Sains Malaysiana 51(11)(2022): 3621-3633
http://doi.org/10.17576/jsm-2022-5111-09

Expression Analysis using Reverse Transcription Quantitative Real-Time PCR (RT-qPCR) Suggests Different Strategies in *Parageobacillus caldoxylosilyticus* ER4B under Exposure to Cold Shock

(Analisis Pengekspresan menggunakan Transkripsi Berbalik Kuantitatif Masa-Nyata PCR (RT-qPCR) Mencadangkan Strategi Berbeza untuk *Parageobacillus caldoxylosilyticus* ER4B di bawah Pendedahan kepada Renjatan Sejuk)

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Received: 29 April 2021/Accepted: 5 July 2022

ABSTRACT

Microorganisms have acquired both common and unique abilities to withstand cold stress on Earth. Many studies on bacterial cold shock have been conducted, however, the majority of the studies were focused on mesophiles and psychrophiles. To date, limited information is available on the response of thermophilic bacteria to cold stress and therefore, it is not known how thermophilic bacteria would respond to different cold shocks. To address this question, the cold shock responses of a thermophilic *Parageobacillus caldoxylosilyticus* ER4B which has an optimal growth temperature at 64 °C were determined using Real-Time PCR (RT-qPCR). When the bacterium was exposed to mild cold shock at 54 °C, the expressions of gene encoding for pyruvate kinase and acetolactate synthase were significantly upregulated, suggesting that more pyruvate molecules were produced to synthesize branched-chain amino acids that could alter the fatty acid profile on the cell membrane. Accumulation of pyruvate in the bacterium could also help to scavenge cold-induced reactive oxygen species (ROS). Meanwhile, exposing the bacterium to extreme cold shock at 10 °C resulted in significant upregulation of genes encoding for γ-glutamylcyclotransferase, cold shock protein B and competence protein ComEA. An increase in these enzymes expression indicated more extreme measures including apoptosis and transformation were adopted during extreme cold shock.

Keywords: Cold shock; cold stress response; *Parageobacillus caldoxylosilyticus*; reverse transcription quantitative real-time PCR (RT-qPCR); thermophilic bacterium

ABSTRAK

Mikroorganisma telah memperoleh ciri umum dan unik untuk bertahan daripada tekanan suhu sejuk di bumi. Banyak kajian mengenai kejutan sejuk bakteria telah dijalankan, namun kebanyakannya memfokuskan kepada bakteria mesofil dan psikofil. Sehingga kini, terdapat maklumat yang terhad mengenai gerak balas bakteria termofilik terhadap tekanan suhu rendah dan sehubungan itu, bagaimana bakteria termofilik bergerak balas terhadap kejutan sejuk yang berbeza masih belum diketahui. Untuk menjawab persoalan ini, gerak balas kejutan sejuk daripada termofilik *Parageobacillus caldoxylosilyticus* ER4B yang mempunyai suhu pertumbuhan optimum pada suhu 64 °C telah ditentukan melalui PCR Masa-Nyata (RT-qPCR). Apabila bakteria terdedah kepada kejutan sejuk 54 °C, pengekspresan gen yang mengekodkan piruvat kinase dan asetolaktat sintase menunjukkan peningkatan transkrip, mencadangkan bahawa lebih banyak molekul piruvat dihasilkan untuk mensintesis asid amino rantai bercabang yang dapat mengubah profil asid lemak pada membran sel. Pengumpulan piruvat pada bakteria juga dapat membantu menghapuskan spesies oksigen reaktif (ROS) yang disebabkan oleh suhu rendah. Sementara itu, pendedahan bakteria kepada kejutan sejuk yang melampau pada 10 °C mengakibatkan peningkatan pengawalaturan pengekodan gen untuk

γ-glutamil siklotransferase, protein kejutan sejuk B dan protein kompetensi ComEA. Peningkatan pengekspresan enzim tersebut menunjukkan pendekatan yang lebih ekstrem termasuk apoptosis dan transformasi dilakukan semasa kejutan sejuk yang melampau.

Kata kunci: Bakteria termofilik; gerak balas tekanan sejuk; kejutan sejuk; *Parageobacillus caldoxylosilyticus*; PCR masa nyata-kuantitatif transkripsi berbalik

Introduction

Temperature is one of the most important elements impacting bacteria's survival, as bacteria from different thermal groups have a specific range of growth temperatures. For instance, mesophilic bacteria grow well at a moderate temperature ranging from 20 °C to 45 °C (Willey, Sherwood & Woolverton 2008), while psychrophilic and thermophilic bacteria can grow only at 20 °C to 0 °C (Morita 1975) and 55 °C to 105 °C (Brock 1986), respectively. However, due to the ever-changing temperature on Earth, these bacteria have developed some unique thermal stress responses to adapt to changes in their growth temperature (Hecker & Völker 2001; Lambros et al. 2021).

While the bacterial response to heat shock has received a lot of attention, research on how bacteria adapt to cold shock is sparse. Cold shock is defined as the period between a temperature drop of more than 10 °C and the organism's return to an exponential growth phase (Smartt 2014). Bacterial cold acclimation and cold adaptation are two common strategies for dealing with cold shock (Morgan-Kiss et al. 2006). Cold acclimation, for example, would allow bacteria to temporarily alter their physical structures or behavior in such a short period that some of them would survive the sudden drop in the surrounding temperature. Cold adaptation then occurs to permanently alter bacterial gene expression patterns, allowing the bacteria to thrive at lower temperatures indefinitely (Bersolin et al. 2006). Currently, various bacterial cold acclimation and adaptation strategies in the event of cold shock have been determined, and it appears that different bacteria have adopted different strategies in response to a different cold shock event. As well-established as it may appear, most studies have primarily focused on psychrophilic and mesophilic bacteria, so our understanding of cold adaptation in thermophilic bacteria remains very limited at the moment.

Parageobacillus caldoxylosilyticus is a Gram-positive, facultative thermophilic bacterium belonging to phylum Firmicutes and the family Bacillaceae. The genus is a unique one, distinct from the *Geobacillus* genus (Aliyu et al. 2016). Members of the *Parageobacillus* genus have Gram-positive cell walls, are rod-shaped, and, most importantly, are thermophilic. In nature, *Parageobacillus* can be found in a variety of mesophilic and thermophilic habitats, such as temperate soils, sea sediment, compost, and hot springs (Lebre et al. 2018; Zeigler 2014). Bacterial species in this genus grow optimally at temperatures ranging from 45 °C to 75 °C, with some able to grow at temperatures as low as 35 °C or as high as 80 °C (Zeigler 2014).

The *P. caldoxylosilyticus* genome sequence has been previously reported and published in proper by Berendsen et al. (2016), whereas the complete genome of this species was established recently using the same type strain ER4B used in this study (Ching et al. 2021). Although the genome data shows some information about the genes and their functions, it is unclear how those genes are regulated when the cells are exposed to lower, less optimal temperatures. Hence, the objective of this study was to determine the gene expression profile of strain ER4B at 54 °C (mild) and 10 °C (extreme) cold shock using reverse transcription-quantitative real-time PCR (RT-qPCR). Based on previous RNA-Seq data (unpublished results), 19 genes were selected for the RT-qPCR gene expression analysis, where 14 of these genes, namely *PK, GPDH, PC, ID, PDE1A, PDE1B, MD, LLD, ALS, IL, PSS, ACAS, SCoA* and *ACoA* (Table 1), were selected to investigate the contribution of pyruvate molecules in cold shock response. Currently, there are not many studies that relate pyruvate to cold adaptation. There is only one study that speculated and reported the effect of endogenous pyruvate concentration on the fatty acid level and subsequently the growth of pathogenic bacterium *Vibrio parahaemolyticu* in cold conditions (Xie et al. 2019).

The other genes, *QO1*, *PutM*, *CspB*, *GGCT,* and *ComEA*, were also chosen for RT-qPCR gene expression investigation to determine their roles in the bacterium's resistance to mild and severe cold shock. Several

cold acclimations, as well as cold adaption strategies adopted by strain ER4B, were proposed in this study. The expression profiles in bacterial cells identified from the RT-qPCR analyses can provide information on putative molecular mechanisms that happened in strain ER4B. The findings of this study contributed to current and existing knowledge about cold shock reactions in thermophiles, not just *P. caldoxylosilyticus*. The proposed cold stress strategies, on the other hand, can be used as benchmarks for comparisons with bacteria from various thermal groups.

Materials and Methods

Source of strain and culture conditions for cold shock

P. caldoxylosilyticus ER4B was isolated from an oil palm empty fruit bunch compost, and the bacterium's identity was determined by aligning its entire genome sequence to bacterial genomes in the Gene Bank (accession number CP040553-CP040554). Strain ER4B was previously determined to have an optimal growth temperature of 64 °C. Strain ER4B was grown in LB broth media in nine conical flasks at their optimal growth temperature, which is 64 °C until it reached the mid-log phase. Subsequently, three of the cultures were exposed to 64 °C, 54 °C, and 10 °C (three replicates each) for 3 h. After 3 h of incubation at various temperatures, 1 volume of cold methanol was then added and mixed well in each replicate to ensure the bacterial cells were frozen for exactly 3 h after the cold shock, and there will be no further change in gene expression (Alles et al. 2017). Next, the mixtures were centrifuged, and the cell pellets obtained were kept at -80 °C before RNA extraction.

RNA extraction

The total RNA was extracted using a phenol-chloroform-based procedure designed to extract total nucleic acid from soil samples (Angel 2012). To produce high-quality RNA samples, genomic DNA contamination was removed using the Qiagen DNase Max® kit, followed by RNA purification using the Qiagen RNeasy Mini Kit. The purified RNA was then evaluated using the Agilent RNA 6000 Nano kit with the Agilent 2100 Bioanalyzer system.

Primer design

Specific primers for RT-qPCR were designed based on the annotated whole-genome sequences (GenBank accession number: CP040553-CP040554). In this study, 20 pairs of specific primers were designed to target reference genes and genes of interest using the Primer3 v.0.4.0 software. The primers were each 20-mers long, and the amplicons ranged in size from 150 bp to 210 bp (Table 1).

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR was conducted using SensiFAST SYBR No-ROX One-Step kit by Bioline. A total of 0.5 ng of total RNA (1 µL) was added to 20 µL master mix containing 10 µL 2× SensiFAST Probe No-ROX One-Step Mix, 1 µL of each primer, 0.4 µL of RiboSafe RNase Inhibitor, 0.2 µL Reverse transcriptase, and 6.4 µL of Diethyl Pyrocarbonate (DEPC) water. The PCR was performed in triplicate for each biological replicate on Bio-Rad CFX96 Touch Real-Time PCR Detection System. A negative control without reverse transcriptase was included in each run. The PCR conditions were 45 °C for 10 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 60 °C (depending on average TM of primers) for 10 s, and 72 °C for 5 s. Melting curves for amplicons were then measured by raising the temperature by 0.5 °C from 55 °C to 95 °C after each run.

RT-qPCR data analysis

The results obtained from RT-qPCR were analyzed using a relative quantification of Livak's method (Livak & Schmittgen 2001). The cycle threshold (CT) values of each gene of interest were normalized against CT values of reference gene *rpoB* to correct the sample-to-sample variation, using 64 °C as calibrator condition. One-way analysis of variance (ANOVA) was performed to evaluate the statistical significance of the expression of each gene under both mild and extreme cold shock. Statistically significant genes then proceeded with posthoc Tukey HSD test (Tukey 1949) using the web-based calculator at (https://astatsa.com/OneWay_Anova_ with_TukeyHSD/) to locate the significant differences ($n=3$, $P<0.05$). Only genes with log2FC above 1.5 or below -1.5 and P value less than 0.05 were considered as significantly up-or down-regulated.

Results and Discussion

The gel electropherogram with two intact (23S and 16S rRNA) bands in each sample (Figure 1), as well as the mass ratio and the RNA Integrity Number (RIN) (Table 2) shows that the RNA samples were sufficiently pure and intact to be used in RT-qPCR for gene expression analysis.

TABLE 1. Details of primer sequences and other information for 20 genes for RT-qPCR

Gene Identifier	Description	Forward primer (5′-3′)	Tm	Reverse primer (5′-3′)	Tm	Amplicon length (bp)
rpoB*	RNA polymerase subunit β	GGTTCGCCGTCTCATTGAAG	56.20	AGCTCACCTTTGTCCATCGA	56.10	186
PK	Pyruvate kinase	ACAATGGGAAAAGAGATGCC	55.98	ACACATCACTTTGCTCTGAC	55.99	194
GPDH	Aerobic glycerol-3-phosphate dehydrogenase	CGTTAAAGCAAGCGATTTAT	55.04	GGACGGGAACATATAGTGAA	55.00	182
PC	pyruvate carboxylase	AGTTTCTTGTATCCGGCAAC	56.05	CCCGTTGATGCGAATATCTT	56.00	188
ID	Isocitrate dehydrogenase	CTTTCGCCAGTTTCCTACAA	55.98	GCTTGAGATTGCCTGCTTTA	56.40	169
PDE1A	Pyruvate dehydrogenase E1 component subunit α	CTTCCACGGCAATCAAATTC	55.62	TCATAGAAATCCCCTTGCGA	55.31	174
PDE1B	Pyruvate dehydrogenase E1 component subunit β	ATACGCTGATTGTCATTCCG	56.03	CCGAGCCTTTATTGGACATT	55.80	195
MD	Malate dehydrogease	GGTGCCTCTTGTTCGTTATT	56.05	ATGATCGCTTCCACCATTTC	56.18	186
LLD	L-lactate dehydrogenase	CTTGGAAATTCAGCGGCTTA	56.41	TACTTCCAATTTCTGCACGG	56.05	186
ALS	Acetolactate synthase	GAATGATGCTGGCAATTTCG	56.07	ATCGTCATCATAAAACCGCC	56.29	186
IL	Isocitrate lyase	AATGAAGGCGATGATTGAGG	55.88	GATTAACACGGTCGGTACAC	56.29	178
PSS	Pyruvate synthase	AATCCGAAGCATTATACCGC	56.08	CTTTAATTCCTTTTGCCCGC	55.89	195
ACAS	Acetyl-coenzyme A synthetase	GAGCTGGGGATGAAATTGTT	55.98	GAACCTGGCTTGATTTCCAT	55.98	199
SCoA	Acetyl-CoA hydrolase	GCAGAATTAGTGCGTTCAGA	56.15	GGTTGTGCTGTGTTCAATTC	55.77	167
ACoA	Acetyl-CoA acetyltransferase	ACAAGACAAATGGGCGTATC	56.12	GACGTATCTTTTCTTGGCGA	55.90	153
CspB	Cold shock protein B	CACTTCACAGCGATCCAAGG	56.00	GGTCCACGGTTTCCTTGAAC	56.10	150
GGCT	γ-glutamylcyclotransferase	CCGTGTGTTTGTGTATGGAA	56.28	TACTTCGCCTTCTTCTTCGA	56.32	153
ComEA	Competence protein ComEA	AGGATTTACAAGGGAAGCAG	55.02	GCTGGGATATTTCTTGCTCT	55.19	182
QO1	Quinol oxidase	TAATGTTTGCTTCTTCCCGA	54.97	CAAAGCACGATAAAGCCAAT	54.94	150
PutM	Putative manganese catalase	ACAGAGGAATTGGCTCATTT	54.90	GAATGTATGTCGCCGTAAAC	54.93	172

*reference gene

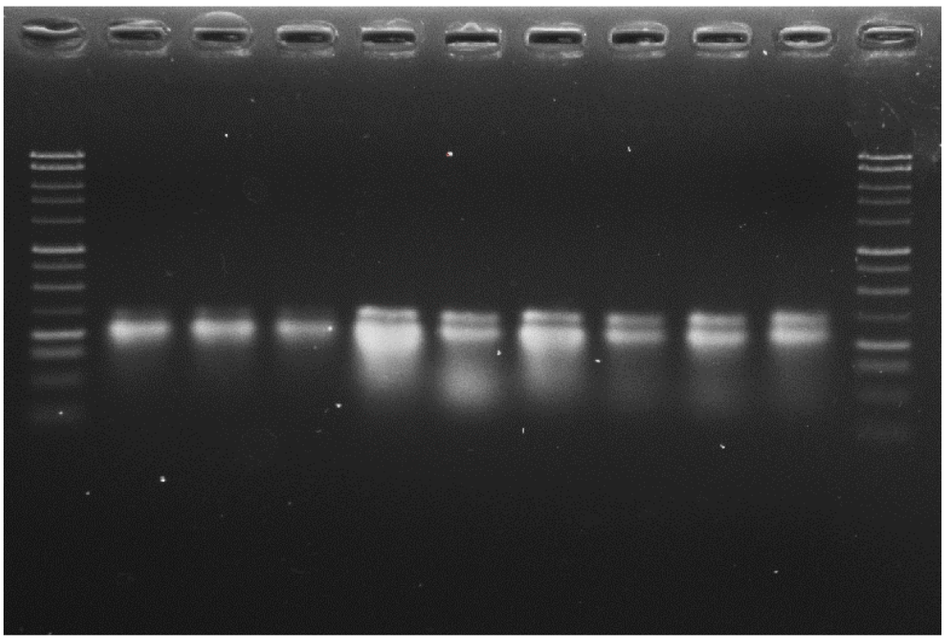

FIGURE 1. Purified *P. caldoxylosulyticus* ER4B total RNA. The 23S rRNA and 16S rRNA is upper and lower bands, respectively. Lanes 1-3 are replicates from the 64 °C control group; lanes 4-6 are replicates from the 54 °C treatment group (moderate cold shock), and lanes 7-9 are replicates from the 10 °C treatment group (extreme cold shock)

TABLE 2. RNA quantitation results generated from Agilent 2100 Bioanalyzer System

Lane	Sample name	RNA concentration (ng/μL)	rRNA mass ratio (23S/16S)	RNA integrity number (RIN)
1	64 °C R1	300	1.9:1	9.8
2	64 °C R2	339	2.0:1	9.6
3	64 °C R3	343	2.0:1	9.5
4	54 °C R1	291	1.7:1	9.5
5	54 °C R2	289	1.9:1	9.4
6	54 °C R3	370	1.8:1	9.4
7	10 °C R1	537	1.8:1	9.2
8	10°C R2	406	1.8:1	9.1
9	10°C R3	370	1.9:1	9.3

As depicted in Figure 2, when strain ER4B was exposed to cold shock at 54 °C, a significant upregulation was observed on *PK* (Log2FC=4.3), *PDE1A* (Log2FC=1.9), *ALS* (Log2FC=5.9), *ACoA* (Log2FC=1.6), *QO1* (Log2FC=4.4) and *PutM* (Log2FC = 6.0); while *GPDH* (Log2FC=-3.8), *IL* (Log2FC=-5.7), *LLD* (Log2FC=-3.0), *PC* (Log2FC=-1.9) and *PS* (Log2FC=-2.7) were significantly downregulated. The remaining genes were considered non differentially expressed as the Log2FC values are within 1.5 and -1.5. As for 10 °C cold shock, *PK* (Log2FC=1.9), *QO1* (Log2FC=3.9), *PutM* (Log2FC=2.0), CspB (Log2FC=2.3), *GGCT* (Log2FC=4.26) and ComEA (Log2FC=1.6) showed significant upregulation; while downregulation occurred on *MD* (Log2FC=-2.9), *LLD* (Log2FC=-1.8), *PS* (Log2FC=-3.2), *ACAS* (Log2FC=-2.6), *SCoA* (Log2FC=-5.2) and *ACoA* (Log2FC=-2.4).

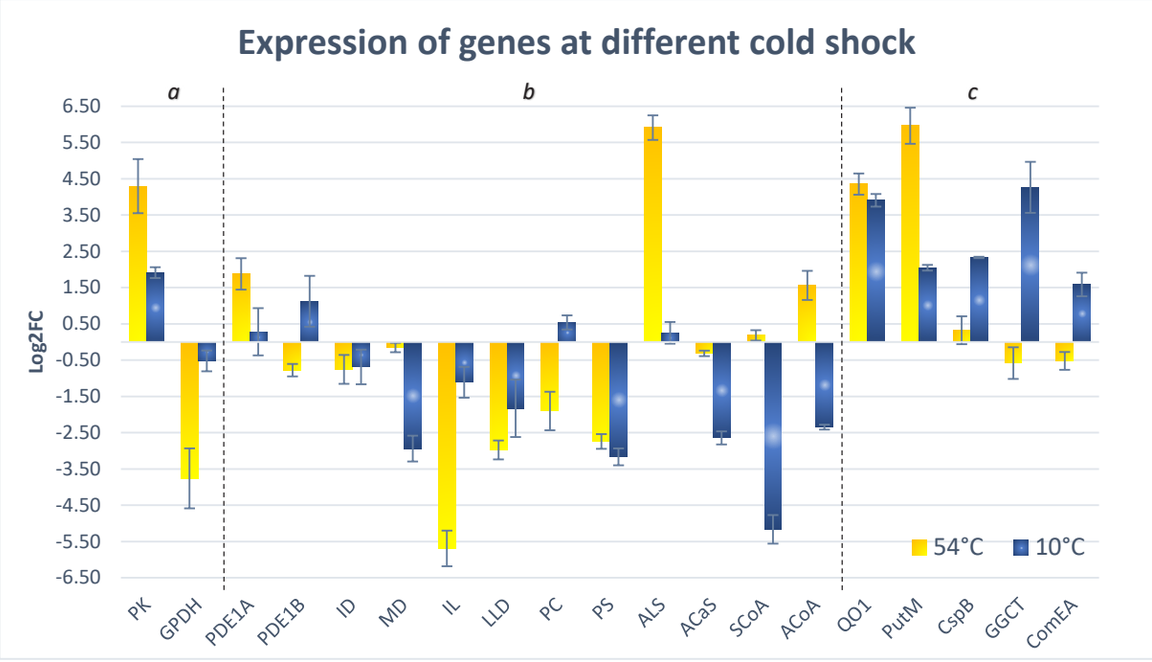

FIGURE 2. Gene expression in response to cold shock at 54 °C and 10 °C. (a) and (b) are related to pyruvate metabolism pathways, where (a) are involved in pyruvate production in glycolysis, and (b) include various pathways or reactions that consume the pyruvate molecules, and (c) the individual genes that have a direct impact on *P. caldoxylosilyticus* ER4B during cold shock

When the cold shock was induced at 54 °C, two genes involved in the glycolysis pathway, which encoded Pyruvate Kinase (*PK*) and Glycerol-3-Phosphate Dehydrogenase (*GPDH*) were significantly upregulated and downregulated, respectively. Pyruvate Kinase (PK) is a rate-limiting enzyme involved in the last step of glycolysis, where the phosphate group of phosphoenolpyruvates was transferred away, generating one molecule of pyruvate (Schormann et al. 2019). Upregulation of the *PK* gene indicated that pyruvate kinase was being produced more than usual (Gray, Tompkins & Taylor 2014; Zhang, St Leger & Fang 2017). As for GPDH, it catalyzes the conversion of dihydroxyacetone phosphate, one of the intermediate products in glycolysis, into glycerol 3-phosphate (Yeh, Chinte & Du 2008). This enzyme uses dihydroxyacetone phosphate as substrate in its reaction, so instead of converting back into glyceraldehyde 3-phosphate which is crucial for pyruvate production, GPDH converts dihydroxyacetone phosphate into glycerol 3-phosphate which is used to

produce glycerol. Downregulation of this enzyme showed that dihydroxyacetone phosphate was most likely to be converted back into glyceraldehyde 3-phosphate via enzyme triosephosphate isomerase (Kulkarni et al. 2017), increasing the substrate for pyruvate synthesis.

The upregulation of *PK* and downregulation of *GPDH* lead to the possibility of increased production of pyruvate when strain ER4B was exposed to mild cold shock. Thus, it is important to investigate the role of pyruvate in bacteria cold stress response. Pyruvate is a key product of several important metabolic pathways (Gray, Tompkins & Taylor 2014) in living organisms; however, its major function to supply energy for living organisms through aerobic respiration in the presence of oxygen is more well known as compared to other functions. It is one of the key products involved in the transition reaction that connects glycolysis to the Tricarboxylic Acid (TCA) Cycle (Su et al. 2018).

In general, an increase in pyruvate should have triggered an increase in the substrate for the TCA cycle, and eventually increase the activity of the TCA cycle, leading to the production of energy in the form of guanosine triphosphate (GTP), reduced nicotinamide adenine dinucleotide (NADH), and reduced flavin adenine dinucleotide (FADH2). However, in this study, there was no clear evidence of pyruvate's conversion into acetyl coenzyme A (acetyl CoA). Pyruvate Dehydrogenase E1 subunit A (PDE1A) and Pyruvate Dehydrogenase E1 subunit B (PDE1B) encoding for 2 subunits of rate-limiting pyruvate dehydrogenase complex converting pyruvate to acetyl CoA (Su et al. 2018). From the results, it can be seen that *PDE1B* did not show significant upregulation (Figure 2) and became the limiting factor of producing functional enzymes. The increase in *PDE1A* is most likely to inhibit the Warburg effect in the cells (Luo et al. 2019). Since the primary source of acetyl CoA is from decarboxylation of pyruvate (Krivoruchko et al. 2014), and there was no evidence of increase in the production of pyruvate dehydrogenase E1 component, it is suggested that there was probably no increase in the substrate molecule acetyl CoA for TCA cycle, hence no significant increase in the TCA cycle activity. Besides, genes encoding two rate-limiting enzymes in the TCA cycle; Isocitrate Dehydrogenase (*ID*) that converts isocitrate to α-ketoglutarate via oxidative decarboxylation, and Malate Dehydrogenase (*MD*) that converts malate to oxaloacetate via oxidation (Zhang, St Leger & Fang 2017) were not differentially expressed as well. This further supported that the TCA cycle activity was not increased. Moreover, the gene of key enzyme Isocitrate Lyase (*IL*) which catalyzes the cleavage of isocitrate into succinate and glyoxylate as the first step of glyoxylate cycle (a variant of TCA cycle) (Kornberg & Krebs 1957; Zhang & Bryant 2015), was significantly downregulated, and this further suggested that demand for pyruvate in the TCA cycle did not increase.

Apart from the TCA cycle, pyruvate is also reported to be important for anaerobic fermentation. Many bacteria can carry out lactic acid fermentation with the enzyme Lactate Dehydrogenase (LLD), as it can catalyze the interconversion of pyruvate to lactate (Suzuki et al. 2019). However, from Figure 2, it can be seen that gene encoding for LLD in strain ER4B was significantly downregulated, and this greatly reduced the possibility of pyruvate being used in the process of fermentation during cold shock at 54 °C. Next, Figure 2 also showed the downregulation of Pyruvate Carboxylase (PC) and Pyruvate Synthase (PSS). PC catalyzes the carboxylation of pyruvate into oxaloacetate (Kiesel et al. 2021), while PSS helps to speed up the conversion of pyruvate into acetyl-CoA (Katsyv et al. 2021). These two enzymes catalyze the reactions that require pyruvate as substrate, and their downregulation highly indicated that pyruvate was not being consumed in the process of pyruvate decarboxylation and ferredoxin oxidoreduction.

Interestingly, unlike other pyruvate consumption genes, the gene encoding for Acetolactate Synthase (*ALS*) showed a very high upregulation. ALS is known to catalyze the condensation of two pyruvate molecules into acetolactate and carbon dioxide, and this conversion is the first step in the biosynthesis of branched-chain amino acids (BCAAs) in many microorganisms and plants (Dezfulian et al. 2017; Franco & Blanchard 2017). The three essential proteinogenic BCAAs (leucine, isoleucine and valine) differ from other essential amino acids in that the aliphatic side chain was formed in a branched structure instead of a straight-chain (Franco & Blanchard 2017). There are a few metabolic and physiological roles filled by BCAA, including regulation of protein synthesis and turnover, energy production and stress signaling (Kimball & Jefferson 2001; Monirujjaman & Ferdouse 2014; Sonenshein 2005), however, BCAAs are particularly important in the biosynthesis of branched-chain fatty acids (BCFAs) in bacteria (Beck 2005; Neinast, Murashige & Arany 2019).

Upregulation of *ALS* during cold shock at 54 °C would lead to the production of BCAAs, and eventually to BCFA. Non-differential expression of the gene encoding

for Acetyl-coenzyme A Synthetase (*ACAS*) and Acetyl-CoA Hydrolase (*SCoA*), as well as the upregulation of Acetyl-CoA Acetyltransferase (*ACoA*), showed an overall increase in coenzyme A, which is known for playing a key role in synthesis and oxidation of fatty acids (Janßen & Steinbüchel 2014; Sibon & Strauss 2016), and this provides support to pyruvate's contribution towards the biosynthesis of BCFAs during cold shock. A significant increase in BCFAs, especially the unsaturated anteiso BCFAs, was observed in bacteria at lower temperatures as reported in other previous studies (Bajerski, Wagner & Mangelsdorf 2017; Klein, Weber & Marahiel 1999; Paton et al. 1978). Besides, BCFAs were also found to be the dominant compound among the cell membrane fatty acid in psychrophiles, stressing the importance of BCFAs to bacterial growth at low temperatures (Hassan et al. 2020).

At low temperatures, phospholipids in the cell membrane have lower kinetic energy and they would get clustered together more closely, causing a decrease in the overall fluidity of the membrane. This would decrease the membrane permeability and potentially restrict the entry of important molecules required for growth into the cells (Los & Murata 2004). It was suggested that BCFAs which are shorter in chain length, have lower melting temperature as compared to their straight-chained counterparts as a result of branching (Zhu et al. 2005), thus, increasing of BCFAs in membrane lipid composition would increase the membrane fluidity at low temperature (Mostofian et al. 2019). Therefore, from the expression analyses of the above-mentioned genes, it is speculated that increasing pyruvate is being channeled into the biosynthesis of BCFA for alteration of cell membrane composition.

On the other hand, when the extreme cold shock was induced at 10 °C, the production of pyruvate is expected to increase, but not as much as that at 54 °C, as *PK* has lower upregulation while *GPDH* was not differentially expressed (Figure 2). Similarly, despite having high likelihood of increasing pyruvate molecules due to upregulation of *PK*, there was no significant increase in activity of TCA and glyoxylate cycles as shown by non-differential expression of *PDE1A, PDE1B*, *IL*, and downregulation of *MD*. Besides, there was no significant upregulation on *LLD, PC,* and *PSS*, suggesting that the expected increase in pyruvate was not meant for the process of fermentation, decarboxylation and ferredoxin oxidoreduction (Figure 2).

However, unlike mild temperature, cold shock at 10 °C did not trigger the upregulation of *ALS*. Not to mention, both *ACAS* and *SCoA* were significantly downregulated, indicating coenzyme A was greatly reduced. These suggested that there was no alteration of membrane lipid composition by BCFAs at this temperature. It is speculated that such a huge drop in growth temperature would cause an extreme cold shock, and it is expected to cause other instantaneous harms which are more serious than the harm caused by membrane fluidity. Membrane lipid alteration most probably could not contribute much to the survival of the bacterium at such extreme cold shock, so the bacterium must have adopted different approaches with the priority to cope with other direct cellular damage caused by cold shock.

Since pyruvate molecules were not involved in any of the pyruvate consumption processes including fermentation, decarboxylation, oxidoreduction, and BCFA biosynthesis as mentioned above, the excess pyruvate was likely being used against reactive oxygen species (ROS). Production of ROS increased at low temperatures due to an increase in oxygen solubility (Chattopadhyay et al. 2011; Tribelli & López 2018), and these ROS would cause several types of intracellular damage, including DNA, lipids and proteins. Therefore, defending against ROS showed to be a major part of cold acclimation in living organisms, including bacteria, at low temperatures (Kloska et al. 2020; Sun et al. 2016; Xie et al. 2019). A similar observation was also reported where pyruvate accumulation occurred when fungus *Metarhizium robertsii* were exposed to thermal shock, postulating the role of pyruvate as being a ROS scavenger (Zhang, St Leger & Fang 2017).

Despite their effectiveness in scavenging cold-induced ROS, pyruvates are not the primary ROS scavenger in most organisms. This can be seen as the gene encoding for Putative Manganese Catalase (*PutM*) was significantly upregulated in both mild and extreme cold shocks. PutM is a non-heme enzyme that decomposes hydrogen peroxide into water and oxygen (Shaeer, Aslam & Rashid 2019). It is known that endogenous hydrogen peroxide (H_2O_2), as one of the main ROS, will increase during cold stress (Fedurayew et al. 2018), and eventually lead to DNA damage and even cell death (Hong et al. 2019). The increase in the expression of *PutM* under cold stress exposure was also reported in *Escherichia coli* and *Synecho*cystis sp. (Fedurayev et al. 2018; Smirnova, Zakirova & Oktyabrskii 2001). Upregulation of *PutM* at both temperatures showed that eliminating H_2O_2 is very important in protecting the cell from oxidative damage caused by ROS. Figure 2 showed that upregulation of *PutM* at 54 °C was almost three times higher than that at 10 °C. It is very likely that the decrease in overall metabolic activities

at 10 °C (Mocali et al. 2017) has limited the production of *PutM*. To keep pace with the threat from ROS, the excess pyruvate may joined in to protect the bacterial cells against oxidative stress.

Besides *PutM*, the gene encoding for Quinol Oxidase (*QO1*) was also found to be upregulated at both mild and extreme cold shock. QO1 is part of the terminal cytochrome bo complex in the bacterial electron transport chain, and it catalyzes the reduction of oxygen into water (Abramson et al. 2000). The upregulation of *QO1* at 54 °C and 10 °C could mean that there was an increase in energy production, however, the results from the above pyruvate metabolism suggested there was no significant increase in the TCA cycle. This showed that other than glycolysis and TCA cycle, strain ER4B had also obtained energy via other secondary pathways, as increasing cellular ATP and ADP are important in protein synthesis and modification, as well as offsetting the kinetic temperature effect (Suyal et al. 2017).

Lower upregulation of *PK* and *PutM*, in addition to the non-differential expression of *ALS*, indicated that strain ER4B did not rely much on either scavenging cold-induced ROS or alteration of membrane composition when exposed to extreme cold shock at 10 °C. In fact, from Figure 2(c), it can be seen that gene encoding for Cold Shock Protein B (C*spB*), γ-glutamylcyclotransferase (*GGCT*), and competence protein ComEA (*ComEA*) were upregulated only at 10 °C but not at 54 °C. CspB is a type of cold shock protein that is commonly found in *Parageobacillus, Bacillus* and a wide range of bacteria species such as *Polaribacter, Caulobacter, Salmonella,* and *Escherichia coli* (Craig et al. 1998; Etchegaray & Inouye 1999; Jung et al. 2018; Mazzon et al. 2012). Like other cold shock proteins, CspB is thought to serve as chaperones that prevent the formation of mRNA secondary structure at low temperature and thus, maintain the process of translation in cells (Keto-Timonen et al. 2016). It is also reported to play an important role in bacteria other than stress response, as some of them are non-cold inducible and they were found to be constantly produced at a certain level for bacterial viability during normal growth (Keto-Timonen et al. 2016). The pre-existing cold shock proteins, including CspB, were thought to be sufficient in maintaining the efficiency of transcription and translation in strain ER4B at 54 °C, however, the previous study reported that some of these cold shock proteins produced at optimum growth temperature are extremely unstable with a half-life as short as 12 seconds during temperature downshift as shown in *E. coli* when exposed to cold shock (Jin, Jeong & Kim 2014; Mitta, Fang & Inouye 1997). Therefore, extreme cold shock at 10 °C might have triggered the production of new CspB with higher stability to replace the other unstable CSPs to maintain the primary structure of bacterial RNAs at lower temperatures.

GGCT is one of the genes that was upregulated at 10 °C instead of 54 °C cold shocks. Similar to eukaryotic GGCT, bacterial GGCT is highly conserved, and it helps catalyze the degradation of GSH by cleaving the molecule into 5-oxoproline and Cys-Gly dipeptide (Kaur et al. 2017). Degradation of GGCT is important in eukaryotes as this is part of the γ-glutamyl cycle which is essential to maintain adequate intracellular glutathione (GSH) level (Orlowski, Richman & Meister 1969). However, unlike eukaryotes, most of the Gram-positive bacteria could not synthesize GSH, so they can only obtain and consume GSH from the growth medium (Pophaly et al. 2012; Smirnova & Oktyabrsky 2005). In this study, glutathione in the LB growth media comes from the yeast extract, and the concentration is very limited. Hence, intracellular glutathione which was degraded by the upregulation of *GGCT* could not be replenished by the limited extracellular glutathione, leading to intracellular glutathione depletion. Depletion of glutathione has been determined to be an important hallmark of apoptosis in response to different stimuli and stresses (Circu & Aw 2008; Franco et al. 2014), so it is highly possible that when cold shock was induced at 10 °C, apoptosis was triggered in the bacteria cells. Apoptosis or programmed cell death (PCD) has been reported in parts of the bacterial cells in the population during thermal stress (Cellini et al. 2001; Nagamalleswari et al. 2017) and it is deduced that the dying cellular debris or components would act as signaling molecules (Fogarty & Bergmann 2015) to alert the surviving cells in the community about the cold stress event through quorum sensing.

In addition, increasing production of 5-oxoproline (also known as pyroglutamate or pyroglutamic acid) entailed from upregulation of *GGCT* is reported to be deleterious especially for prokaryotes (Niehaus et al. 2017). Besides causing several problems such as metabolic acidosis, neurological disorders and breakdown of antioxidant defenses in mammals, 5-oxoproline is also found to effectively inhibit the growth of prokaryotes, particularly thermophiles and acidophiles (Park, Ryu & Lee 2003; Park, Lee & Ryu 2001). So, the presence of 5-oxoproline in large amounts would eventually lead to serious growth inhibition in strain ER4B during extreme cold shock. Both apoptosis and growth inhibition caused by upregulation of *GGCT*

is expected to be a mechanism for long-time survival of the rest of the population (Allocati et al. 2015). In contrast, *GGCT* was not differentially expressed at 54 °C and this further exhibited GGCT-induced apoptosis and growth inhibition as an extreme measure taken by strain ER4B in response only to the cold shock at 10 °C.

At the same time, *ComEA* was also observed to be upregulated only at 10 °C but not at 54 °C. ComEA is a competent protein that is essential for DNA uptake and bacterial transformation (Provvedi & Dubnau 1999). Recent studies showed that ComEA was localized into the periplasm of bacteria, binding to exogenous DNA from the outer membrane and transporting them across the inner membrane (Taton et al. 2020). Unlike all the other genes that contributed to cold acclimation of strain ER4B, upregulation of *ComEA* showed that strain ER4B was trying to scavenge useful exogenous DNA that might help the bacterium to achieve cold adaptation through transformation. It is hypothesized that at 54 °C, cold acclimation strategies could help strain ER4B to grow and survive in response to the relatively minor temperature downshift, however, it was very hard for strain ER4B to cope with extreme cold shock at 10 °C through temporary cold acclimation alone, so it might be a better idea to adopt cold adaptation traits from the surroundings for long term survival.

Conclusion

Gene expression analysis using RT-qPCR showed that strain ER4B adopted different strategies in response to both mild and extreme cold shock. When exposed to mild cold shock at 54 °C, the production of pyruvate molecules was speculated to increase significantly. While the expression of other genes involved in the consumption of pyruvate remained unchanged or downregulated, *ALS* showed high upregulation. This suggested that pyruvate produced was used in BCFA synthesis, in which the BCFA is needed for alteration of the phospholipid membrane composition during mild cold shock. Besides, the increasing pyruvate molecule, together with the increasing manganese catalase, is expected to act as ROS scavengers to remove harmful H_2O_2 that was produced during 54 °C cold shocks. On the other hand, the result of this study proposed that extreme cold shock at 10 °C has triggered several extreme measures, including apoptosis, growth inhibition, as well as transformation. These strategies are expected to be very crucial for the survival of strain ER4B under cold stress in a long run.

Acknowledgements

The funding support from the Ministry of Science, Technology, and Innovation (MOSTI), Malaysia, under the Antarctica Flagship Programme (Sub-Project 1: Grant no. FP1213E036) is gratefully acknowledged.

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