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ORIGINAL PAPER

Transcriptomic responses of haloalkalitolerant bacterium *Egicoccus halophilus* EGI 80432^T to highly alkaline stress

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Abstract

The haloalkalitolerant bacterium *Egicoccus halophilus* EGI 80432^T exhibits high adaptability to saline–alkaline environment. The salinity adaptation mechanism of *E. halophilus* EGI 80432^T was fully understood based on transcriptome analyses and physiological responses; however, the alkaline response mechanism has not yet been investigated. Here, we investigated the alkaline response mechanism of *E. halophilus* EGI 80432^T by a transcriptomic comparison. In this study, the genes involved in the glycolysis, TCA cycle, starch, and trehalose metabolism for energy production and storage, were up-regulated under highly alkaline condition. Furthermore, genes responsible for the production of acidic and neutral metabolites, i.e., acetate, pyruvate, formate, glutamate, threonine, and ectoine, showed increased expression under highly alkaline condition, compared with the control pH condition. In contrast, the opposite results were observed in proton capture or retention gene expression profiles, i.e., cation/proton antiporters and ATP synthases. The above results revealed that *E. halophilus* EGI 80432^T likely tended to adopt an "acidic metabolites production" strategy in response to a highly alkaline condition. These findings would pave the way for further studies in the saline–alkaline adaptation mechanisms of *E. halophilus* EGI 80432^T, and hopefully provide a new insight into the foundational theory and application in ecological restoration with saline–alkaline strains.

Keywords *Egicoccus halophilus* · Transcriptomic comparison · Alkaline response

Abbreviations

CPA	Cation/proton antiporter
NCBI	National Center for Biotechnology Information
SRA	Sequence Read Archive
SNP	Single-nucleotide polymorphisms
SD	Shine–Dalgarno
DEG	Differentially expressed gene
FPKM	Fragments per kilobase of transcript sequence per millions of base pairs
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
qPCR	Quantitative real-time PCR

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cDNA Complementary DNA
TCA Tricarboxylic acid

Introduction

Typical saline–alkaline environments like saline–alkaline lakes and soil harbor a number of halo(alkali)philic and halo(alkali)tolerant microorganisms (Banciu and Sorokin 2013). To thrive in those habitats, microorganisms deploy some adaptive strategies to cope with highly saline and/or alkaline stress. Microorganisms adopt the "salt-in-cytoplasm" strategy and the "compatible solute" strategy to withstand the salt stress (Chen et al. 2020, 2021). Besides, to overcome the burden of alkaline pH, microorganisms rely on some mechanisms of cytoplasmic pH homeostasis, e.g., the capture or retention of proton by the primary proton pumps (e.g., ATP synthase) and secondary active transporters [e.g., monovalent cation/proton antiporters (CPA)]; the production of acidic metabolites (e.g., acetate, pyruvate, and glutamate) through carbohydrate and amino acid metabolism; the modification of cell membrane by the alteration in membrane fatty acids components; the changes of secondary cell wall polymer containing various negatively charged residues, which favor H^+ accumulation and deter OH^- penetration (Slonczewski et al. 2009; Guo et al. 2019; Mamo 2020).

The class *Nitriliruptoria*, a higher taxon of phylum *Actinobacteria*, has six culturable members, namely *Nitriliruptor alkaliphilus* ANL-iso2^T, *Euzebya tangerina* F10^T, *Euzebya rosea* DSW09^T, *Euzebya* sp. DY32-46, *Egicoccus halophilus* EGI 80432^T, and *Egibacter rhizosphaerae* EGI 80759^T, which exhibit great adaptability to various high-salt environments (Sorokin et al. 2009; Kurahashi et al. 2010; Zhang et al. 2016a, b; Yin et al. 2018; Xu et al. 2019). The genomic features playing a role in the adaptation to high-salt environments in *Nitriliruptoria* were analyzed by a comparative genomics approach (Chen et al. 2020). The research revealed that a similar synthesis systems of solutes, namely trehalose, glutamine, glutamate, and proline, were present in *Nitriliruptoria*. On the other hand, the specific mechanisms likely contributing to withstand various salt environments were found in each member of *Nitriliruptoria* species, including K^+ influx and efflux, betaine and ectoine synthesis, and compatible solutes transport. Chen et al. (2021) performed physiological and transcriptomic analysis to reveal the salinity adaptation strategy in *E. halophilus* EGI 80432^T. They proposed that *E. halophilus* EGI 80432^T adopted the "salt-in-cytoplasm" strategy and the "compatible solute" strategy in response to moderate salinity condition, while the "compatible solute" strategy acted as a dominant strategy to withstand high salt stress. It is noteworthy that *E. halophilus* EGI 80432^T is a haloalkalitolerant bacterium isolated from saline–alkaline soil. The salt-tolerant mechanism of *E. halophilus* EGI 80432^T was elucidated by Chen et al. (2021), but the alkaline response mechanism of *E. halophilus* EGI 80432^T still remains unknown.

Here, we tried to elucidate the alkaline response mechanism of *E. halophilus* EGI 80432^T by comparing the transcriptome profile under highly alkaline condition with a control condition. We are confident that our research would be helpful for deeply understanding the adaptation mechanism of *E. halophilus* EGI 80432^T to the saline–alkaline environment, and provide a theoretical support for its application in environmental domination.

Materials and methods

Strains and culture conditions

E. halophilus EGI 80432^T (= CGMCC 1.14988^T = KCTC 33612^T) grown in the pH between pH 8.0 and pH 10.0, and optimally at pH 8.0–9.0, was isolated from a saline–alkaline soil in Xinjiang Province, north-west China (Zhang et al. 2016a). The strain was maintained on modified marine 2216E agar (Difco, Sparks, MD, USA) supplemented with 2% NaCl (w/v) and pH adjusted to 8.0 at 30 °C (Zhang et al. 2016a). The cells from modified marine 2216E agar were transferred to 50 mL fresh modified marine 2216E liquid medium in 250 mL Erlenmeyer flasks and incubated for 3 days at 30 °C with 150 rpm shake. The pre-cultures were used as inocula for the study.

Alkaline pH stress experiment and sample preparation

The pre-cultures were transferred to fresh modified marine 2216E liquid medium with different pH, namely pH 8.0 (control check, CK) and pH 10.0 (high alkali, HA), and cultured at 30 °C with shake of 150 rpm. The cultures grown to the mid-exponential growth phase in different alkaline conditions were harvested by centrifuging at 5,000 *g* for 10 min and washed three times with ddH_2O for subsequent transcriptomic analysis.

RNA-seq sample preparation and transcriptome sequencing

Six RNA samples obtained from cells grown under control (pH 8.0) and high alkali (pH 10.0) treatments with three biological replicates were used to generate sequencing libraries. Total RNA per sample was extracted with a modified RNeasy midi kit (Qiagen Science, CA, USA) and treated with RNase-free DNase I (TaKaRa, China) to remove genomic DNA. Subsequently, the extracts were monitored on 1% agarose gels and checked with

the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). The quantity and quality of RNA were measured using Qubit® 2.0 fluorometer with Qubit® RNA assay kit (Life Technologies, CA, USA), and Agilent Bioanalyzer 2100 system with the RNA Nano 6000 assay kit (Agilent Technologies, CA, USA), respectively. Sequencing libraries were constructed using NEBNext® Ultra™ Directional RNA library prep kit (NEB, USA) and sequenced on an Illumina Hiseq 2500 platform at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China).

RNA-seq data analysis

Raw reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Bioproject: PRJNA718721 and the accession numbers: SRR14116862-SRR14116867). The raw data of fastq format were firstly processed through in-house perl scripts to remove reads containing adapter, ploy-N, and low-quality reads. The quality of clean data was assessed with Q20 and Q30 (Table 1). The high-quality clean data were aligned to *E. halophilus* EGI 80432^T genome using Bowtie2-2.2.3 (Langmead and Salzberg 2012). The identification of novel genes and prediction of gene structure were performed by Rockhopper (McClure et al. 2013). The single-nucleotide polymorphisms (SNP) calling was performed by GATK (McKenna et al. 2020). Subsequently, the Shine–Dalgarno (SD) sequence and terminator sequence were predicted by RBSfinder and TransTermHP, respectively (Suzek et al. 2001; Kingsford et al. 2007). Finally, we used IntaRNA and RNAfold to predict the sRNA targets and RNA secondary structures, respectively (Hofacker and Stadler 2006; Busch et al. 2008).

Differentially expressed genes (DEGs) analysis and annotation

To estimate the levels of gene expression, the read numbers mapped to each gene were counted by HTSeq v0.6.1, and the effect of sequencing depth and gene length for each gene was calculated based on the fragments per kilobase of transcript sequence per millions of base pairs sequenced (FPKM) (Trapnell et al. 2010). Subsequently, we used the DESeq R package (1.18.0) to analyze the DEGs between the control group and the highly alkaline group (Anders and Huber 2013). In this study, we performed three biological replicates per group, such that the DEGs were identified with an adjusted P value < 0.05 (Anders and Huber 2013). Finally, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs identified between two groups were implemented by the GOseq R package and KOBAS software, respectively (Mao et al. 2005; Young et al. 2010).

Validation of RNAseq data by quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to validate the RNA-seq data. Six randomly selected genes, namely ELR47_RS03560, ELR47_RS05540, ELR47_RS09510, ELR47_RS09675, ELR47_RS11550, and ELR47_RS14440, were used as target genes, and the chaperonin Cpn60 gene as an internal control. The primers used in this study were generated with DNAMAN 6.0 software and listed in Supplementary Table S1. The treated RNA (1 ng/μL) per sample was used to synthesize complementary DNAs (cDNAs) by the Hifair™ II super mix plus gDNA digester with the Oligo(dT) (Yeasen, China). The qPCR reaction was performed in the mixture containing Hieff® qPCR SYBR®

Table 1 Summary of RNA-sequencing and assembly results

Sample name[a]	CK-1	CK-2	CK-3	CK_group	HA-1	HA-2	HA-3	HA_group
Raw reads	18,580,600	15,358,214	16,776,214	16,905,009	33,696,494	26,238,266	33,726,920	31,220,560
Clean reads	18,488,732 (99.51%)	15,086,069 (98.23%)	16,652,445 (99.26%)	16,742,415 (99%)	33,160,745 (98.41%)	25,624,772 (97.66%)	33,354,850 (98.9%)	30,713,455 (98.32%)
Multiple mapped	579,489 (3.12%)	780,026 (5.08%)	630,028 (3.76%)	663,181 (3.99%)	996,244 (2.96%)	1,386,829 (5.29%)	1,015,432 (3.01%)	1,132,835 (3.75%)
Uniquely mapped	17,909,243 (96.39%)	14,306,043 (93.15%)	16,022,417 (95.51%)	16,079,234 (95%)	32,164,501 (95.45%)	24,237,943 (92.38%)	32,339,418 (95.89%)	29,580,620 (94.57%)
Q20 (%)	97.51	97.38	97.46	97.45	97.37	97.40	97.35	97.37
Q30 (%)	93.44	93.24	93.34	93.34	93.13	93.17	92.96	93.08
GC content (%)	70.56	71.15	71.17	70.96	71.68	70.73	71.38	71.26
Genes	3767	3833	3833	3840	3825	3822	3819	3837

CK control check, *HA* high alkali

[a]CK and HA-1, -2, -3 represent three biological replicates of *E. halophilus* EGI 80432^T under optimum pH and high pH conditions, respectively

Green master mix (Yeasen, China), a forward/reverse primer, a cDNA template, and ddH_2O, with the procedure: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min, on a QuanStudio3 real-time PCR system (Applied Biosystems, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of randomly selected genes (Livak and Schmittgen 2001). Three biological and three technical replicates were performed for the control and highly alkaline groups.

Results

Transcriptome sequencing and assembly analysis

To investigate the response mechanism to high pH shock in *E. halophilus* EGI 80432^T, six libraries were generated and sequenced from cells grown under pH 8.0 (control check, CK) and pH 10.0 (high alkali, HA) conditions. Table 1 shows the results obtained from RNA-sequencing and assembly. After removing reads containing adapter, ploy-N, and low-quality reads, average 99% and 98.32% clean reads were obtained from average 16,905,009 and 31,220,560 raw reads generated for control and highly alkaline conditions, respectively. Moreover, average 16,079,234 (95%) and 29,580,620 (94.57%) reads were uniquely mapped to *E. halophilus* EGI 80432^T reference genome and assembled into 3,840 and 3,837 genes. To validate the RNA-seq data reliability, we randomly selected 6 genes, ELR47_RS03560, ELR47_RS05540, ELR47_RS09510, ELR47_RS09675, ELR47_RS11550, and ELR47_RS14440, and performed the qPCR analysis. The qPCR results were in agreement with RNA-seq data (Supplementary Fig. S1).

Differentially expressed genes (DEGs) analysis and annotation

The overall transcription levels of genes were quantified by the FPKM metrics (Trapnell et al. 2010), and DEGs were identified with the standard threshold of P value < 0.05 (Anders and Huber 2013). Compared with the control, 1,129 genes were identified as DEGs at highly alkaline treatment, including 536 genes up-regulated and 593 genes down-regulated (Fig. 1a, Table 2). Furthermore, 733 DEGs (355 genes up-regulated and 378 genes down-regulated) and 606 DEGs (303 genes up-regulated and 303 genes down-regulated) were functionally annotated with GO and KEGG database, respectively (Table 2). According to KEGG pathway enrichment analysis, DEGs were classified into seventeen functional categories and mainly involved in the categories including "carbohydrate metabolism", "energy metabolism", "nucleotide metabolism", "amino acid metabolism", "metabolism of cofactors and vitamins", "membrane transport", and "signal transduction" (Fig. 1b).

Response of carbohydrate metabolism to highly alkaline stress

The highly alkaline environment significantly affected the expression of genes involved in carbohydrate metabolism, i.e., glycolysis, tricarboxylic acid cycle (TCA cycle), starch, and trehalose metabolism (Fig. 2). Six unigenes encoding enzymes, including fructose-1,6-bisphosphatase II, fructose-bisphosphate aldolase II, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphopyruvate hydratase, and pyruvate dehydrogenase E1, involved in glycolysis, were up-regulated under pH 10.0 condition. As for TCA cycle, four genes encoding fumarate hydratase class II, aconitate hydratase, and succinate-CoA ligase subunit alpha/beta, were up-expressed under highly alkaline condition. Similarly, the genes responsible for trehalose synthesis, i.e., *treS* (trehalose synthase), *treY* (malto-oligosyltrehalose synthase), and *treX* (glycogen debranching enzyme), showed positive response under highly alkaline shock. Moreover, genes required to synthesize starch from amylose (ELR47_RS05355) and the degradation of starch to dextrin (ELR47_RS11515) were highly expressed in highly alkaline stress.

Response of proton transport to highly alkaline stress

Proton capture or retention, performed by the primary proton pumps (e.g., respiratory chain complexes) and secondary active transporters [e.g., monovalent cation/proton antiporters (CPA)], is one major microbial strategy of maintaining intracellular pH homeostasis under a high pH environment (Slonczewski et al. 2009; Mamo 2020). The present work demonstrated up-regulated genes involved in the respiratory chain complexes (complex I, complex III, and complex IV) such as NADH–quinone oxidoreductase subunit I, cytochrome b subunit, cytochrome c1 subunit, and cytochrome c oxidase subunit I/II/III (Fig. 3). The main function of these genes is the proton production and translocation (Papa et al. 1994). In contrast, gene encoding ATP synthase for proton influx was down-regulated under high pH condition (Fig. 3).

The monovalent cation/proton antiporters regulate the influx of proton and the efflux of cations (Krulwich et al. 2011). Those antiporters were categorized into two superfamilies, the CPA families [CPA1, CPA2, and CPA3 (also known as Mrp-type)] and the Nha (Na^+/H^+ antiporter) families (NhaA, NhaB, NhaC, and NhaD) (Krulwich et al. 2009; Ito et al. 2017). Here, one Na^+/H^+ antiporter gene (ELR47_RS08060) and two cation/proton antiporter

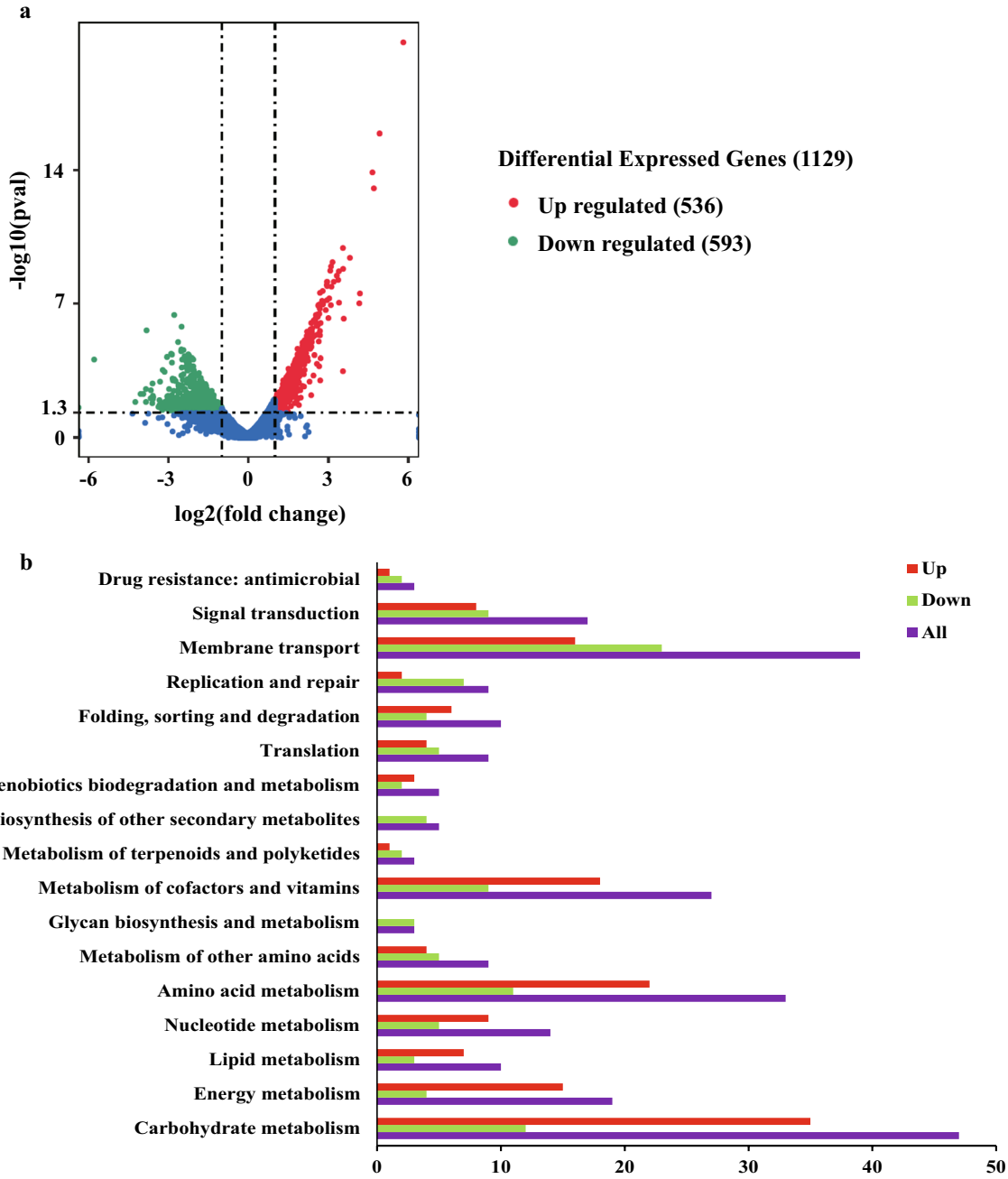


Fig. 1 Distribution of differentially expressed genes (DEGs) under highly alkaline condition compared with control condition (pH 10.0 vs pH 8.0). **a** Volcano plots of DEGs. **b** Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of DEGs

Table 2 Summary of DEGs of *E. halophilus* EGI 80432^T transcriptome in response to high alkali stress

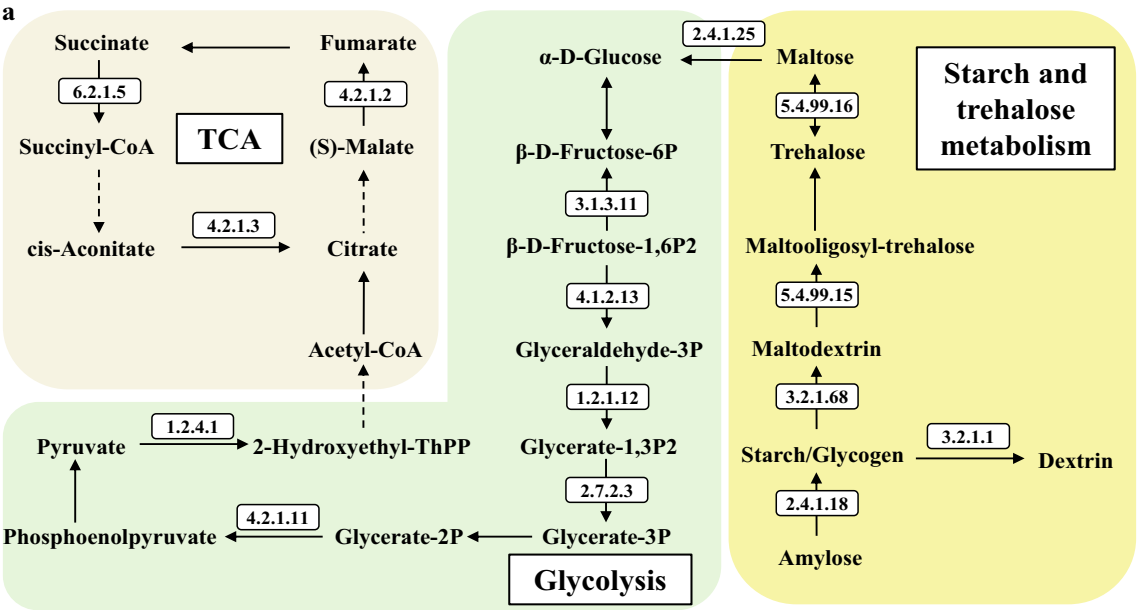
	HA vs CK		
	All	Up	Down
DEG	1129	536	593
DEG_GO	733	355	378
DEG_KEGG	606	303	303

CK control check, *HA* high alkali

genes (ELR47_RS01215 and ELR47_RS08070) were down-expressed under higher alkaline condition (pH 10.0) (Fig. 3).

These findings suggested that *E. halophilus* EGI 80432^T likely decreased the proton capture or retention under highly alkaline condition (pH 10.0), compared with the control pH condition (pH 8.0).

a



b

Descriptions	Locus_tag	EC numbers	Putative Function	CK	HA
Glycolysis	ELR47_RS03990	3.1.3.11	Fructose-1,6-bisphosphatase II		
	ELR47_RS07380	4.1.2.13	Fructose-bisphosphate aldolase II		
	ELR47_RS09380	1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase		
	ELR47_RS09385	2.7.2.3	Phosphoglycerate kinase		
	ELR47_RS16800	4.2.1.11	Phosphopyruvate hydratase		
	ELR47_RS00695	1.2.4.1	Pyruvate dehydrogenase E1		
Citrate cycle (TCA cycle)	ELR47_RS03785	4.2.1.2	Fumarate hydratase class II		
	ELR47_RS09730	4.2.1.3	Aconitate hydratase		
	ELR47_RS03675	6.2.1.5	ADP-forming succinate-CoA ligase subunit beta		
	ELR47_RS03680	6.2.1.5	Succinate-CoA ligase subunit alpha		
Starch and trehalose metabolism	ELR47_RS05320	2.4.1.25	4-Alpha-glucanotransferase		
	ELR47_RS05360	5.4.99.16	Trehalose synthase		
	ELR47_RS05325	5.4.99.15	Malto-oligosyltrehalose synthase		
	ELR47_RS05330	3.2.1.68	Glycogen debranching enzyme		
	ELR47_RS11515	3.2.1.1	Alpha-amylase		
	ELR47_RS05355	2.4.1.18	1,4-Alpha-glucan branching enzyme		

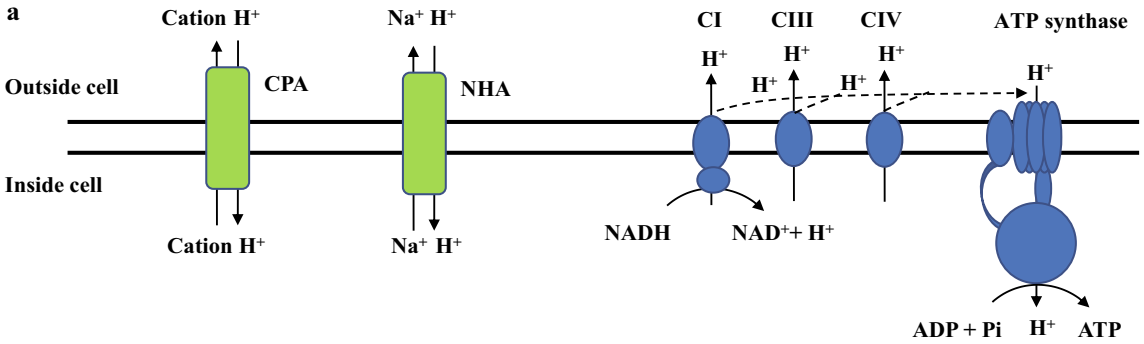
Down-regulated NS Up-regulated

Fig. 2 The DEGs involved in carbohydrate metabolism. **a** Pathways for carbohydrate metabolism in *E. halophilus* EGI 80432^T. **b** Heat maps of the different expression levels of genes responsible for carbohydrate metabolism in *E. halophilus* EGI 80432^T under highly alkaline condition. *CK* control check (pH 8.0) and *HA* high alkali (pH 10.0). The information of genes involved in carbohydrate metabolism is shown in the Supplementary Table S2

Response of organic acid metabolism to highly alkaline stress

The production of acidic metabolites that lower internal pH is another microbial strategy of maintaining intracellular pH homeostasis under a high pH environment (Slonczewski et al. 2009; Mamo 2020). We analyzed and compared the expression of genes involved in the production of common organic acids in *E. halophilus* EGI 80432^T under highly alkaline treatment (pH 10.0) with those in the control treatment (pH 8.0) (Fig. 4a, c). Figure 4a shows that the highly alkaline stress showed positive effect on the production of acetate, which was performed by two pathways. One was performed by three up-regulated genes encoding pyruvate dehydrogenase, dihydrolipoyl dehydrogenase, and acetyl–CoA synthetase. The other one was performed by aldehyde dehydrogenase and alcohol dehydrogenase. Under highly alkaline condition, aldehyde dehydrogenase was encoded by two up-regulated genes (ELR47_RS06225 and ELR47_RS13150) and one down-regulated gene (ELR47_RS16010), and alcohol dehydrogenase was encoded by two up-regulated genes (ELR47_RS12650 and

a



b

Descriptions	Locus_tag	Putative Function	CK	HA
NHA	ELR47_RS08060	Na^+/H^+ antiporter		
CPA	ELR47_RS01215	Cation/proton antiporter		
	ELR47_RS08070	Cation/proton antiporter		
CI	ELR47_RS02730	NADH-quinone oxidoreductase subunit I		
CIII	ELR47_RS12625	Cytochrome b subunit		
	ELR47_RS12615	Cytochrome c1 subunit		
CIV	ELR47_RS17970	Cytochrome c oxidase polypeptide I		
	ELR47_RS01470	Cytochrome c oxidase polypeptide I		
	ELR47_RS17975	Cytochrome c oxidase polypeptide II		
	ELR47_RS01475	Cytochrome c oxidase polypeptide II		
	ELR47_RS17965	Cytochrome c oxidase polypeptide III		
ATP synthase	ELR47_RS05885	ATP synthase		

Down-regulated NS Up-regulated

Fig. 3 The DEGs involved in proton transport. **a** Schematic diagram of proton transport in *E. halophilus* EGI 80432^T. **b** Heat maps of the different expression levels of genes responsible for proton transport in *E. halophilus* EGI 80432^T under highly alkaline condition. *CK* control check (pH 8.0), *HA* high alkali (pH 10.0), *CI* complex I, *CIII* complex III,and *CIV* complex IV. The information of genes involved in proton transport is shown in the Supplementary Table S2

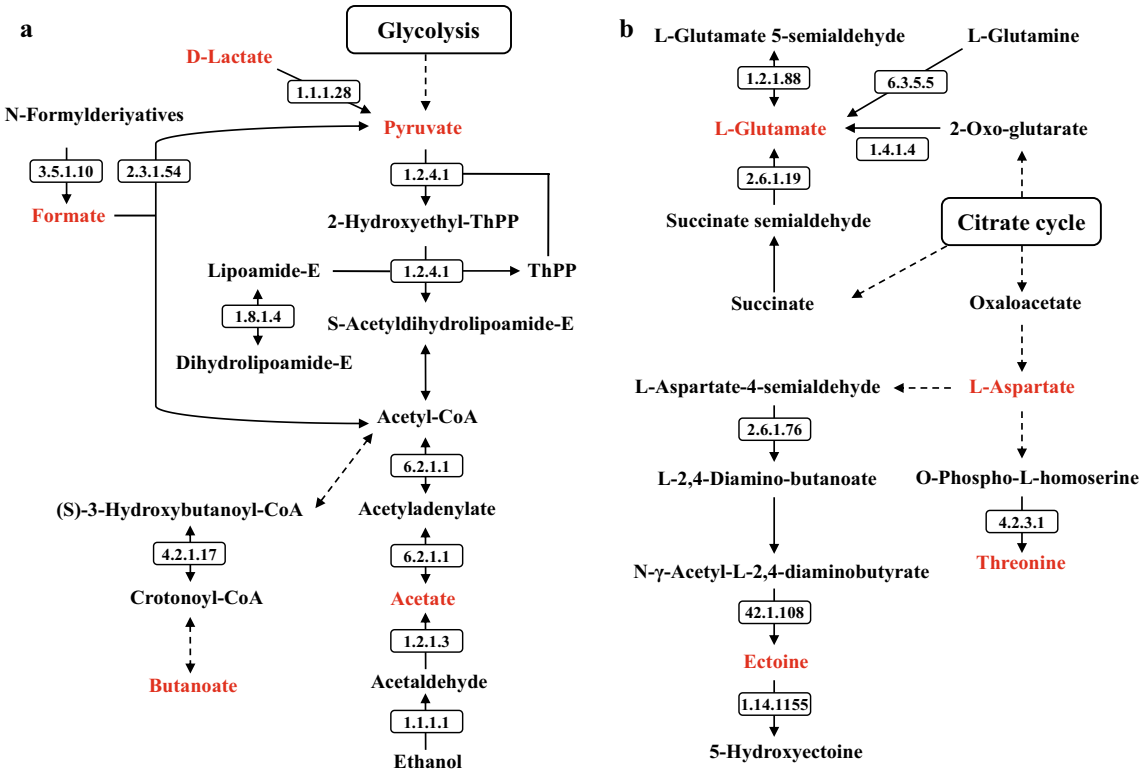
ELR47_RS17805) and two down-regulated genes (ELR47_RS08185 and ELR47_RS13890). In addition, genes encoding formate C-acetyltransferase and D-lactate dehydrogenase responsible for the pyruvate production from formate and lactate, respectively, were up-regulated under the pH 10.0 condition. The enhanced expression of a gene that encodes formyltetrahydrofolate deformylase involved in the formate synthesis and the decreased expression of a gene that encodes enoyl-CoA hydratase responsible for butanoate production were found under highly alkaline treatment.

We further analyzed the expression of genes involved in the acidic amino acid metabolism in *E. halophilus* EGI 80432^T under highly alkaline condition (Fig. 4b, d). No significant difference was found in the expression of genes involved in the aspartate synthesis between highly alkaline treatment and control treatment. However, the highly alkaline stress led to the positive regulation in glutamate synthesized by genes encoding glutamate dehydrogenase, L-glutamate gamma-semialdehyde, carbamoyl-phosphate synthase, and aminotransferase. Surprisingly, the highly alkaline treatment also played a positive role in the gene encoding threonine synthase required for threonine synthesis. Furthermore, three ectoine and 5-hydroxyectoine synthesis genes encoding diaminobutyrate acetyltransferase, ectoine synthase, and ectoine hydroxylase were increased expression under high pH condition.

The above results revealed that *E. halophilus* EGI 80432^T likely increased the production of acidic and neutral metabolites, i.e., acetate, pyruvate, formate, glutamate, threonine, ectoine, and 5-hydroxyectoine, in response to highly alkaline condition (pH 10.0).

Discussion

The development and popularization of next-generation sequencing provided more possibility to investigate the adaptation mechanism of microorganisms at various levels, e.g., gene, genome, and transcriptome (Cheng et al. 2016; Chen et al. 2020; Shu et al. 2020). Recently, RNA-sequencing, an effective method to evaluate the gene expression of organisms at various stage and state, was used to reveal the molecular mechanism of environmental stress response in microorganisms, e.g., temperature, salt, and pH



c

Locus_tag	EC numbers	Putative Function	CK	HA
ELR47_RS03720	1.1.1.28	2-hydroxyacid dehydrogenase		
ELR47_RS00695	1.2.4.1	Pyruvate dehydrogenase		
ELR47_RS11300	1.8.1.4	Dihydrolipoyl dehydrogenase		
ELR47_RS08125	6.2.1.1	Acetyl-coenzyme A synthetase		
ELR47_RS06225	1.2.1.3	Aldehyde dehydrogenase		
ELR47_RS13150	1.2.1.3	Aldehyde dehydrogenase		
ELR47_RS16010	1.2.1.3	Aldehyde dehydrogenase		
ELR47_RS12650	1.1.1.1	Alcohol dehydrogenase		
ELR47_RS08185	1.1.1.1	Alcohol dehydrogenase		
ELR47_RS13890	1.1.1.1	Alcohol dehydrogenase		
ELR47_RS17805	1.1.1.1	Alcohol dehydrogenase		
ELR47_RS08685	2.3.1.54	Formate C-acetyltransferase		
ELR47_RS15915	3.5.1.10	Formyltetrahydrofolate deformylase		
ELR47_RS09675	4.2.1.17	Enoyl-CoA hydratase		

d

Locus_tag	EC numbers	Putative Function	CK	HA
ELR47_RS06680	1.4.1.4	Glutamate dehydrogenase		
ELR47_RS12425	1.2.1.88	L-glutamate gamma-semialdehyde dehydrogenase		
ELR47_RS10780	6.3.5.5	Carbamoyl-phosphate synthase large subunit		
ELR47_RS10785	6.3.5.5	Carbamoyl-phosphate synthase small subunit		
ELR47_RS13155	2.6.1.19	Aminotransferase class III-fold pyridoxal phosphate-dependent enzyme		
ELR47_RS17550	4.2.3.1	Threonine synthase		
ELR47_RS14550	23.1.178	Diaminobutyrate acetyltransferase		
ELR47_RS14540	42.1.108	cupin domain-containing protein		
ELR47_RS14535	1.14.1155	ectoine hydroxylase		

Down-regulated NS Up-regulated

Fig. 4 The DEGs involved in the production of acidic metabolites. Pathways for common organic acids production (**a**) and acidic amino acid metabolism (**b**) in *E. halophilus* EGI 80432^{T}. Heat maps of the different expression levels of genes responsible for common organic acids production (**c**) and acidic amino acid metabolism (**d**) in *E. halophilus* EGI 80432 ^{T} under highly alkaline condition. *CK* control check (pH 8.0) and *HA* high alkali (pH 10.0). The information of genes involved in acidic metabolites metabolism is shown in the Supplementary Table S2

(Raymond-Bouchard and Whyte 2017; Liang et al. 2020; Songserm et al. 2020; Chen et al. 2021). In this study, the alkaline response mechanism of haloalkalitolerant bacterium *E. halophilus* EGI 80432^T was investigated by comparing the transcriptome profile under highly alkaline condition (pH 10.0) with control condition (pH 8.0). The clean reads were efficiently mapped to *E. halophilus* EGI 80432^T reference genome (control condition for 95% and highly alkaline condition for 94.57%). Furthermore, 1129 DEGs (536 genes up-regulated and 593 genes down-regulated) were identified with the standard threshold of P value < 0.05 (Anders and Huber 2013).

It is a well-known fact that energy is required when microorganism adapt the environmental stress. The expression of genes responsible for energy production was significantly affected by environmental stress (Raymond-Bouchard and Whyte 2017; Wang et al. 2019; Songserm et al. 2020). The up-regulated genes involved in glycolysis and TCA cycle, which are the major pathways for ATP generation, were reported in *E. halophilus* EGI 80432^T withstanding salt stress (Chen et al. 2021). Our transcriptomic analyses showed that the high alkali treatment also showed a positive effect on glycolysis and TCA cycle in *E. halophilus* EGI 80432^T. Furthermore, we found that the expression of genes involved in the synthesis of trehalose and starch, which are used as the main source of carbon and energy, was increased under highly alkaline condition. These findings suggested that *E. halophilus* EGI 80432^T likely increased energy production and reserve compounds synthesis to survive and grow in a highly alkaline environment. Previous researches proposed that trehalose was used not only as a source of carbon and energy but also as a stress protectant to cope with various environmental stresses, such as the compatible solute for coping with high salt environments (Wang et al. 2019; Chen et al. 2021) and the thermoprotectant for withstanding to high temperature environments (Reina-Bueno et al. 2012; Liu et al. 2019). The elevated expression of genes in trehalose synthesis pathways under highly alkaline condition suggested that trehalose may act as stress protectant in the highly alkaline response of *E. halophilus* EGI 80432^T.

Even in a high pH environment, most microbes tend to maintain their cytoplasmic pH close to neutral (Slonczewski et al. 2009). For this, maintaining a relatively higher intracellular concentration of H^+ is necessary. One important component of the cell membrane that contributes to cytoplasmic pH homeostasis is the primary proton pump, such as the respiratory system (Hicks and Krulwich 1995). Lewis et al. (1983) proposed that the proton translocation concomitant with respiration, and the H^+ was extruded with a high H^+/O ratio in the alkaliphiles. In the respiratory system of alkaliphiles, the NADH dehydrogenase was believed to be a proton--translocating complex (Hicks and Krulwich 1995), and the cytochrome c was thought to have a function in the transfer of electron-coupled H^+ and the storage of electron and H^+ for ATP production (Matsuno and Yumoto 2015). In *E. halophilus* EGI 80432^T, the expression of genes encoding NADH–quinone oxidoreductase subunit I and cytochrome c1 subunit under pH 10.0 condition were higher than those under pH 8.0 condition, suggesting that *E. halophilus* EGI 80432^T likely transferred and accumulated a large number of electron and H^+ on the outer surface of membrane under higher alkaline condition. Researches proposed that ATP synthase utilize the electrochemical gradients of H^+ on the surface of membrane to synthesize ATP and transfer H^+ into the cytoplasm (Hicks et al. 2010). Here, the expression of ATP synthase gene was decreased under pH 10.0 condition, suggesting that the transfer of H^+ to the cytoplasm by ATP synthase was lowered when *E. halophilus* EGI 80432^T under higher alkaline condition.

The monovalent cation/proton antiporters that perform the exchange of the intracellular cations (e.g., Na^+, Li^+, and K^+) and the extracellular H^+ are thought to be a very crucial mechanism for proton capture (Mamo 2020). The monovalent cation/proton antiporters were encoded by diverse genes (Krulwich et al. 2009). However, not all the antiporter genes show differential expression during an alkaline pH environment. *Desulfovibrio vulgaris* possesses several putative Na^+/H^+ antiporter genes, but only one (DVU3108) was up-regulated in response to high alkali stress (Stolyar et al. 2007). Furthermore, the study of Cheng et al. (2016) revealed that the antiporter genes exhibited different transcriptional profiles under alkaline conditions. Compared with the pH 8.0 condition, antiporter genes, *mrpA*, *mrpD*, *mrpE*, *nhaD2*, and *nhaP*, were down-regulated in *Halomonas* sp. Y2 in response to higher alkaline condition (pH 10.17), whereas opposite results were detected in genes, *mrpB*, *mrpC*, *mrpF*, *mrpG*, and *nhaD1*. Fifteen putative monovalent cation/proton antiporter genes were detected in *E. halophilus* EGI 80432^T (Chen et al. 2020). However, only three antiporter genes exhibited decreased expression in response to higher alkaline condition (pH 10.0), suggesting that the proton capture by monovalent cation/proton antiporters was low in *E. halophilus* EGI 80432^T in response to a higher alkaline condition.

It is known that metabolic processes are significantly affected by extracellular pH. Under an alkaline environment, cells produce acidic compounds to maintain intracellular pH homeostasis (Mamo 2020). The increased production in lactate, formate, acetate, and butanoate were observed in *Fusobacterium nucleatum* cultured at pH 8.2 compared to pH 7.4 (Chew et al. 2012). Here, the transcription levels of genes involved in the synthesis of acidic end products from carbohydrate metabolism, i.e., acetate, pyruvate, and formate, exhibited increasing trends in *E. halophilus* EGI 80432^T in response to higher alkaline condition. High pH

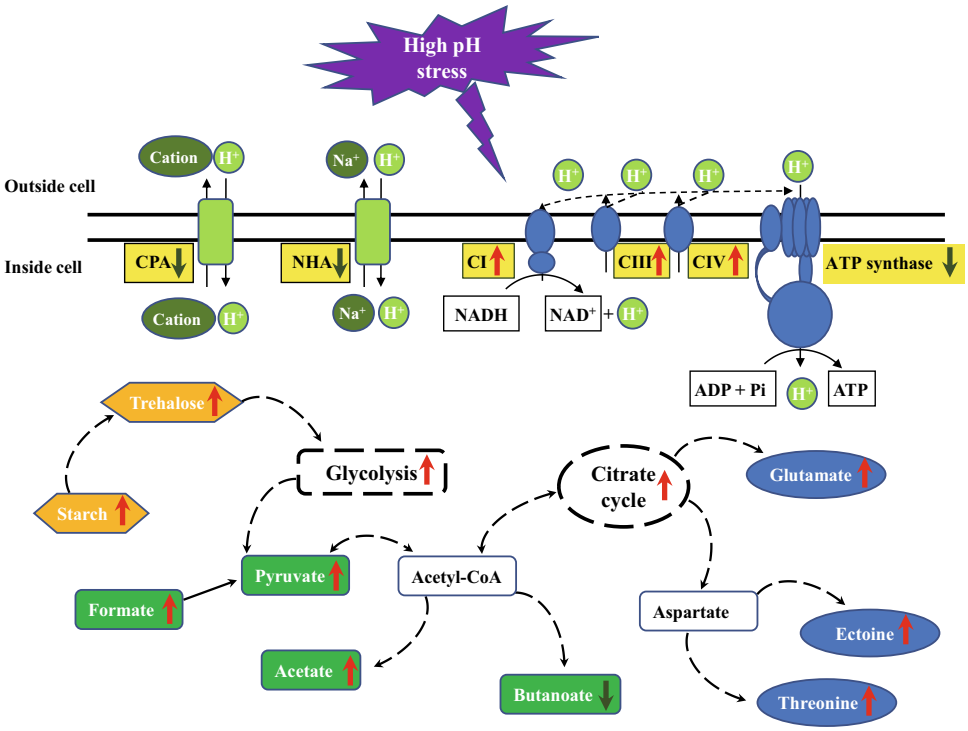


Fig. 5 Schematic diagram of mechanisms responsible for high pH stress in *E. halophilus* EGI 80432^T. Significantly up-regulated genes involved in the pathways were marked with red arrows and down-regulated genes involved in the pathways were marked with green arrows. *CPA* cation/proton antiporter, *NHA* Na^+/H^+ antiporter, *CI* complex I, *CIII* complex III, and *CIV* complex IV

favors the production of acid substances from carbohydrate and amino acid metabolism (Mamo 2020). The intracellular glutamate content of *Streptomyces hygroscopicus* in alkaline pH treatment was higher than the control (Jiang et al. 2018). The study of Chew et al. (2012) reported that the expression of glutamate dehydrogenase responsible for glutamate biosynthesis was significantly increased in *F. nucleatum* under alkaline condition. The positive effects in the four pathways for glutamate biosynthesis, namely glutamate dehydrogenase, L-glutamate gamma-semialdehyde, carbamoyl-phosphate synthase, and aminotransferase, were detected in *E. halophilus* EGI 80432^T under higher alkaline condition. Moreover, the elevated expression of genes responsible for the synthesis of threonine, ectoine, and 5-hydroxyectoine, which were used for *E. halophilus* EGI 80432^T withstanding higher salinity stress (Chen et al. 2021), was observed under highly alkaline stress, indicating that these compounds were responsible for *E. halophilus* EGI 80432^T in response to a higher alkaline environment.

In summary, we compared the transcriptome of *E. halophilus* EGI 80432^T under highly alkaline condition (pH 10.0) with control condition (pH 8.0), and proposed the putative mechanism of *E. halophilus* EGI 80432^T in response to higher alkaline shock (Fig. 5). The energy production pathways, i.e., glycolysis and TCA cycle, and storage pathways, i.e., starch and trehalose metabolism, were extremely active, when *E. halophilus* EGI 80432^T was cultured under higher alkaline condition, indicating that *E. halophilus* EGI 80432^T may produce and store a large amount of energy to cope with higher alkaline environment. In some alkaliphilic and alkalitolerant microorganisms, the monovalent cation/proton antiporters and ATP synthase are the effective channels for trapping proton (Slonczewski et al. 2009; Mamo 2020). However, they likely were not the major strategy for *E. halophilus* EGI 80432^T in response to higher alkaline condition, since the gene expression of cation/proton antiporters and ATP synthases was decreased under pH 10.0 condition. On the other hand, the acidic metabolites are thought to increase the cytoplasmic H^+ concentration, which would relieve the burden of capturing proton to the cytoplasm (Mamo 2020). The higher gene expression was observed in the production of acidic and neutral metabolites, i.e., acetate, pyruvate, formate, glutamate, threonine, and ectoine, when *E. halophilus* was incubated under highly alkaline condition (pH 10.0), revealing that under higher alkaline condition, *E. halophilus* EGI 80432^T likely tended to produce acidic metabolites to adjust the intracellular pH change.

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Author contributions DDC, WSS, and WJL conceived and designed the research. DDC conducted the experiments, analyzed the data, and wrote the original draft. YHL, SW, and BBL analyzed the data and modified the first draft of this manuscript. AM and SXG conducted the review and editing. DDC, YHL, HCJ, and WJL provided funding. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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