Complex gene response of herbicide-resistant *Enterobacter* strain NRS-1 under different glyphosate stresses

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Abstract

Knowledge of biological evolution and genetic mechanisms is gained by studying the adaptation of bacteria to survive in adverse environmental conditions. In this regard, transcriptomic profiling of a glyphosate-tolerant *Enterobacter* strain NRS-1 was studied under four different treatments to investigate the gene-regulatory system for glyphosate tolerance. A total of 83, 83, 60 and 74 genes were up-regulated and 108, 87, 178 and 117 genes down-regulated under 60-NPG, 110-NPG, NaCl (355 mM) and HCl (pH 4.46) stress treatments, respectively. Complex gene network was identified to be involved in regulating tolerance to glyphosate. This study revealed that NRS-1 has gained glyphosate tolerance at the cost of osmotic and acidic resistance. The 25 differentially expressed genes are reported to may have partly changed the function for providing resistance to glyphosate directly, among them genes *metK*, *mtbK*, *fdnG* and *wzb* that might detoxify/degrade the glyphosate. However, under 110-NPG condition, NRS-1 might have utilized economical and efficient ways by depressing its metabolism and activity to pass through this stress. Hence, the present study provides insights into the genes involved in glyphosate tolerance, which can be effectively utilized to engineer herbicide-resistant crop varieties after their proper validation to manage weed growth.

Keywords *Enterobacter* strain · Glyphosate · RNA-seq · Acid and osmotic stress · Adaptation mechanism

Introduction

The genus *Enterobacter* belongs to the family *Enterobacteriaceae* that are ubiquitous in nature as well as possess remarkable ability to resist various environmental adverse conditions. Antibiotic resistance is the most concerning characteristic, and many antibiotics such as ampicillin,

amoxicillin, amoxicillin–clavulanate, tetracycline and ertapenem have been reported to have no effect against *Enterobacter* (Mezzatesta et al. 2012; Paauw et al. 2009; Chang et al. 2013; Matar 2017), and this resistance may occur either naturally or acquired after encountering a challenge. Similarly, metal-tolerant *Enterobacter* strains were identified in metallic-polluted surroundings (Chien et al. 2013; Nakahara et al. 1985; Jaroslwiecka and Piotrowska-Seget 2014; Chen et al. 2017). Many *Enterobacter* strains were found living in extreme temperature, osmotic stress and unfavorable pH conditions (Jha et al. 2011; Roumagnac et al. 2012; Andrés-Barrao et al. 2017). In addition, certain strains have been also reported to survive in high concentrations of herbicides such as glyphosate and paraquat (Perez et al. 2012a, b; Kryuchkova et al. 2014).

Obviously, *Enterobacter* employs various mechanisms to survive under unfavorable conditions. For example, biofilms or cell wall with lipopolysaccharide helps cells to endure stress (Chang et al. 2013; Jaroslwiecka and Piotrowska-Seget 2014); reducing membrane permeability through balancing porins is another vital strategy (Lavigne et al. 2013); efflux pumps and transporters ease the stress on cell (Perez

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et al. 2012a, b); enzymes like C-P lyase and β -lactamase can destroy or convert toxic substances to non-toxic (Kryuchkova et al. 2014; Yang et al. 2012); the target genes of a drug also contribute to the resistance (Mezzatesta et al. 2012); energy storage is also important when cells encounter stress (McMeechan et al. 2005). Hence, these bacterial strains may utilize multiple mechanisms to resist environmental stress.

Glyphosate is *N*-(phosphonomethyl)-glycine, and its isopropylamine salt is used as the commercial product Roundup® (Monsanto Company). This compound is a broad-spectrum non-selective herbicide, and is widely used in modern agriculture. All the crop plants are sensitive to this herbicide, and considerable efforts have been made to develop genetically engineered glyphosate-resistant crops for controlling weed growth. Glyphosate-resistant crops represent more than 80% of the 120 million ha of transgenic crops grown annually worldwide, and the resistant genes of microbes have been mostly used (Duke and Powles 2009). Although the antimicrobial activity of this herbicide is well reported, many strains such as *Bacillus*, *Pseudomonas* and *Agrobacterium* are resistant to this drug/herbicide (Arfarita et al. 2013). Many studies have been used to investigate the glyphosate-resistance mechanisms, such as enzymes C-P lyase, glyphosate oxido-reductase, glyphosate acetyltransferase, glycine oxidase and phosphotransferase can use glyphosate as P, C or N resource (Dick and Quinn 1995; Pollegioni et al. 2011; Zhan et al. 2018). The mutant target gene of glyphosate that encodes the 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*) also provides resistance to glyphosate (Funke et al. 2009). However, the global response of the microbe against this drug remains unclear.

In this regard, “omics”-based technologies such as RNA sequencing (RNA-seq) is an attractive and powerful way to explore the functional genes and pathways that are altered under environmental stresses (Liu et al. 2015). In our previous study, we identified glyphosate-tolerant *Enterobacter* strain, namely *NRS-1*, which grows well under 60-NPG (pH 4.46), HCl (pH 4.46) and NaCl (355 mM) treatments. It was selected from soil microbe mixtures. Its tolerance to high glyphosate concentration might be controlled by a variety of responding genes (Fei et al. 2013). Since the stress under different glyphosate concentrations are related to different aspects, we performed RNA-seq analysis to identify and analyze the differentially expressed genes (DEGs) of *NRS-1* to reveal its adaptation and survival strategies under different glyphosate concentrations as well as simulated osmotic and acidic stresses. This study provides insights into new resistance genes as well as their regulatory mechanism that can be used to engineer herbicide-resistant crops.

Materials and methods

Bacterial strain and growth condition

Glyphosate-resistant *NRS-1* strain, belonging to the genus *Enterobacter* of Gram-negative bacteria, was cultured in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at a temperature of 30 °C with shaking at 200 rpm for 12 h. Bacterial cells were divided into several portions and harvested by centrifugation (5000 rpm, 5 min). Fresh liquid medium was added to the sediments.

Growth of *NRS-1* under various stresses

Bacterial broth was used to observe the growth tendency of *NRS-1* strain under four different treatments, viz., 110 g L⁻¹ glyphosate (110-NPG; pH 3.9), 60 g L⁻¹ (60-NPG; pH 4.46), HCl (pH 3.9) and NaCl (430 mM), and the growth was compared with the control that did not experience any stress. The 60-NPG was a middle concentration of glyphosate treatment for *NRS-1*, and the treatments of 110-NPG, HCl (pH 4.46) and NaCl (355 mM) are all critical doses for this strain. Cell density was recorded as the OD₆₀₀ (OD means optical density) after 2 h intervals. Wild-type strain (not resistant to glyphosate) was treated in the same manner as *NRS-1*, and each experiment was performed in triplicate.

Experimental design and preparation

For RNA-seq experiments, bacterial cultures were grown to an optical density of OD₆₀₀ = 0.6–0.8 nm, and treatments of 60-NPG, 110-NPG, HCl (pH 4.46) and NaCl (355 mM) were given for 20 min (the molar concentration and acidity of HCl treatment were the same as that of 60-NPG). Samples of stressed and non-stressed cells were collected and washed in phosphate-buffered saline (PBS). For RNA extraction, TRIzol Reagent procedure (Life Technologies, USA) was used. The RNA extract was treated with DNase I to eliminate genomic DNA contamination. The quality and concentration of the RNA samples were checked using a Nanodrop UV spectrophotometer and RNA Nano chip on an Agilent Bioanalyzer 2100. The same protocol was used to prepare RNA samples for quantitative real-time PCR (qRT-PCR).

Illumina-based transcriptome profiling and data analysis

Libraries of cDNA were prepared according to the standard Illumina instructions. The GenRead rRNA Depletion kit (QIAGEN) was used to remove/eliminate the rRNA, which accounts for more than 80% of the total RNA. Ambion RNA

fragmentation kit (Life Technologies) was used for mRNA fragmentation, and random hexamers were used to synthesize double-stranded cDNA. The QiaQuick PCR extraction kit (QIAGEN) was used to purify the cDNA products. After end repair, poly-A tailing and adapter ligation, the prepared short fragments were further purified by agarose gel electrophoresis for size selection. Final libraries were generated by the last step of PCR, and the quality was analyzed on an Agilent 2100 Bioanalyzer and the ABI step-one plus Real-Time PCR system. Sequencing was performed on an Illumina HiSeq 2000.

To reduce the effect of low-quality nucleotides, the raw reads were cleaned by the Illumina GASAVA-1.8 Fastq Filter, and adapter sequences, sequences with too many unknown nucleotides and reads with Q value ≤ 20 were removed. Based on the overlapping area, the qualified reads were de novo assembled using the short read assembly program Trinity to generate contigs or unigenes (Grabherr et al. 2011). Assembled genes that were too short were deleted.

The optimal generated results were annotated to determine the molecular function, metabolic process and cellular component using the non-redundant protein (Nr) and non-redundant nucleotide (Nt) databases, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases using BLASTN, BLASTX (Altschul et al. 1997), and BLAST2GO (Conesa et al. 2005). Sequences without annotation information were not used for further analysis.

Differentially expressed genes (DEGs) were the transcripts that showed comparative altered expression between stressed and non-stressed samples. They were identified by R in Bioconductor (Anders and Huber 2010; Rapaport et al. 2013) and analyzed using edge R with TMM normalization (Robinson et al. 2009; Kadota et al. 2012). The DEGs with a fold change ≥ 2 or ≤ -2 were considered for further analysis, and the data with shared annotations were combined for each experiment. Heat map were created in HemI (Deng et al. 2014). Differentially expressed genes (DEGs) data obtained from the above four different treatments have been deposited in the NCBI database (Accession codes: SRR6749573–SRR6749578).

Expression validation by qRT-PCR

The qRT-PCR analysis was performed to validate the RNA-seq transcriptome profiling experiments. A total volume of 10 μ g of RNA was used for reverse transcription with PrimeScript RT Master Mix Perfect Real-Time (TaKaRa, Japan). Eight genes from different samples were selected randomly, and the 16S *rDNA* was used as a reference standard. Specific primer sets were designed by Primer3 software (Table S1) (Untergasser et al. 2012). Real-time quantitative gene amplification was performed according to the SYBR Premix Ex

Taq (TaKaRa, Japan) protocol on a Bio-Rad iCycler iQ5. Each experiment was performed in triplicate, and the relative gene expression levels were determined by the $2^{-\Delta\Delta C_t}$ method (Herrmann 1995).

Data availability

The data are not publicly available due to copyright issues. However, the data set can be obtained from the corresponding author through e-mail.

Results

Growth of *NRS-1* under different stresses

The growth of *NRS-1* strain was compared with the control, and the results revealed that *NRS-1* growth was severely affected under 110-NPG stress with very low recovery rate (Fig. S1A). In addition, the growth of *NRS-1* was also critically reduced under the treatments of HCl (pH 3.9) and NaCl (430 mM), especially with acidic stress. However, there was little inhibition of *NRS-1* growth under a stress of 60-NPG (pH 4.46) (Fig. S1A), and the strain grew well under the simulated osmotic (355 mM NaCl) and acidic (pH 4.46 HCl) stresses corresponding to the 60-NPG condition (Fei et al. 2013).

Growth curves of the wild-type *Enterobacter* strain indicates that it could not survive either under 60-NPG or 110-NPG stresses (Fig. S1B), and suggested that *NRS-1* has gained glyphosate resistance through long-term selection. Therefore, being a type of *Enterobacter*, *NRS-1* has the remarkable ability to acquire glyphosate resistance and may have surprising ways to withstand glyphosate shock. Moreover, the growth of wild-type *Enterobacter* was superior compared to *NRS-1* strain under both NaCl (430 mM) and HCl (pH 3.9) stresses (Fig. S1), which suggests that *NRS-1* had osmotic- and acid-resistance traits before the selection, but had lost them during its adaptation to glyphosate stress.

Global gene expression pattern of *NRS-1* under different stress treatments

According to the N50 and Q20 values of RNA-seq data, the transcripts were of high quality, and the coverage was sufficient to identify all genes in the microbe (Table S2, S3 & S4). By comparing the datasets of treated and untreated samples, a handful of differentially regulated transcripts were uncovered. After removing all the sequences that have not been annotated and selecting only the significantly altered genes (fold change ≥ 2 or ≤ -2), we identified 238 (60 activated, 178 repressed), 191 (74 activated, 117 repressed), 191 (83 activated, 108 repressed) and 170 (83 activated, 87

repressed) DEGs under NaCl (355 mM), HCl (pH 4.46), 60-NPG and 110-NPG treatments, respectively, which were used for further investigation/studies (Table S4; Fig. 1). In NaCl (355 mM) and HCl (pH 4.46) treatments, the down-regulated genes were comparatively high compared to up-regulated genes; however, such differences were not observed under 60-NPG and 110-NPG stresses (Fig. 1).

To validate the reliability of RNA-seq data, we used qRT-PCR analysis to check the expression levels of randomly selected eight genes, viz., *osmY*, *fusA*, *proS*, *cirA*, *yedK*, *dadA*, *pflB* and *citF* under different treatments. The results showed that genes, viz., *yedK*, *citF*, *pflB* and *proS* were down-regulated, whereas *dadA* and *cirA*, were up-regulated, and genes *fusA* and *osmY* showed different expression under 60-NPG and 110-NPG, but the trend of gene expression was the same in both qRT-PCR and RNA-Seq analysis (Fig. 2). Hence, the gene expression analysis by RNA-seq was reliable.

All the DEGs were classified according to the gene ontology (GO), and nine different functional categories were identified that showed a significant number of up-regulated and down-regulated genes, viz., cellular process, establishment of localization, metabolic process, single-organism processes, cell membrane, membrane part, binding, catalytic activity and transporter activity (Fig. S2). The largest group of down-regulated genes was related to carbohydrate metabolism, energy production and conversion as well as cell motility. In addition, the Venn diagram revealed the difference in gene utilization under different stresses (Fig. 3a, b). For example, 11 up-regulated and 65 down-regulated genes were the same under 60-NPG and NaCl (355 mM)

treatments, and similarly 53 up-regulated and 75 down-regulated genes were the same under 60-NPG and HCl (pH 4.46) treatments (genes in 60-NPG were associated with induced osmotic and acidic stresses). In addition, 30 activated and 25 repressed genes were specifically responsive to 60-NPG (genes only responded to glyphosate pressure). Furthermore, under two different concentrations of glyphosate (60-NPG and 110-NPG), 30 up-regulated and 45 down-regulated genes were the same, whereas more than 50% of DEGs were different (Fig. 3a, b) and a lot of pathways were the same in the two samples (Fig. S2). Hence, the efficiency and economy of genes might explain the difference in the two degrees of glyphosate stresses.

Heat map provided an overview of the fold changes of DEGs, and the results of the map are shown in Fig. 3c. The expression of most DEGs was in the range of -4.426 to 1.448 , which is unlike the RNA-seq data of plants or animals, where the change of DEGs is small. This might be attributable to the uncomplicated genome structure as well as metabolic systems of the bacteria and its quicker adaptation to various environments compared to other organisms. Moreover, if there are more number of DEGs with large fold change, more energy and time will be consumed for recovery after the cell enters a new balance, which is uneconomic and ineffective for the microorganism.

Gene response of the shikimate pathway to stresses

The shikimate pathway is the only known route for the biosynthesis of aromatic compounds that consist of essential amino acids and secondary metabolites, which can provide

Fig. 1 Number of differentially expressed genes (DEGs) in *NRS-1* under NPG-60, 110-NPG, NaCl (355 mM) and HCl (pH 4.46) stresses

Fig. 2 qRT-PCR gene expression analysis of eight randomly selected genes under different stresses. **a** Expression profiles of selected transcripts under 60-NPG and 110-NPG. **b** Expression profiles of selected transcripts under 60-NPG, NaCl (355 mM) and HCl (pH 4.46) stresses. *, **, represent significance at $P \leq 0.05$ and $P \leq 0.01$, respectively

defense to the cell against various external stresses such as ultraviolet light, insect pests and abiotic stress (Herrmann 1995; Sherman and Vaughn 2018). This pathway is controlled by seven genes, viz., *aroG*, *aroB*, *aroQ*, *aroE*, *aroK*, *aroA* and *aroC* that work together for this route. The pathway is the only target of herbicide glyphosate and blocks it by restraining the function of the enzyme coded by the *aroA*

gene which is involved in the important step of this pathway for the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) (Yi et al. 2007; Zabalza et al. 2017), and hence this blockage causes critical damage.

The results of the present study revealed that the expression of many genes involved in the shikimate pathway are changed under the following four treatments, viz., NaCl

Fig. 3 The number of differentially expressed genes (DEGs) and their fold change analysis under 60-NPG, 110-NPG, NaCl (355 mM) and HCl (pH 4.46) stresses. **a** Venn diagram of up-regulated genes. **b** Venn diagram of down-regulated genes. **c** Heat map of transcriptomic data under four different stresses

Table 1 Differentially expressed genes (DEGs) of the shikimate pathway that change their expression under four different stresses used in the present study

S. no.	Treatment	Gene symbol	Fold change	Up/down	Description
1	NaCl (355 Mm)	<i>aroE</i>	− 19.0934	Down	Shikimate dehydrogenase
2	HCl (pH 4.46)	<i>aroE</i>	− 28.1729	Down	Shikimate dehydrogenase
3	60-NPG	<i>aroE</i>	− 29.3511	Down	Shikimate dehydrogenase
		<i>aroG</i>	2.8139	Up	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive
		<i>aroK</i>	− 2.0554	Down	Shikimate kinase 1
4	110-NPG	<i>aroE</i>	− 30.7108	Down	Shikimate dehydrogenase
		<i>aroQ</i>	− 13.9631	Down	3-Dehydroquinate dehydratase

(355 mM), HCl (pH 4.46), 60-NPG and 110-NPG (Table 1; Fig. 4). In all these four treatments, gene *aroE* was down-regulated with high fold change values (> 19), and the transcript of this gene has been previously reported to be subjected to feedback inhibition by shikimate (Krämer et al. 2003). This suggests that shikimic acid might be accumulated in the cell under adverse stress conditions, and thus the downstream routes (routes which are directly associated with the shikimate pathway, including tyrosine metabolism and phenylpropanoid biosynthesis) might be blocked. By searching the downstream genes of the shikimate pathway

in datasets of transcriptome, we reported that six and four genes were repressed by NaCl (355 mM) and HCl (pH 4.46), respectively; however, only fewer genes were affected by 60-NPG and 110-NPG treatments (Table S5 & S6). These results indicate that under osmotic and acidic stresses, many genes are down-regulated that may induce the accumulation of shikimate, and hence the decrease in *aroE* expression. However, in case of glyphosate shock, the glyphosate-resistance mutant gene *aroA* of *NRS-1* might be the principal reason for low shikimate accumulation, because this mutant gene has reduced affinity for glyphosate and the pathway

Fig. 4 Shows protection mechanisms used by the bacteria under various stresses through differentially expressed genes (DEGs) in the shikimate pathway. Gene symbol in red, blue and black represent up-regulated, down-regulated and unchanged genes, respectively. NPG, 60-NPG and 110-NPG represent glyphosate, 60 gL⁻¹ of glyphosate and 110 gL⁻¹ glyphosate, respectively. Pale yellow dotted lines and solid lines represent shikimate feedback regulation, the accumulation

of shikimic acid by NaCl (355 mM), HCl (pH 4.46) and glyphosate stresses through distinct causes. Dark yellow dotted line and solid line represent parts of the shikimate pathway of *NRS-1*, which responds to 110-NPG and 60-NPG. Red bold solid line: glyphosate regulates the sensor gene directly. Red dotted line and solid line: two different regulatory mechanisms of sensors for 110-NPG and 60-NPG, respectively

is not completely blocked, leading to little shikimate accumulation as well as change in *aroE* mRNA level (Fei et al. 2013).

Moreover, *NRS-1* strain showed an entirely different response under 60-NPG and 110-NPG treatments for shikimate pathway genes, such as the expression of *aroE*, *aroG* and *aroK* were changed in the case of 60-NPG, whereas under 110-NPG the expression of *aroE* and *aroQ* was changed (Table 1; Fig. 4). Vinella et al. (1996) reported that reduced *aroK* could promote cell division, and this action was beyond the shikimate pathway. It was also revealed that blocked *aroQ*, which was not regulated by aromatic acid, had an unfavorable influence on growth (Simmons et al. 1997; Kim et al. 2006). Hence, the function of the two genes is closely related to the growth of *NRS-1* under 60-NPG and 110-NPG treatments. So, they were thought to have functions beyond the shikimate pathway and may be associated with the growth under the stress.

The gene *aroG* was up-regulated under 60-NPG, and responded only to this stress. It was reported that *aroG* was activated in response to the deficiency of aromatic acids (Doroshenko et al. 2010); however if 60-NPG treatment affected the synthesis of aromatic acids, the expression of *aroG* gene would also be changed in the case of 110-NPG stress. Thus, searching all upstream DGEs of the shikimate pathway, the down-regulated transcript *gap2* showed a specific response to 110-NPG, and this gene is involved in the carbon fixation pathway in prokaryotes. This information

leads us to speculate that *gap2* and *aroG* might be associated with the flow of carbon, and proteins (sensors) might exist directly and specifically corresponding to different concentrations of glyphosate. Hence, the microorganism may exploit *aroG* and *aroK* to rapidly recover from damage under 60-NPG treatment, and the changes in *gap2* and *aroQ* under 110-NPG appeared disadvantageous for the growth of *NRS-1*. These reactions might be some specific strategies of the bacterium to manage extreme glyphosate pressure and allow full preparation for late reproduction. Thus, the changes of the above genes might be related to the growth of microorganism during stress (Fig. S1).

Expression response of *NRS-1* under 60-NPG and NaCl stresses

The *NRS-1* exhibited unique response to 60-NPG compared to NaCl (355 mM) treatment (Table S4). Firstly, under 60-NPG stress, the expression of many genes were up-regulated that enhanced the utilization of energy, nutrition, fatty acids (*tesB*), putrescine (*puuE*), amino acid (*sdaB*) and saccharide (*ptxR* and *rhaB*) by the cell. In contrast, NaCl (355 mM) treatment reduced the expression of genes that suppressed the usage of various substances such as glucoside (*albglA*), arabinose (*araC*), dipeptide (*ddpA*), unsaturated fatty acids (*fadJ/H*), urea (*hpxO*), lichenin (*licC*) and trehalose (*treA*). Hence, the discrepancy of amino acid accumulation under these two stress conditions was great. Moreover,

genes in 60-NPG were well distributed in both repressed and activated DEGs, the *gltI* regulates amino acid absorption and *tktB* reinforces the synthesis of aromatic amino acids. However, NaCl (355 mM) mainly negatively modulated the genes, such as *ilvA*, *pheA* and *trpB* (Table S5).

Genes related to the iron (Fe) ion were greatly elevated under 60-NPG stress such as *entE/F/S*, *exbB*, *feoB*, *feoA/B* and *HI-0362*, and these genes are involved in the assimilation of this ion. In addition, genes, viz., *iutA* and *sufD/S*, are also up-regulated under 60-NPG stress that shaped the protein of Fe–S, which is the cofactor of many enzymes. The structure of Fe–S is involved in almost all bioprocesses such as electron transport, redox reactions, protein stabilization and DNA synthetic repair (Zeng et al. 2007). However, the cell balanced the gain and loss of the ferric ion under NaCl (355 mM) stress, such that the expression levels of *efe* and *cycA* were increased, whereas for genes *cysA*, *fet* and *feoA* it was reduced.

Bacterial biofilms are polymers consisting of DNA, RNA, protein, ions and polysaccharides; they protect cells from harm (Penaloza-Vazquez et al. 2010). Under 60-NPG stress, only few transcripts involved in biofilm formation were altered (*ycgF* was induced, *yafK* was suppressed), whereas *clpVI*, *fimD*, *lsrB* and *yhbT* genes involved in the formation of biofilm were down-regulated by NaCl (355 mM). Unlike NaCl (355 mM), 60-NPG led to an increased expression of genes, viz., *otsA*, *proP* and *treS*, that are closely related to the absorption and synthesis of osmoprotectants such as trehalose and proline, which could stabilize as well as protect the cell membrane and protein structure and maintain the health of the bacteria (Wolf et al. 2003). Moreover, NaCl (355 mM) increased the expression of 30S (*rpsA/B/H/I/K/N*) and 50S (*rplC/D/E/M*) ribosomal proteins, DNA polymerase (*rpoC*) and elongation factors (*fusA*, *tufI*); and the synthesis (*ddC*) and transport (*plaP*) of polyamines, which in turn regulated the permeability of the membrane as well as induced the function of nucleotides and protein (Hobley et al. 2014). Yet, related processes were negatively affected by glyphosate.

In conclusion, *NRS-1* lives in the soil where osmotic force is common, and thus the bacterial cell is more familiar to osmotic pressure than glyphosate. Hence, the bacteria utilize genes, energy and substances as less as possible under NaCl (355 mM) stress, and enable mRNAs with powerful function to live easily through the stress. In comparison, under 60-NPG, the more serious the stress, the more genes are induced to guard the organism.

Expression response of *NRS-1* under 60-NPG and HCl stresses

Although the acidity of 60-NPG and HCl (pH 4.46) treatments was the same, a lot of differences appeared in terms

of DEGs (Table S5). Firstly, in 60-NPG stress, many genes such as *ostA*, *proP*, *ybaY*, and *yeiQ* were triggered that participate in anti-osmosis. In addition, this stress also induces genes that are involved in cell detoxification such as *terC* (bacteriostatic agent), *smvA* (excreted drugs) and *ynfC* (participated in detoxification); all these genes help to improve the endurance of the bacterium. Expression of many genes, viz., *dsdA*, *HI-1286*, *mutS*, *rdgC* and *xthA*, that are associated with DNA and protein repair systems, was decreased. These were the underlying factors of evolution that allows acclimation of the bacterial cell, and these processes are important for the survival of the cell. However, under HCl (pH 4.46) treatment these changes were not obvious.

The transcripts of genes such as *bamC*, *lgt*, *mrcA*, *murQ*, *yoaE* and *ytfL* showed a reduced response under acidic treatment (HCl), and these genes influenced the plasmalemma as well as cell wall permeability. However, such changes were not clear under 60-NPG. Being the outermost guard of the cell, the cell membrane or wall not only assists in maintaining the cell shape, but also protects the cell and provides communication; therefore, increasing or maintaining the expression levels of related mRNAs is important (Chahboune et al. 2005). However, the growth status of *NRS-1* was better in response to HCl (pH 4.46) treatment relative to 60-NPG; therefore, the down-regulated DEGs under acidic stress might be optional, the cell could save more resource and the growth was not affected. These results clearly showed that the more severe the glyphosate stress, the more complex were the processes employed by *NRS-1*, and these findings were similar to the above comparison of gene expression response of *NRS-1* under NaCl (355 mM) and 60-NPG. Hence, the cell will efficiently and economically utilize genes to confront familiar stress.

Expression response of *NRS-1* under 60-NPG and 110-NPG stresses

A number of identical and distinct DGEs were identified under two different concentrations of glyphosate (Table S5 and S6).

Similar reactions of *NRS-1* for 60-NPG and 110-NPG

Many DEGs were common in 60-NPG and 110-NPG treatments, and the most striking genes among them were trans-regulatory factors such as *arcA*, *arcB*, *fdhF*, *mppA*, *ompC*, *pckA* and *trkA*, and all these were inhibited by 60-NPG and 110-NPG stresses. The controlled *arcA* and *arcB* could increase the formation of the biofilm (Dai 2010); reduced *fdhF* function ensured the balance of energy and redox reactions (Lu et al. 2009; Trchounian et al. 2017); *mppA* and *trkA* negatively affected antibiotic and drug resistance (Grkovic et al. 2001; Castaneda-Garcia et al. 2011); *ompC*

encoded the porin of the cell, which acts as a transporter and its reduced expression would help to reduce drug absorption (Lin et al. 2014); *pckA* gene is involved in the metabolic pathway that consumes large amounts of energy, and its low expression is better for long-time survive in unfavorable conditions (Riordan et al. 2007). In addition, other common DEGs identified that are involved in anti-oxidant activity, acid or drugs ejection, ionic balance and energy absorption were all enhanced leading to adaptation to glyphosate stress.

Distinct reactions of NRS-1 for 60-NPG and 110-NPG

The number of DEGs that were different under 60-NPG and 110-NPG treatments was large compared to common DEGs. In 60-NPG, many genes involved in the enhanced utilization of saccharide and many amino acids like serine (*sdaB*) and arginine (*astD*, *astC*) were up-regulated, whereas genes, viz., *priB*, *rimM*, *malT*, *DBP2*, *helD* and *senI*, were down-regulated to inhibit protein synthesis under 60-NPG stress. However, in the case of 110-NPG treatment, the cell focused on utilizing sugars (*aglA*, *malP*, *malS*, *wzb*), and the protein synthesis was fully utilized. In addition, the expression of genes encoding RNA helicase (*hrpA*), DNA-binding protein (*hupB*), transcription factors (*RRT15* and *cytR*) and translation initiation protein (*infC*) was increased to reinforce the protein synthesis. The comparison led us to conclude that the less severe the glyphosate stress, the more economic methods will the cell employ to obtain the substance. This could provide energy and source of carbon to the cell, and the cell will absorb substances rather than synthesize to save energy and extend its life (Alonso Casajus et al. 2006; Wang and Wise 2011).

Furthermore, 60-NPG will activate genes that will lead to increased protection of the cell at the membrane and cell wall level. For example, *ycfS* gene was induced and is closely related to saccharin synthesis, directly used in the construction of the cell wall (Cossu et al. 2012), whereas *mreC* gene was down-regulated, which is a determinative factor for morphology, and its reduced expression initiates a protective change in the cell wall (Rogers et al. 2007). In addition, trypsinase was down-regulated to weaken the digestion of materials (Demaude et al. 2009). However, under 110-NPG stress weaker protection exists, for example, two drug efflux pump genes were down-regulated and more glyphosate would have entered into the cell; this situation was more serious than in 60-NPG. Two-component system is crucial for response to various surroundings, and the related genes such as *narJ/X/K/H/G*, *cheZ* and *uhpA* were up-regulated in 110-NPG; many of these genes were repressed in 60-NPG. Hence, this system might be unique for *NRS-1* to obtain relief from the extreme pressure and to give the cell a greater chance to survive.

The transcripts, viz., *entS*, *entE*, *fepA*, *fepB*, *feoB* and *sufD*, which are related to the ferric ion absorption and the Fe-S center synthesis revealed increased expression level in 60-NPG, whereas only few such genes were induced in 110-NPG. This metal ion usually acts as an active center of enzymes, and the differences here suggested that the activity of enzymes were low in 110-NPG, and the growth of the cell might be extremely slow (the same result as the growth curve in Fig. S1). Therefore, reducing the metabolism and activity of the cell could allow the cell to endure the extreme stress and relieve the damage from the drug.

Discussion

The *NRS-1* strain belongs to the genus of Gram-negative bacteria called *Enterobacter*, and has the remarkable feature of resistance to various environmental surroundings. Mechanisms at various levels would help the cell to successfully protect it against adverse conditions. During the process of selection, *NRS-1* strain has gained tolerance to high concentration of glyphosate, but at the cost of losing acidic and osmotic resistance. Hence, some changes must have occurred in the metabolic pathways or physical methods that lead to protection of *NRS-1* against glyphosate directly or indirectly, and these changes were heritable. What changes happened in *NRS-1* when it encountered glyphosate? How was the cell resistant to different doses of glyphosate? RNA-seq analysis was used to get a detailed explanation.

What changes happened in NRS-1 when it encountered glyphosate?

Information from the growth of *NRS-1* suggested that the expression of several mRNAs might have changed or shared part of the function to support the glyphosate resistance, and this resistance was gained at the cost of osmotic and acidic resistance (Fig. S1). According to this speculation, DEGs of *NRS-1* under the four different treatments, viz., 60-NPG, 110-NPG, NaCl (355 mM) and HCl (pH 4.46), were compared to identify the genes which might have changed the function. The 22 activated and 43 repressed genes were common among the DEGs in these four treatments, and only the genes associated with the resistance of the cell were selected for further exploration, which finally included 22 up-regulated and 3 down-regulated genes (Table 2). Among these 25 DEGs, the expression of 10 transcripts were affected by all the four treatments (60-NPG, 110-NPG, HCl and NaCl); the transcript of only one gene was influenced under three treatments (60-NPG, 110-NPG and NaCl); and the expression of 14 genes was changed in the 60-NPG, 110-NPG and HCl treatments (pH 4.46). Hence, this gene expression distribution is in agreement with the growth of the bacterial cell

Table 2 List of common DEGs that show the same expression pattern under different stress treatments used in the present study

S.No.	Treatments	Gene symbol	Up/down	Main function in KEGG/NCBI database
1	NaCl (355 mM) HCl (pH 4.46) 60-NPG 110-NPG	<i>cyoA</i>	Up	ATP
		<i>cyoB</i>	Up	ATP
		<i>glpD</i>	Up	Glycerophospholipid metabolism
		<i>gntT</i>	Up	Gluconate transporter, gluconate metabolism
		<i>aceE</i>	Up	TCA
		<i>aceF</i>	Up	TCA
		<i>mgo</i>	Up	TCA
		<i>rlmF</i>	Up	rRNA modification
		<i>metK</i>	Up	Cysteine and methionine metabolism
		<i>mppA</i>	Down	ABC transporters, beta-lactam resistance
2	NaCl (355 mM) 60-NPG 110-NPG	<i>mutS</i>	Down	Replication and repair
3	HCl (pH 4.46) 60-NPG 110-NPG	<i>tehA</i>	Up	Transporters, tellurite resistance
		<i>iutA</i>	Up	iron complex outer membrane receptor
		<i>yqfA</i>	Up	Pore-forming toxins
		<i>ycgF</i>	Up	Putative amino acid efflux protein ,transcription activator antirepressor
		<i>ybdL</i>	Up	Lysine biosynthesis, peptidoglycan biosynthesis
		<i>mtnK</i>	Up	Cysteine and methionine metabolism
		<i>cirA</i>	Up	Ferric iron-catecholate outer membrane transporter
		<i>HI_0362</i>	Up	Iron transport protein
		<i>mrcB</i>	Up	Peptidoglycan biosynthesis
		<i>fdhD</i>	Up	Formate dehydrogenase
		<i>ibpB</i>	Up	Lipopolysaccharide biosynthesis, molecular chaperone
		<i>arnB</i>	Up	Cationic antimicrobial peptide (CAMP) resistance, lipopolysaccharide biosynthesis
		<i>ybiB</i>	Up	Pili
		<i>ompC</i>	Down	Pore protein, beta-lactam resistance

under different stresses, and the ability of the cell to resist acidic condition was attenuated more seriously than NaCl (355 mM) resistance.

The KEGG database was used to find the function of these 25 genes and to identify the ways in which they could directly help to provide resistance against glyphosate (Table 2; Fig. 5). Genes related to energy synthesis, rRNA modification, peptidoglycan biosynthesis, replication and repairing were ruled out because they seemed to be not directly involved in glyphosate resistance; however, genes encoding proteins that are associated with transport, amino acids synthesis, lipopolysaccharide synthesis and pore formation were more likely to be involved in providing resistance against glyphosate directly, and the possible relationships with glyphosate are depicted in Fig. 5. Our results revealed that the expression of many genes involved in the amino acid synthesis such as *fdhD*, *metK* and *mtnK* were increased under glyphosate treatment, and these amino acids function to detoxify and degrade glyphosate. In addition, the degraded glyphosate will be effluxed by the cell

through membrane micropores which are actively induced by the *nyqfA* gene. The transporters encoded by genes such as *mppA*, *tehA* and *ycgF* combined with glyphosate to move it out of the cells and modify in the lipid bilayer in the form of lipopolysaccharides (LPSs). Transporters, viz., *mppA*, *tehA*, *ycgF* and pore protein (*ompC*), function in drug efflux and are defined as multidrug-resistance proteins (Grkovic et al. 2001; Viveiros et al. 2005; Liu et al. 2012; Correia et al. 2017). Hence, during the evolution of *NRS-1*, these proteins might prefer to pump the glyphosate out of the cell.

The bacterial toxin gene (*yqfA*) forms pores in the membrane and induces the death of the host cell (Dupont et al. 2007; von Rhein et al. 2009; Mašlaňová et al. 2018), but *NRS-1* distinctly stimulates this gene to form pores and might act as a glyphosate sensor which could rapidly induce stress signaling pathways in glyphosate stress. Lipopolysaccharides (*ibpB*, *arnB*) are the structures of the cell that communicate with the environment through structural modification (Lloret et al. 1995; De Castro et al. 2008), and the transcripts of *ibpB* and *arnB* are thought to sense the

Fig. 5 Transcripts that respond to glycosylate specifically, with the hypothesis of their associated drug-resistance mechanism. Glycosylate is found in cells which induce genes, viz., *fdhD*, *metK* and *mtuK*, leading to accumulation of aromatic amino acids; however, these amino acids detoxify and degrade glycosylate. The degraded

glycosylate is effluxed in the cell by membrane micropores which are actively induced by the *yqfA* gene. The transporters such as *mppA*, *tehA* and *yegF* combine with glycosylate to flow out of the cells and are then modified in the lipid bilayer in the form of lipopolysaccharides (LPSs)

glycosylate stress. Furthermore, genes, viz., *metK* and *mtuK*, participate in the synthesis of methionine and its derivatives, which mainly function as antioxidants and detoxify xenobiotics (Luo and Levine 2009; Nandi et al. 2005). However, the functions of methionine derivatives such as glutathione and polyamines are diverse and powerful, but in our case detoxification might be the most important function in glycosylate resistance. Formate dehydrogenase (*fdhD*) is a membrane-binding protein that functions to expel acids or protons out of cell and provides resistance against organic acids (Jormakka et al. 2003; Wilks et al. 2009). In addition, it degrades the pollutant formaldehyde (Roca et al. 2008), and *fdhD* works in glycosylate resistance and expels or degrades the glycosylate.

How was the cell resistant to different dose of glycosylate?

Different responses of *NRS-1* to 60-NPG and 110-NPG were examined to study the distinctive mechanisms of resistance for the two varying doses of glycosylate. The DEGs that were different in 60-NPG and 110-NPG treatments with

active roles were selected (up-regulated and repressed genes), and the DEGs of the two glycosylate treatments that were identical to DEGs in HCl (pH 4.46) and NaCl (355 mM) were removed to avoid the specific influence of osmotic or acidic stress. Hence, in this way we found a total of 23 and 42 DEGs in 60-NPG and 110-NPG, respectively (Table S5).

However, more genes and processes were induced by 110-NPG relative to 60-NPG treatment (Fig. 6). Under 60-NPG stress, more Fe was imported which is a cofactor of many enzymes (Zeng et al. 2007). These enzymes function in the cell substance metabolism that is the source of carbon and nitrogen to be used for the biosynthesis of fatty acids and lipoproteins, which are central components of the cell membrane. Hence, *NRS-1* took full advantage from a series of processes to protect the structure and function of the plasmalemma as well as to withstand unfavorable environments. At the same time, chaperones were up-regulated to stabilize the macromolecules and ensure the normal operation of the cell (Len et al. 2004; Bhat et al. 2017).

Moreover, under 110-NPG stress *bax* and *cytR* genes were up-regulated, leading to lipopolysaccharide degradation and

Fig. 6 Diagram showing different responses of *NRS-1* strain to two different concentrations of glyphosate. This figure shows that the number of genes induced under 60-NPG is less, while 110-NPG is extremely adverse and activates more pathways. In 60-NPG, glyphosate stimulates cells to transport Fe intracellularly, which acts as cofactors of enzymes involved in metabolic metabolism. In catabolism, it provides cells with C and N sources, synthetic fatty acids, lipoprotein and other cell membrane components, so microbes use membrane integrity and function to resist external stress. In 110-NPG, the most obvious up-regulated genes were *bax* and *cytR*, which led to the inhibition of the degradation for the lipopolysaccharide and

transcription process. However, other side of the cell is engaged in absorption of exogenous N as well as degradation of substances such as carbohydrates, nucleosides to provide N and C source for others cell; synthesis of amino acids for transcriptional and translation process; transcriptional and translation products can relieve membrane damage, to help cells re-film assembly. At the same time, 110-NPG induced two formate dehydrogenase genes and *wzb* (phosphate monostearate hydrolase) to speculate that it can degrade glyphosate. This series of changes reflects the positive effect of *NRS-1* on inhibitory regulation or injury in extreme stress response and mitigation

transcription inhibition. This in turn triggered a series of protective actions, such as cells assimilated more N sources from the outside environment, substances inside the cell (sugar and nucleosides) were broken down into C and N sources, which could be used to synthesize amino acids, and all sources were used in transcription and translation processes. Other protective actions included the expression of many transcription factors and inhibition of ribosome degradation, which were also triggered to safeguard the transcription and translation processes; products of the transcription and translation were finally involved in membrane assembly to relieve the loss of the structure. In addition, 110-NPG induced phosphate ester hydrolase (*wzb*), hydrolase and formate dehydrogenase, which might be associated with glyphosate degradation.

Lastly, our result revealed that many genes involved directly or indirectly in drug resistance showed different expressions with 60-NPG and 110-NPG treatments such as

drug efflux pump genes, two-component genes, ferric ion absorption and Fe-S center synthesis. This difference might be the possible reason for the differential resistance of *NRS-1* strain under different doses of glyphosate.

Conclusion

To understand how *NRS-1* did confront the glyphosate and what changes have occurred during the evolution of this bacterial strain; what was the difference under two different concentration of glyphosate; the RNA-seq gave the powerful explanation here, and many genes could have provided glyphosate tolerance directly are interesting to be further studied. These genes after successful validation can be potential candidates for engineering glyphosate-resistant crop plants to reduce production losses that result due to weeds.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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