CO_2 (Ninomiya et al. 2009) among other factors can stimulate EPS synthesis.

In LAB, EPSs play an important role in protecting the cells from harsh environmental conditions (Nguyen et al. 2020). In addition, EPSs are also involved in the formation of biofilm and adhesion (Caggianiello et al. 2016) as well as in determining cell interaction characteristics (Lee et al. 2016). Nonetheless, the structure and biological function of EPSs may vary depending on environmental conditions (Vu et al. 2009) that related to the transcriptional levels of *eps* gene cluster (Boels et al. 2003).

EPS synthesis in LAB is a complex process involved in the regulation of expression of EPS-related genes (Zeidan et al. 2017). Furthermore, changing environmental conditions can alter the expression level of these genes (Wu and Shah 2018). Therefore, in this study, we evaluated the impact of various environmental challenges on the expression levels of genes involved in EPS synthesis in *L. plantarum* VAL6. The overall aim of the study was to clarify the potential relationships between these regulatory systems and environmental stresses.

Materials and methods

Bacterial strain and culture conditions

L. plantarum VAL6 was obtained from Department of Biotechnology, An Giang University, Vietnam National University Ho Chi Minh City, Vietnam. To perform microbial cell culture for this study, *L. plantarum* VAL6 was grown in Man–Rogosa–Sharpe medium (MRS medium) (De Man et al. 1960). *L. plantarum* VAL6 was stored at −80 °C in solution containing 30% glycerol; it was rehydrated in MRS broth with 2% (v/v) inoculum, followed by incubating at 37 °C for 24 h, agitation rate was set up to 250 rpm under aerobic facultative condition.

Bioreactor operating conditions for stress treatments

All the cultures were carried out in 5-L bioreactors (BIOSTAT, Sartorius Stedim Biotech, Germany). Briefly, 5 L of MRS medium was inoculated with 100 mL of bacterial culture (*L. plantarum* VAL6 was cultivated in MRS medium and incubated at 37 °C for 24 h to obtain an optical density at 595 nm (OD 595) of 1.5 which corresponds to approximately 10^8 CFU/mL). The pH was maintained to 6.8 by regularly adding 10 M sodium hydroxide (NaOH), the temperature was kept at 37 °C, and agitation rate was set up to 250 rpm. After 24 h of culture, stress treatments were then performed independently:

For thermal stress, the culture was treated with high temperature either 42 and 47 °C for 7 h and the pH was maintained to 6.8. The time was calculated when the bioreactor reached the required stress temperature.

For pH stress, the culture was treated with pH conditions either pH 3 and 8 for 7 h by adding 10 M phosphoric acid (H_3PO_4) and the temperature was kept at 37 °C. The time was calculated when the bioreactor reached the required pH.

For NaCl stress, the culture was treated by adding NaCl at 4, 7, and 10% (w/v) concentrations for 7 h. The pH was maintained to 6.8 and the temperature was kept at 37 °C.

The non-stress control of these treatments was simultaneously carried out in another bioreactor, where the pH was maintained to 6.8, the temperature was maintained at 37 °C for the entire time.

In the case of CO_2 treatment, right after inoculation, CO_2 was continuously supplied at the rate 250 cm^3/min for 4, 8, and 24 h. The pH was maintained to 6.8 and the temperature was kept at 37 °C. The non-stress control of this treatment was also carried out at the same time without CO_2 supplement.

Extraction of total RNA and synthesis of first-strand cDNA

Total RNA of cells (For conditions of stress and non-stress, cells were collected at 1, 3, 5, and 7 h. For CO_2 treatments, cells were collected at 24 h after inoculation) was extracted according to the instructions of TRIzol reagent (Invitrogen, UK). RNA was treated with RQ1 RNase-free DNase (Promega, USA) to remove contamination of chromosomal DNA. Qualitative test of RNA at 260 and 280 nm was found to be more than 1.8 using a NanoDrop DeNovix DS-11 Spectrophotometer (DeNovix, USA). The first-strand cDNA was synthesized according to the instructions of the GoScript Reverse Transcription System Kit (Promega, USA).

Design and synthesis of primers

The reference gene was selected from the housekeeping gene, i.e., 16S rRNA. Primers of the six target genes were designed for quantitative real-time polymerase chain reaction (qPCR) analysis based on the genome sequence of *L. plantarum* WCFS1 (Genbank: AL935263.2) using Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were designed with sequence length from 18–23 nucleotides, coupling temperature (T_m) from 57–60 °C and GC rate not more than 70% (Table 1). The primers were synthesized by The Shanghai Brilliance Biotechnology Co. Ltd.

Table 1 The primers used for the amplification

Gene	Primer name	Primer sequence (5′→3′)	T_m (°C)	Product length (bp)
glmU	Sense	ATGCAACAACGGATCAACGC	60.1	475
	Antisense	CGACATTCGTGTGGTGCTTG	60.1	
pgmB1	Sense	TCGATGGCGTTATCACGGAC	60.3	423
	Antisense	CGGGATCAGGTTTACCGTGT	59.8	
cps4E	Sense	AGTTGACTTCTGACGGTCCC	59.3	449
	Antisense	AACCGTTCCCATCAGCATCT	59.4	
cps4F	Sense	CTGGGGCTTTACTCCTGGTG	60.0	452
	Antisense	CCCCAACGTCCGATTGAGAA	60.0	
cps4H	Sense	TTTGCTTTGGTCATGCTGGC	60.0	596
	Antisense	TCGGACGTTCCGAACCAAAT	60.0	
cps4J	Sense	ACGGCTCGATTTTTAGGGCT	59.8	480
	Antisense	GACGCTCATTGCGATTGGTG	60.3	
16s RNA	Sense	GCATTAAGCATTCCGCCTGG	60.0	183
	Antisense	ACCTGTATCCATGTCCCCGA	60.0	

qPCR experiment

The qPCR experiment was performed on an Liberty16 mobile real-time PCR system (Ubiquitome Limited, New Zealand), according to the instructions of the SensiFAST SYBR No-Rox Kit (Bioline, Meridian Bioscience, USA). The reaction mixture contained 10 µL of SensiFAST SYBR No-Rox Solution (2×), 7.4 µL of cDNA template, 0.8 µL of forward primer (10 µM), 0.8 µL of reverse primer (10 µM), and 1 µL of molecular grade water. As a negative control for all qPCR experiments, cDNA was replaced with RNA (without the reverse transcription step as template). The reaction for each gene was carried out in triplicate in individual qPCR reactions. The PCR amplification conditions were denaturation at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Fluorescence performance was obtained at the end of each thermal cycle. The $2^{-\Delta\Delta Ct}$ method was used to calculate the changes in gene expression (Livak and Schmittgen 2001). Expression values were obtained by log2 of the ratio between cells under stress and without stress (Luiz de Freitas et al. 2020).

Statistical analysis

The experiments were repeated three times. All the data were expressed as means ± standard deviations. Significance of difference was evaluated with one way ANOVA, followed by Tukey' test procedure to identify statistically significant differences at the 95% confidence interval.

Results

The expression of genes involved in EPS synthesis under thermal stress

The effect of high-temperature stresses at 42 and 47 °C on the expression of genes involved in EPS synthesis in *L. plantarum* VAL6 was investigated via RNA sequencing. Through analysis and comparison of the gene expression profile of the stressed *L. plantarum* VAL6 and the control at 37 °C, we found that *cps4E*, *cps4F*, and *cps4J* genes were significantly ($p < 0.05$) upregulated in response to thermal stress. Also, the exposure time played an important role in the regulation of these genes (Fig. 1).

The expression level of *cps4E* (Fig. 1c) peaked at 5 h after thermal stress with log2 (Fold change) = 4.8 and 5 under 42 and 47 °C treatments, respectively. Similar to *cps4E*, the log2 (Fold change) at the same time of *cps4F* (Fig. 1d) was 4.5 under 42 °C and 5 under 47 °C. However, the expression levels of two genes decreased after exposure to thermal stress for 7 h. Meanwhile, the expression level of *cps4J* was steadily increased over time of stress and peaked after 7 h of treatment (log2 (Fold change) ~ 4.2) (Fig. 1f).

The expression of genes involved in EPS synthesis under acid and alkaline stress

In this work, the response of *L. plantarum* VAL6 to acid and alkaline stress via the transcriptional analysis of genes involved in EPS synthesis was also studied. The results disclosed that *cps4H* was significantly ($p < 0.05$)

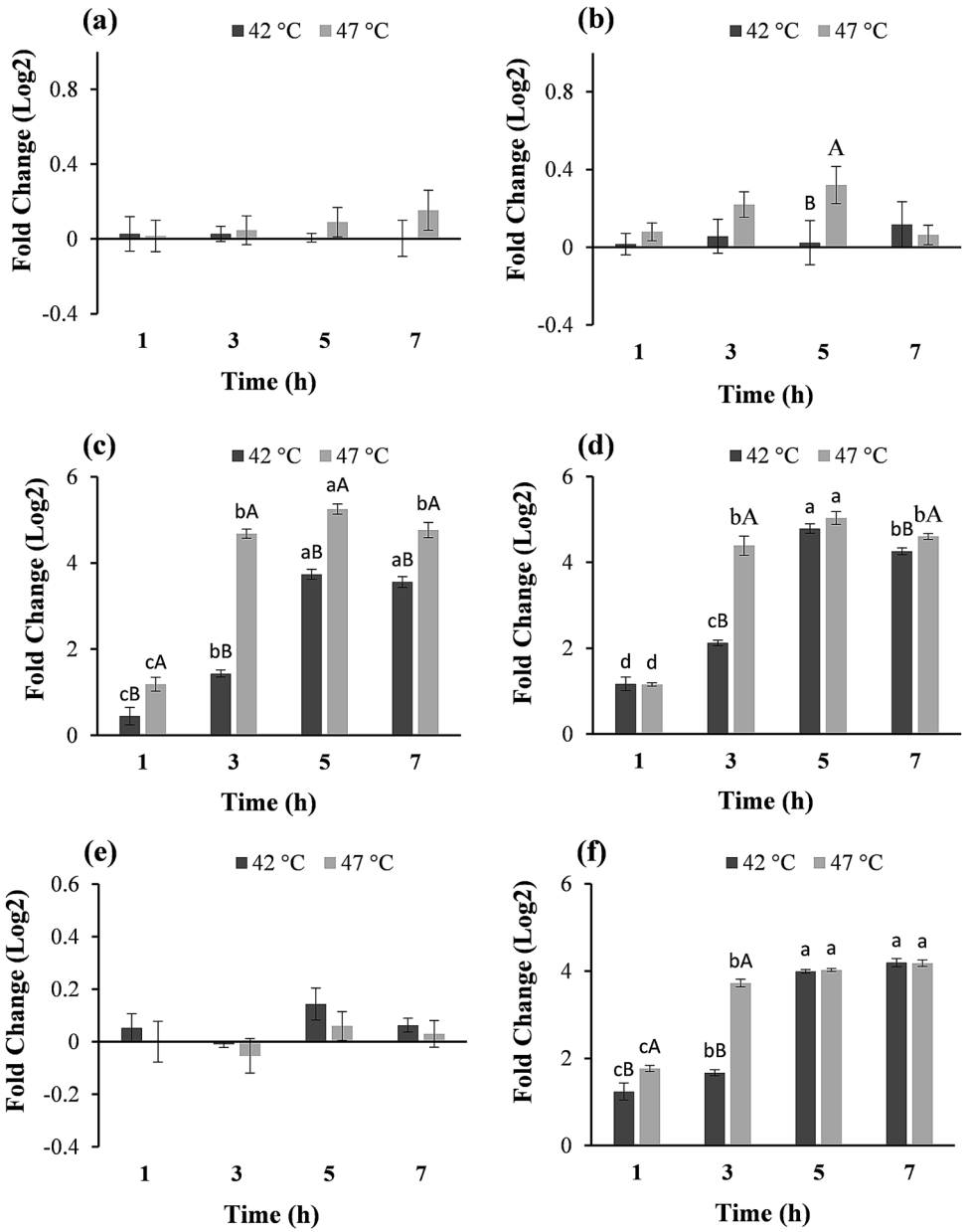

Fig. 1 The expression of genes involved in EPS synthesis under stress at 42 and 47 °C: **a** *glmU*, **b** *pgmB1*, **c** *cps4E*, **d** *cps4F*, **e** *cps4H*, and **f** *cps4J*. Expression values were obtained by log2 of the ratio between cells under stress and without stress. Different superscript uppercase letters indicates statistically significant differences between treatments at the same sample collection time and different superscript lowercase letters indicate significant differences throughout time for each separate treatment by Tukey' test ($p < 0.05$). Error bars indicate standard error ($n = 3$)

upregulated in exposure to acid at pH 3. Meanwhile, *cps4E* and *cps4F* were significantly ($p < 0.05$) upregulated in response to alkaline stress condition at pH 8 (Fig. 2).

Under stress at pH 3, there was a great increase in the expression level of *cps4H* (log2 (Fold change) ~ 2) (Fig. 2e), but the expression levels of *glmU*, *pgmB1*, and *cps4E* decreased (Fig. 2a–c). In the case of stress at pH 8, log2 (Fold change) of *cps4F* increased steadily from 1 at 1 h to 1.72 at 7 h (Fig. 2d). As a similar pattern with *cps4F*, the expression level of *cps4E* was also rose gradually during the time of stress, although this difference was not statistically significant ($p > 0.05$) (Fig. 2c).

The expression of genes involved in EPS synthesis under NaCl stress

We also investigated the effect of the addition of NaCl at different concentrations on the expression of genes involved in EPS synthesis in *L. plantarum* VAL6. The overall results indicated that the expression of *glmU*, *pgmB1*, *cps4H*, and *cps4J* genes were significantly ($p < 0.05$) downregulated in exposure at 7 and 10% NaCl (Fig. 3).

In addition, an increase in the expression level of *cps4E* was detected at 1 h of 10%-NaCl treatment and at 5 h of 4%-NaCl treatment (Fig. 3c). Similarly, *cps4F* was

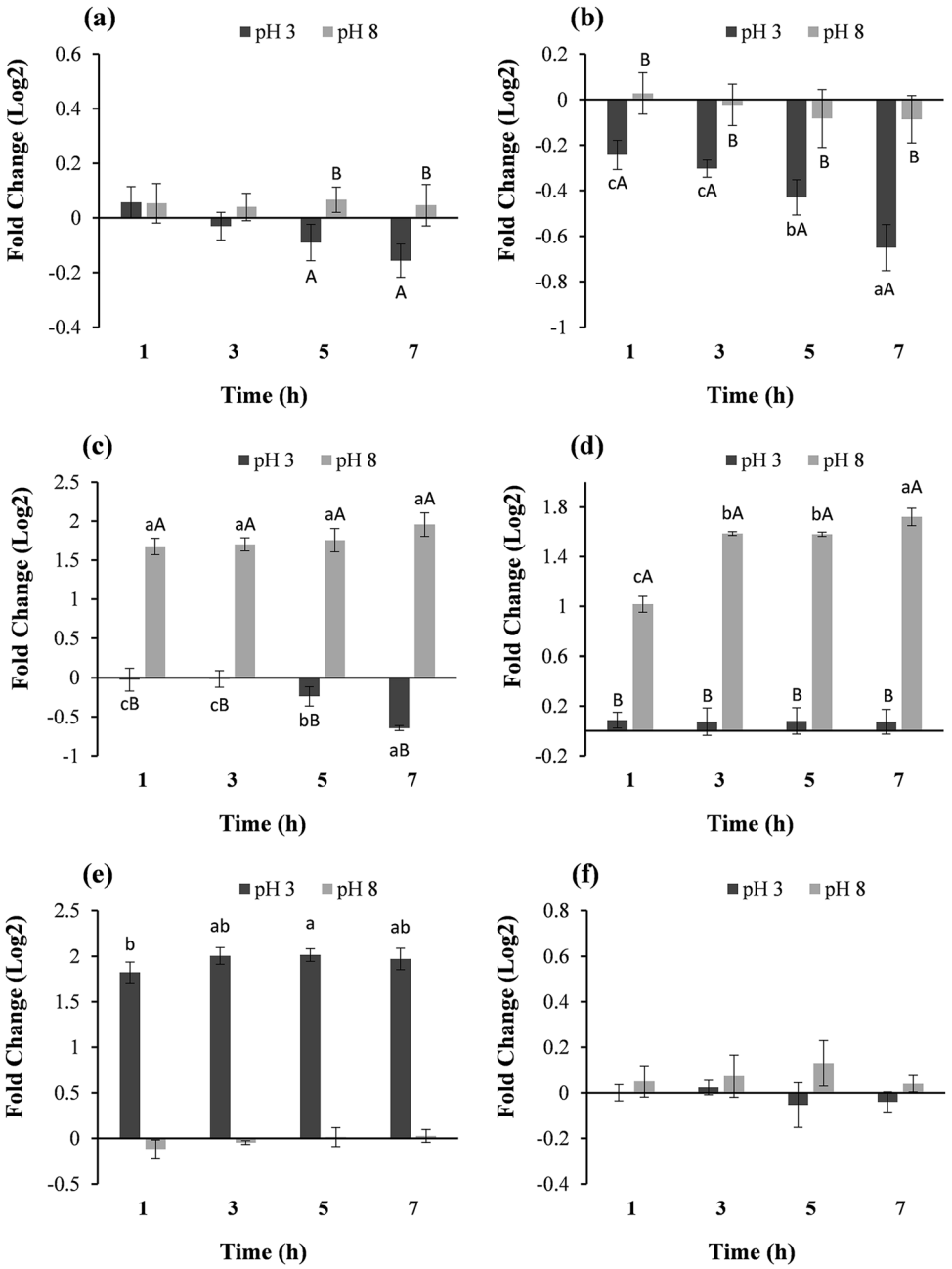

Fig. 2 The expression of genes involved in EPS synthesis under acid at pH 3 and alkaline at pH 8 stress: **a** *glmU*, **b** *pgmB1*, **c** *cps4E*, **d** *cps4F*, **e** *cps4H*, **f** *cps4J*. Expression values were obtained by log2 of the ratio between cells under stress and without stress. Different superscript uppercase letters indicates statistically significant differences between treatments at the same sample collection time and different superscript lowercase letters indicate significant differences throughout time for each separate treatment by Tukey' test ($p < 0.05$). Error bars indicate standard error ($n = 3$)

upregulated at 1 h of 10%-NaCl treatment and at 5 h of 4%-NaCl treatment, respectively (Fig. 3d).

The expression of genes involved in EPS synthesis under CO_2 intensification culture

Unlike other stress treatments, CO_2 intensification culture increased the expression of all tested genes. Furthermore, with the exception of *cps4E*, the other genes were upregulated with increasing time of CO_2 supplementation (Fig. 4). The expression level of *cps4E* was highest upon exposure to 8-h CO_2 treatment with log2 (Fold change) = 0.89, while its expression returned to log2 (Fold change) = 0.58 upon exposure to 24-h CO_2 treatment (Fig. 4c).

Discussion

Changes in environmental conditions may alter extracellular polysaccharide production (Lloret et al. 1998) and induce the synthesis of new type of EPSs in bacteria (Nandal et al. 2005). In addition, the overexpression of a certain gene involved in EPS synthesis can increase or decrease the level of a specific sugar component in EPSs (Nguyen et al.

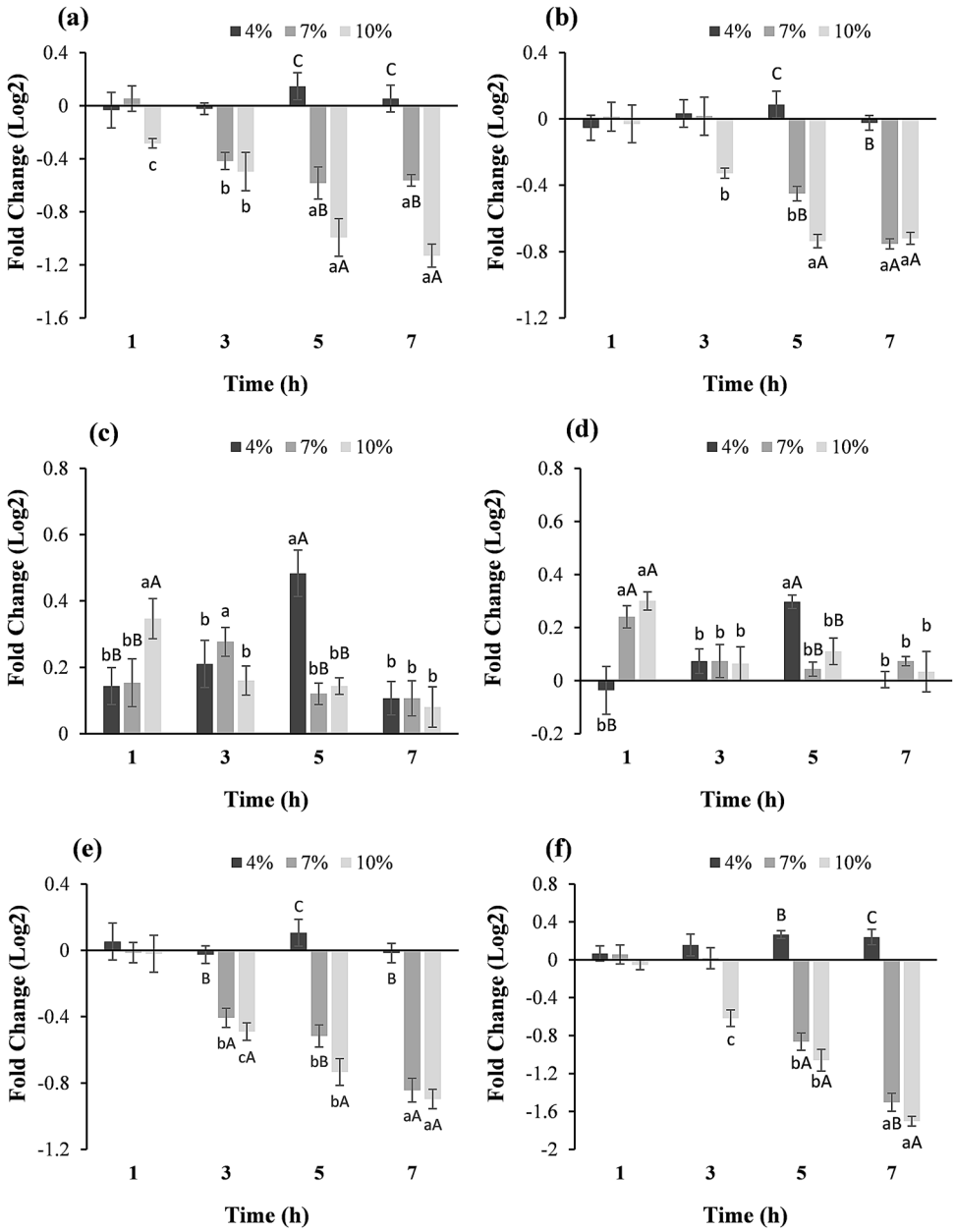

Fig. 3 The expression of genes involved in EPS synthesis under NaCl stress: **a** *glmU*, **b** *pgmB1*, **c** *cps4E*, **d** *cps4F*, **e** *cps4H*, and **f** *cps4J*. Different superscript uppercase letters indicates statistically significant differences between treatments at the same sample collection time and different superscript lowercase letters indicate significant differences throughout time for each separate treatment by Tukey' test ($p < 0.05$). Error bars indicate standard error ($n = 3$)

2021). Therefore, changes in expression level of *eps* gene under environmental stresses leads to differences in yield and monosaccharide composition in EPSs.

In this study, *glmU* was only upregulated under CO_2 intensification culture but downregulated under exposure to pH 3 and NaCl concentrations at 7 and 10%. In comparison with a previous study in *Lactobacillus vini*, *glmU* was also downregulated under acid stress conditions (Mendonca et al. 2019). The enzymes encoded by *glmU* are glucosamine-1-phosphate N-acetyltransferase converting glucosamine-1-phosphate to N-acetylglucosamine-1-phosphate and N-acetylglucosamine-1-phosphate uridyltransferase catalyzing the formation of UDP-N-acetyl-D-glucosamine from N-acetylglucosamine-1-phosphate (Li et al. 2011). UDP-N-acetyl-D-glucosamine is one of the essential precursors of the peptidoglycan structure of cell wall (Chapot-Chartier and Kulakauskas 2014) and the synthesis of repeating units of EPSs (Zeidan et al. 2017). Altogether, we could conclude that exposing *L. plantarum* VAL6 to CO_2 intensification culture impacts on the regulation of *glmU*, possibly leading to an increase in the synthesis of major component of bacterial cell wall and in the content of *N*-acetyl-D-glucosamine in EPSs.

The *pgmB1* encodes for β-phosphoglucomutase which catalyzes the interconversion of D-glucose 6-phosphate and D-glucose 1-phosphate to form beta-D-glucose 1,6-(bis)

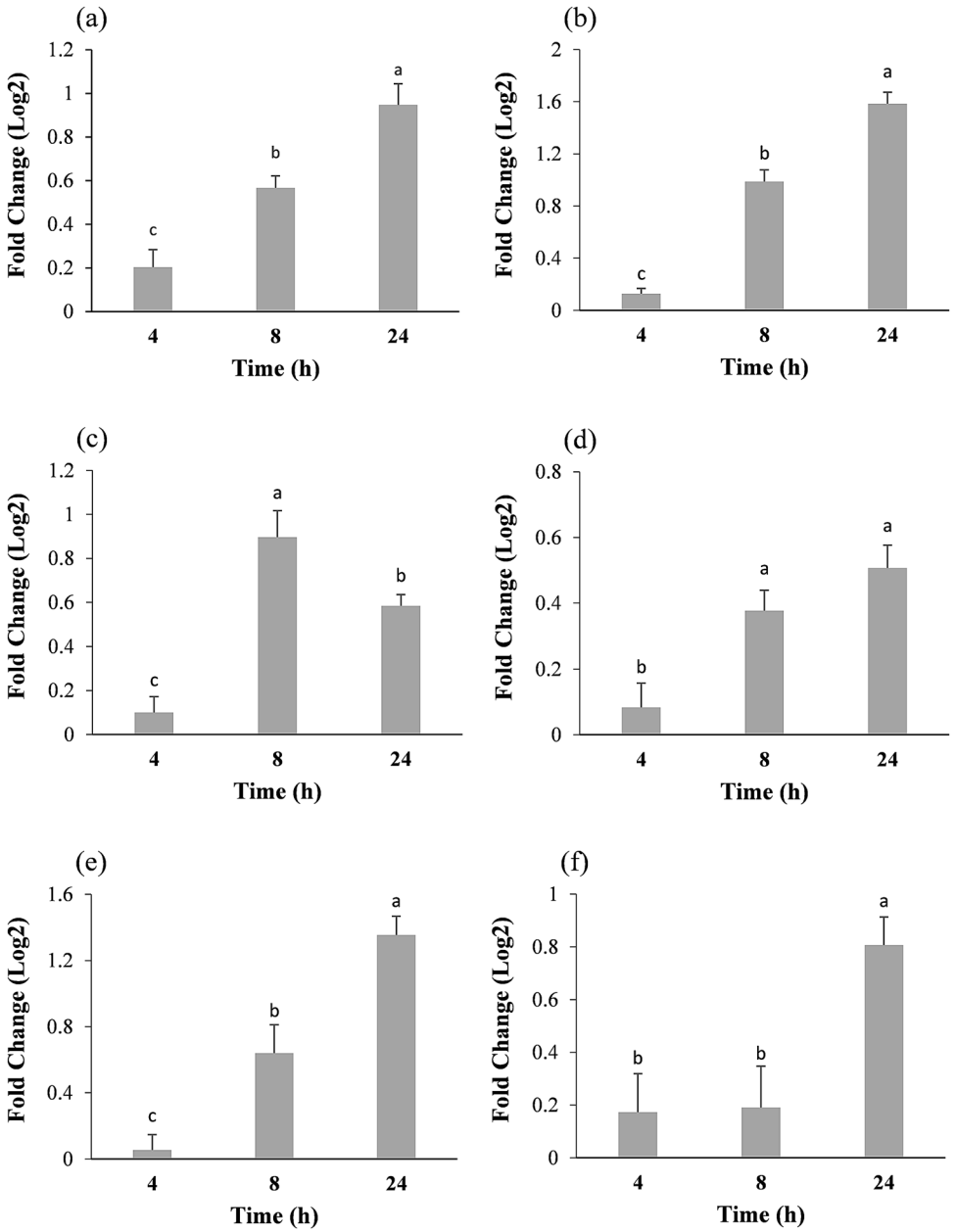

Fig. 4 The expression of genes involved in EPS synthesis under CO_2 intensification culture for 4, 8, and 24 h: **a** *glmU*, **b** *pgmB1*, **c** *cps4E*, **d** *cps4F*, **e** *cps4H*, and **f** *cps4J*. Different letters indicate significant differences between treatments by Tukey' test ($p < 0.05$). Error bars indicate standard error ($n = 3$)

phosphate. *pgmB1* plays an important role in the formation of sugar nucleotides as UDP-glucose (Li et al. 2019). Thus, the overexpression of *pgmB1* may result in increased glucose content in EPS composition. In our study, the expression of *pgmB1* increased when *L. plantarum* VAL6 was exposed to CO_2 intensification culture but decreased in exposure to stresses at pH 3 and at 7 and 10% NaCl. These results were inconsistent with the study of Li et al. (2019) in *L. plantarum* FS5-5, *pgmB* expression increased in response to high salt stress (> 6%) but remained unalterably in response to low salt stress (< 6%) (Li et al. 2019). The difference in the stress method could be the cause of this discrepancy. While Li et al. (2019) supplement NaCl right after inoculation, our study stressed after culture for 24 h.

Among the upregulated genes, *cps4E* and *cps4F* encode for glycosyltransferases which transport sugar nucleotides to a lipid carrier to form repeating units (Zeidan et al. 2017). In *Streptococcus thermophilus* Sfi6, *epsE* (*cps4E*) encodes for a priming galactosyl-1-phosphate transferase and *epsF* (*cps4F*) encodes for a galactosyltransferase which adds the branching α-1,6-galactose (Stingele et al. 1996). In *Lactobacillus johnsonii* FI9785, *epsE* (*cps4E*) encodes for a glycosyltransferase which adds galactose-1-phosphate to the lipid carrier (Dertli et al. 2013). In this study, with the exception of stress at pH 3, other treatment conditions could enhance

Table 2 Effect of environmental challenges on the mRNA expression of genes involved in EPS synthesis

Stress	Expression of gene[a]					
	glmU	*pgmB1*	*cps4E*	*cps4F*	*cps4H*	*cps4J*
Temperature at 42 °C	–	–	↑	↑	–	↑
Temperature at 47 °C	–	–	↑	↑	–	↑
Acid at pH 3	↓	↓	↓	–	↑	–
Alkaline at pH 8	–	–	↑	↑	–	–
NaCl at 4%	–	–	↑	↑	–	↑
NaCl at 7%	↓	↓	↑	↑	↓	↓
NaCl at 10%	↓	↓	↑	↑	↓	↓
CO_2 intensification for 4 h	↑	↑	↑	↑	↑	↑
CO_2 intensification for 8 h	↑	↑	↑	↑	↑	↑
CO_2 intensification for 24 h	↑	↑	↑	↑	↑	↑

[a]↑ Upregulation; ↓ downregulation; – non-alteration

the expression of these two genes. Taken together, it was suggested that EPSs isolated from *L. plantarum* VAL6 under environmental stress may be rich in galactose. In addition, simultaneous changes in expression of *cps4E* and *cps4F* under environmental challenges may elicit the possibility of an association between the two genes.

The *cps4J* encoding for flippases which are responsible for taking repeating units and transporting them across the cytoplasmic membrane (Zeidan et al. 2017) was upregulated under stresses of temperature at 42 and 47 °C, and CO_2 intensification culture. Meanwhile, the overexpression of *cps4H* encoding for enzymes which polymerize repeating units (Zeidan et al. 2017) was detected under stress at pH 3 and CO_2 intensification culture. Thus, the enzymes encoded by *cps4J* and *cps4H* are exclusively involved in the secretion of EPSs, which demonstrated that the increase in

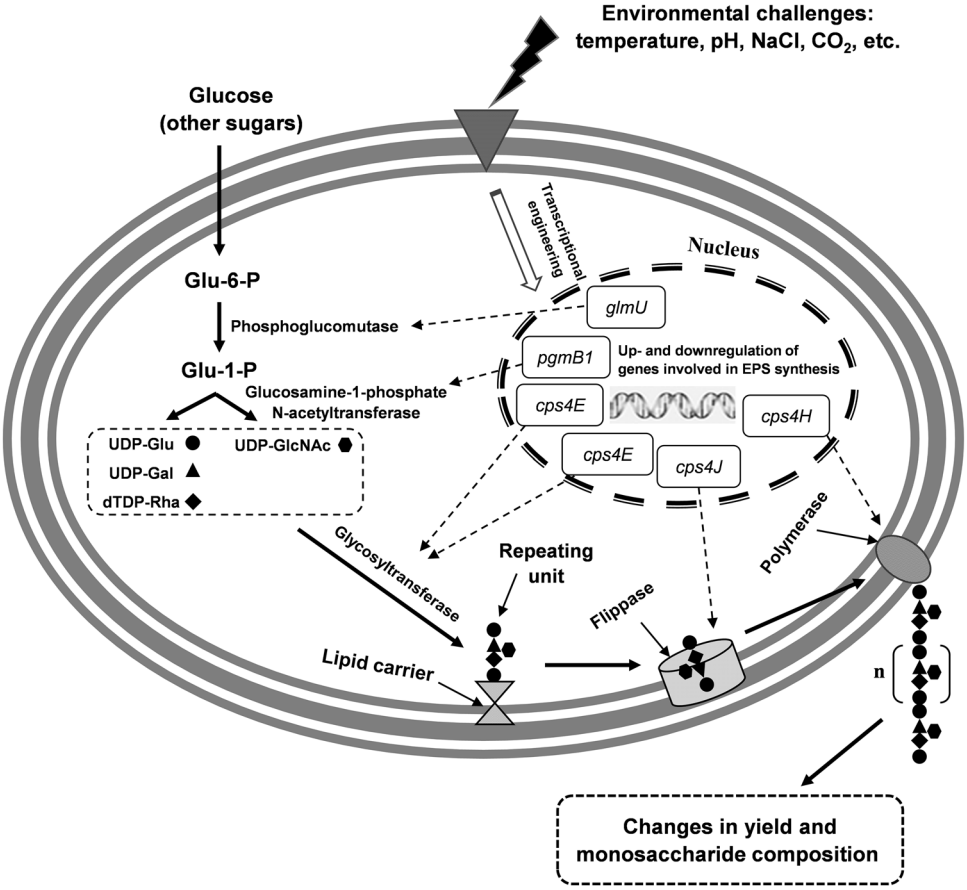

Fig. 5 Expression of genes involved in EPS synthesis in response to environmental stresses. The reactions are catalyzed by enzymes encoded by the genes in italic: Phosphoglucomutase (*galU*), Glucosamine-1-phosphate N-acetyltransferase (*pgmB1*), Glycosyltransferase (*cps4E*, *cps4F*), Flippase (*cps4J*), Polymerase (*cps4H*). Glu-6-P, glucose-6-phosphate; Glu-1-P, glucose-1-phosphate; UDP-Glu, uridine diphosphate glucose; UDP-Gal, uridine diphosphate galactose; dTDP-Rha, thymidine diphosphate rhamnose; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine

the expression levels of two genes do not change the sugar composition of the obtained EPSs, but it was related to the resulting EPS yield.

Conclusion

To survive and adapt to environmental challenges, microorganisms have developed complex mechanisms at physiological and molecular levels (Guan and Liu 2020). Our results revealed that different environmental conditions can alter the expression level of genes involved in EPS synthesis including *glmU*, *pgmB1*, *cps4E*, *cps4F*, *cps4H*, and *cps4J*. Based on achieved results, we propose a profile for the changes in the expression of these genes in *L. plantarum* VAL6 by applying environmental challenges (Table 2). The expression of these genes may lead to changes in the monosaccharide composition of EPSs (Fig. 5). However, it is necessary to further study the expression levels of the respective proteins and the analysis of EPS composition, such as mass spectrometry and other techniques, to identify monosaccharides to better understand the potential relationships between these regulatory systems and environmental stresses.

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Author contributions Conceptualization, T.-S.L, P.-T.N., T.-T.N., and H.-T.N.; methodology, T.-S.L, P.-T.N., and T.-T.N.; investigation, T.-S.L, P.-T.N., S.-H.N.-H., T.-P.N., M.-N.T., and T.-T.N.; data curation, T.-S.L, P.-T.N., T.-U.N.-T., M.-C.N., Q.-K.H., and H.-T.N.; writing—original draft preparation, T.-S.L, P.-T.N., and H.-T.N.; writing—review and editing, T.-S.L, P.-T.N., M.-C.N., Q.-K.H., and H.-T.N.; supervision, M.-C.N., Q.-K.H., and H.-T.N.; funding acquisition, H.-T.N. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest The authors have no financial conflicts of interest to declare.

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