Identification of regulated genes conferring resistance to high concentrations of glyphosate in a new strain of Enterobacter

Yun-Yan Fei, Jun-Yi Gai & Tuan-Jie Zhao

Soybean Research Institute/National Center for Soybean Improvement/National Key Laboratory for Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing, China

Correspondence: Tuan-Jie Zhao, Soybean Research Institute, Nanjing Agricultural University, Weigang 1 Hao, Xuanwu District, Nanjing 210095, Jiangsu Province, China.
Tel.: + 86 25 84399531; fax: + 86 25 84395331; e-mail: tjzhao@njau.edu.cn

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Enterobacter; glyphosate tolerance; microarray; differentially expressed gene.

Abstract
Glyphosate is a widely used herbicide that inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity. Most plants and microbes are sensitive to glyphosate. However, transgenic-resistant crops that contain a modified epsps obtained from the resistant microbes have been commercially successful and therefore, new resistance genes and their adaptive regulatory mechanisms are of great interest. In this study, a soil-borne, glyphosate-resistant bacterium was selected and identified as Enterobacter. The EPSPS in this strain was found to have been altered to a resistant one. A total of 42 differentially expressed genes (DEGs) in the glyphosate were screened using microarray techniques. Under treatment, argF, sdhA, ivbL, rrfA-H were downregulated, whereas the transcripts of speA, osmY, pflB, ahpC, fusA, deoA, uxaC, rpoD and a few ribosomal protein genes were upregulated. Data were verified by quantitative real-time PCR on selected genes. All transcriptional changes appeared to protect the bacteria from glyphosate and associated osmotic, acidic and oxidative stresses. Many DEGs may have the potential to confer resistance to glyphosate alone, and some may be closely related to the shikimate pathway, reflecting the complex gene interaction network for glyphosate resistance.

Introduction
Commerically available glyphosate, also known as Round-up®, is a broad-spectrum, non-selective organophosphate herbicide. Originally, all crops were sensitive to glyphosate; however, transgenic glyphosate-resistant crops including soybean, corn, cotton, potato and rape seed have become widespread and have led to glyphosate becoming one of the most popular herbicides available. Glyphosate reversibly inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) activity, after which EPSPS cannot be imported into chloroplasts, blocking the downstream synthesis of aromatic amino acids and secondary metabolites from chorismate, and enabling efficient weed control (Peñaloza-Vazquez et al., 1995; Yan et al., 2011).

EPSPS has been classified into three distinct classes. Class I is naturally glyphosate-sensitive, but its resistance can occur through site-directed mutagenesis. Three positions are considered to be important: Ala to Gly at position 80–120, Asp to Gly at position 120–160 and Thr to Ala at position 170–210. Class II naturally sustains efficient catalysis at high concentrations of glyphosate, and is only found in bacteria (Funke et al., 2009). Class III is found in Pseudomonas putida, and confers natural resistance to glyphosate. This class of genes shares < 30% identity with class I and class II enzymes (Sun et al., 2005).

The aroA (cp4-epsps) from Agrobacterium tumefaciens CP4 confers glyphosate resistance. Its product reduces the affinity of EPSPS for glyphosate, and therefore does not affect downstream pathways. Many other resistant proteins, including C-P lyase, glyphosate oxido-reductase, glyphosate acetyltransferase and glycine oxidase, induce resistance by degrading glyphosate (Pollegioni et al., 2011), most of these originate from microorganisms such as Escherichia sp., Azotobacter sp., P. fluorescens and Ochrobactrum anthropin (Moneke et al., 2010).

The evolution of glyphosate resistance in bacteria is a complex event, as it responds not only to toxicity from glyphosate itself but also to many other stresses induced by glyphosate, such as oxidative stress (Romero et al.,...
2011). Therefore, glyphosate-resistant bacteria may also be resistant to other herbicides, pesticides, bactericides or other stresses. The differentially expressed genes (DEGs) of Escherichia coli in glyphosate stress affect many systems, including amino acid metabolism, central carbon metabolism and cell motility (Lu et al., 2013). As such, the elucidation of the bacterial mechanism of glyphosate resistance may help to reveal new genes that confer resistance, leading to the discovery of potentially novel resistance pathways.

We identified a strain of Enterobacter isolated in China that exhibited resistance to glyphosate. Further, we obtained the transcriptional profiles of Enterobacter exposed to the herbicide. The aim of this study was to gain insights into the inherited changes in epsps and other non-target genes that confer glyphosate resistance in NRS-1.

Materials and methods

Isolation of the microorganism and the culture conditions

Taking into consideration that selective forces can contribute to microbial community adaptive evolution, a gradually increasing concentration of glyphosate in enrichment culture was used. Samples were collected from 5–25-cm depths of lawn soil at Nanjing Agricultural University. There were no records of the use of commercialized glyphosate in that soil. Bacteria were shaken (200 r.p.m.) in Luria–Bertani (LB) broth at 30 °C. Liquid LB cultures were supplemented with increasing concentrations of commercialized glyphosate (10 mg L\(^{-1}\) to 60 g L\(^{-1}\)) to acclimatize the microorganisms. The colonies growing in the presence of 60 g L\(^{-1}\) glyphosate were used for subsequent experiments. The wild-type strain was also obtained from the resampled soil.

To further investigate the growth reaction of the resistance strain and its wild type to possible stresses in 60 g L\(^{-1}\) glyphosate concentration, the strains were also cultured in LB with simulated stress treatments of 355 mM NaCl (osmotic shock) and pH 4.46 HCl (acidic shock). The OD\(_{600}\) values of the bacteria were measured to construct growth curves, and the initial concentration of bacteria was measured at OD\(_{600}\) = 0.01–0.03.

Microscopy of the bacteria

To identify the sample, Gram stain tests were performed under light microscopy (Beveridge & Schultze-Lam, 1996). Transmission electron microscopy (TEM) studies were also performed: bacteria were cultivated to an OD\(_{600}\) of 0.6–0.8, centrifuged (1500 g for 5 min at 4 °C), and the supernatant washed twice with phosphate-buffered solution (PBS) before samples were immediately fixed with 2.5% (v/v) glutaraldehyde prepared in PBS. The TEM testing was performed as previously described (Hajmeer et al., 2006).

Phylogenetic analysis of the organism

Modified three-step PCR amplifications were used to obtain the 16S rDNA and RNA polymerase beta-subunit encoding gene (rpoB) (Mollet et al., 1997; Miller et al., 2009). PCR products were sent to Invitrogen for sequencing. Other sequences were derived from the European Molecular Biology Laboratory, and phylogenetic analysis of the alignments and construction of the phylogenetic trees were performed with MEGA software, version 5 (Tamura et al., 2011). The clustalw alignment program and neighbor-joining (NJ) algorithm were applied, and the resulting tree topology was tested by bootstrap analysis based on 1000 re-samplings.

Studies of EPSP synthase

A PCR program using primers epsps–F, 5’-ATGGAATT CCTGACGTACAACTGATGTTACGGTTACAC C-3’, and epsps–R 5’-ATGTCTCGAGGCCGAGGTCTG ATGCATCAGCAGC-3’ was conducted to clone epsps, using the KOD-plus polymerase (Toyobo) and PCR conditions: 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 58 °C for 30 s and 68 °C for 2 min, and extension at 68 °C for 10 min. PCR products were sent to Invitrogen for sequencing.

Several epsps nucleotide and amino acid sequences were downloaded from NCBI, including both glyphosate-sensitive and glyphosate-resistant sequences, and studied using the same procedures used for 16S rDNA and rpoB.

DNA microarray experiments and data analysis

Bacteria for gene chips were incubated in LB in the presence and absence of 60 g L\(^{-1}\) glyphosate. At an OD\(_{600}\) of 0.6–0.8, total RNA was extracted from cells, and the concentration and quality of the RNA was checked by spectrophotometric measurement and gel electrophoresis. The Affymetrix E. coli Genome 2.0 Array was employed, and the reverse-transcription of RNA, cDNA fragmentation, labeling and hybridization were performed according to the manufacturer’s protocol. The normalization and comparison of the raw microarray data was accomplished using the robust multichip analysis (RMA) and significance analysis of microarrays (SAM) software. Molecule annotation and cluster analysis was performed according to the directions of CAPITALBIO MOLECULE ANNOTATION SYSTEM.
V3.0 and CLUSTER software (Irizarry et al., 2003). All experiments were done in triplicate with independent RNA samples. DEGs were analyzed in Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) to determine their effects on glyphosate.

**Verification of identified DEGs**

To confirm the results of the microarray data, quantitative real-time PCR (qPCR) was performed. Briefly, RNA from the samples was used for the reverse-transcription reaction according to PrimeScript RT Master Mix Perfect Real-Time (TaKaRa). PCR reactions were carried out using SYBR Premix Ex Taq (TaKaRa), and 16S rDNA was used as a reference gene. The primers of the selected genes are listed in Table 1. The relative mRNA levels from qPCR were analyzed using the equation \(2^{-\Delta\Delta Cq}\) of treatment/\(\Delta Cq\) of control (Pfaffl, 2001). Transcriptional changes of *epsps* were also detected by qPCR analysis.

**Results and discussion**

**Isolation of a new bacterial strain, NRS-1, with glyphosate resistance**

Most of the sampled microorganisms could not survive under high concentrations of glyphosate. However, a few clones were tolerant up to 60 g L\(^{-1}\) of glyphosate, and a novel bacterial clone named NRS-1 was isolated. Morphological identification of NRS-1 showed that it was a Gram-negative bacterium with a short rod and peritrichous, allowing motility (Supporting Information, Fig. S1).

Many studies have shown that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria, as their cell walls contain unique lipopolysaccharides that create a barrier against hydrophobic substances and macromolecules. However, this allows the slow penetration of antibiotics such as glyphosate and can promote the generation of resistance (Helander et al., 2010).

**Table 1. Primers used in PCR experiments**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fusA</em>-F</td>
<td>5'-CGTGCAGAAAGTACCGATGT-3'</td>
</tr>
<tr>
<td><em>fusA</em>-R</td>
<td>5'-CCACCTTGTAGTGCTGTTGAT-3'</td>
</tr>
<tr>
<td><em>deoA</em>-F</td>
<td>5'-CGCTGCTCACCGATATGAAC-3'</td>
</tr>
<tr>
<td><em>deoA</em>-R</td>
<td>5'-ATCAGCATCTCCACGCACA-3'</td>
</tr>
<tr>
<td><em>osmY</em>-F</td>
<td>5'-GCGTTCTGGGTAGCGGTTCT-3'</td>
</tr>
<tr>
<td><em>osmY</em>-R</td>
<td>5'-TTTCCCCCTTCGGTGTTG-3'</td>
</tr>
<tr>
<td>16S rRNA-F</td>
<td>5'-AGCAGCAACCTTTATCCTTGT-3'</td>
</tr>
<tr>
<td>16S rRNA-R</td>
<td>5'-ATCCCCACCTTCCAGTTT-3'</td>
</tr>
<tr>
<td><em>epsps</em>-F</td>
<td>5'-AGAGGTGGAAAGGGCCGAAG-3'</td>
</tr>
<tr>
<td><em>epsps</em>-R</td>
<td>5'-GCCACCAACAGAAGGCACA-3'</td>
</tr>
</tbody>
</table>

In addition, Gram-negative bacteria have been shown to be able to metabolize antibiotics (Sutherland, 1964).

**Molecular identification of the bacterial strain NRS-1**

The 16S rDNA sequence comprising 1360 nucleotides was determined and compared with sequences of some members of the family *Enterobacteriaceae*. An NJ tree was also formed (Fig. S2a). The NRS-1 strain was grouped closely with *E. cancerogenus* and *E. asburiae* with 98% and 99% sequence similarity, respectively.

The tree obtained from *rpoB* is an alternative accepted tool for the genotypic identification of *Enterobacteriaceae* (Adékambi et al., 2009). The NJ analysis in Fig. S2b shows that NRS-1 was clustered closely to *E. cloacae*, *E. dissolvens*, *E. kobei*, *E. hormaechei* and *E. asburiae* with 98% sequence similarity. The results of the 16S rDNA and *rpoB* sequence analysis and phenotypic characterization indicated that NRS-1 is a species in the genus *Enterobacter*. Such species are widely encountered all over the world (Wang et al., 2010) and many of them have adapted to various hostile surroundings. Some of the *Enterobacter* species utilize glyphosate as their sole source of phosphorus, nitrogen or carbon, and can inactivate glyphosate (Quinn et al., 1988; KrzyskoLupicka & Orlik, 1997). Some exhibit exceptionally higher levels of resistance to glyphosate compared with other bacteria (Ahemad & Khan, 2010).

**Characteristics of the *epsps* gene in NRS-1**

As shown in Fig. 1, the nucleotide sequence of *epsps* cloned from NRS-1 was clustered with glyphosate-sensitive strains. However, the translated sequence differed from the Class I EPSP synthases at position 179, where a conserved Thr was mutated into an Ala, conferring glyphosate resistance to the NRS-1 EPSPS. This result suggests that the *Enterobacter* strain has adapted to high glyphosate concentrations, and that the *epsps* gene is involved in *Enterobacter* survival. At the same time, comparison of key transcriptomic data between control and treated groups indicated that *epsps* transcription was up-regulated by 2.7-fold. As the fold change increase in *epsps* expression was not striking, it is plausible that other genes or pathways could be important in the mechanism of glyphosate resistance.

**Growth reaction of the strains under different stresses**

Both NRS-1 and wild-type *Enterobacter* grew well in LB with NaCl and HCl stress treatments (Fig. 2). It appears...
that the NRS-1 and wild-type bacteria have adapted to osmotic and acidic stresses (355 mM NaCl, pH 4.46 HCl) when the two strain types were compared under stress. Wild-type Enterobacter grew better than NRS-1 in the initial period, but NRS-1 quickly caught up and surpassed the wild type. This was an interesting phenomenon, which may have been caused by the change in NRS-1 metabolism or phenotype during culturing in glyphosate. The wild-type strain was completely depressed when cultured with glyphosate but NRS-1 grew readily in glyphosate. It is said that the toxicity of glyphosate was the main stress, and resistant epsps or many other genes related to glyphosate may be the reason for the survival of NRS-1 when exposed to high concentrations of glyphosate. **Identification of DEGs in response to high glyphosate concentrations**

In our transcriptional profiling studies, a total of 42 DEGs, 25 genes and 17 hypothetical proteins, were found in response to high concentrations of glyphosate (Table S1, Table 2). Bacteria under glyphosate stress may also suffer osmotic, acidic and oxidative shock stress induced by high concentrations of glyphosate and it is therefore possible that the genes might involve pathways that regulate these cellular stresses. Three genes, fusA, deoA and osmY, were selected for quantitative real-time PCR assays (Fig. 3). The results showed that expression trends of these genes were consistent with those detected by microarray analysis. **Downregulated genes in response to glyphosate treatment**

We observed reductions in the expression of ornithine carbamoyltransferase 2, chain F (argF), succinate dehydrogenase flavoprotein subunit (sdhA), ilvB operon leader peptide (ivbL) and 5S ribosomal RNA (rrfA-H), with fold
The identified DEGs on the GeneChip and their known roles in responses to some stresses

Table 2. The identified DEGs on the GeneChip and their known roles in responses to some stresses

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene title (symbol)</th>
<th>Fold change</th>
<th>Reported responses to the stresses possibly associated with high dosage of glyphosate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1765382_s_at</td>
<td>Ornithine carbamoyltransferase 2, chain F (argF)</td>
<td>−2.08</td>
<td>Repress expression to protect cells in osmotic stress (Tan &amp; Cooke, 1978; Vissers et al., 1982; Hajmeer et al., 2006)</td>
</tr>
<tr>
<td>1762750_s_at</td>
<td>Succinate dehydrogenase flavoprotein subunit (sdhA)</td>
<td>−2.04</td>
<td>Downregulated in the presence of glyphosate, oxidative stress (Anthony, 1978; Franchini &amp; Egli, 2006)</td>
</tr>
<tr>
<td>1766154_s_at</td>
<td>ilvB operon leader peptide (ivbL)</td>
<td>−2.12</td>
<td>Attenuated in osmotic, acidic or oxidative shock (Tsui &amp; Freundlich, 1985; Álvarez-Ordóñez et al., 2010; Senouci-Rezkallah et al., 2011)</td>
</tr>
<tr>
<td>1763897_s_at</td>
<td>30S ribosomal RNA (rrfA-H)</td>
<td>−4.76</td>
<td>Downregulated in the presence of oxidative stress (Gérard et al., 1999; Schultz, 2003; Bearson et al., 2009)</td>
</tr>
<tr>
<td>1765138_s_at</td>
<td>Arginine decarboxylase (speA)</td>
<td>+2.04</td>
<td>Against reactive oxygen, acid, high salt and osmotic stresses and various other environmental challenges (Jantaro et al., 2006; Pan et al., 2006; Burrell et al., 2009; Bearson et al., 2010)</td>
</tr>
<tr>
<td>1767463_s_at</td>
<td>Periplasmic protein (osmY)</td>
<td>+2.04</td>
<td>Hyperosmotically-induced (Oh et al., 2000)</td>
</tr>
<tr>
<td>1767207_s_at</td>
<td>Formate acetyltransferase 1 (pfB)</td>
<td>+2.08</td>
<td>Reassert the redox balance and provide energy in stresses (Sun et al., 2012)</td>
</tr>
<tr>
<td>1767961_s_at</td>
<td>Alkyl hydroperoxide reductase subunit C (ahpC)</td>
<td>+2.12</td>
<td>An antioxidant, against multiple stresses like heat, salt, pesticides (carbofuran), herbicides (paraquat), methyl tert-butylether (Mishra et al., 2009; Wasim et al., 2009)</td>
</tr>
<tr>
<td>1761999_s_at</td>
<td>Elongation factor G (fusA)</td>
<td>+2.27</td>
<td>Increase expression in acid stress (Len et al., 2004)</td>
</tr>
<tr>
<td>1760243_s_at</td>
<td>Thymidine phosphorylase (deoA)</td>
<td>+2.32</td>
<td></td>
</tr>
<tr>
<td>1760728_s_at</td>
<td>Glucuronorate isomerase (uxaC)</td>
<td>+4.54</td>
<td></td>
</tr>
<tr>
<td>1765431_s_at</td>
<td>RNA polymerase sigma factor RpoD (rpoD)</td>
<td>+2.27</td>
<td>Upregulated in high osmotic and heat-shock treatment (Klančnik et al., 2006; Zhang &amp; Baseman, 2011)</td>
</tr>
<tr>
<td>1760752_s_at</td>
<td>50S ribosomal protein L2 (rplB)</td>
<td>+2.50</td>
<td>Increased in stressful environments (Schnier, 1987; Mikulik et al., 2001)</td>
</tr>
<tr>
<td>1761917_s_at</td>
<td>50S ribosomal protein L4 (rplD)</td>
<td>+2.43</td>
<td></td>
</tr>
<tr>
<td>1767821_s_at</td>
<td>50S ribosomal protein L5 (rplE)</td>
<td>+2.27</td>
<td></td>
</tr>
<tr>
<td>1761244_s_at</td>
<td>50S ribosomal protein L22 (rplV)</td>
<td>+2.04</td>
<td></td>
</tr>
<tr>
<td>1762454_s_at</td>
<td>50S ribosomal protein L23 (rplW)</td>
<td>+2.70</td>
<td></td>
</tr>
<tr>
<td>1767321_s_at</td>
<td>50S ribosomal protein L24 (rplX)</td>
<td>+3.03</td>
<td></td>
</tr>
<tr>
<td>1762933_s_at</td>
<td>30S ribosomal protein S7 (rpsG)</td>
<td>+2.63</td>
<td></td>
</tr>
<tr>
<td>1767291_s_at</td>
<td>30S ribosomal protein S10 (rpsJ)</td>
<td>+2.12</td>
<td></td>
</tr>
<tr>
<td>1760745_s_at</td>
<td>30S ribosomal protein S12 (rpsL)</td>
<td>+2.63</td>
<td></td>
</tr>
<tr>
<td>1768397_s_at</td>
<td>30S ribosomal protein S17 (rpsQ)</td>
<td>+2.77</td>
<td></td>
</tr>
<tr>
<td>1761856_s_at</td>
<td>30S ribosomal protein S19 (rpsS)</td>
<td>+2.22</td>
<td></td>
</tr>
<tr>
<td>1766433_s_at</td>
<td>ncRNA (rprA)</td>
<td>+2.17</td>
<td>A regulatory non-coding RNA in adaptive responses (McCullen et al., 2010)</td>
</tr>
<tr>
<td>1762381_s_at</td>
<td>misc RNA (spf)</td>
<td>+2.85</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Fold-changes between microarray and qPCR methods. Array data, black columns; qPCR data, white columns. *P ≤ 0.05, **P ≤ 0.01 using Student’s t-test.

Transcription profiles of glyphosate-resistant Enterobacter

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concentrations of glyphosate. As a membrane-bound protein, \textit{sdhA} is located on the cell surface (Johnson \textit{et al.}, 2004) and its suppression affects the ability of glyphosate to cross cell membranes. A reduction in \textit{sdhA} can also limit the growth rate of cells, which can help \textit{Enterobacter} to survive under stress (Anthony, 1978).

The level of products of \textit{ivbL} and 5S ribosomal RNA was found to respond to various environmental stress; the expression of \textit{ivbL} was easily attenuated by osmotic, acidic or oxidative shock (Tsui \\& Freundlich, 1985; Álvarez-Ordóñez \textit{et al.}, 2010; Senouci-Rezkallah \textit{et al.}, 2011), and 5S ribosomal RNA was downregulated when cells encountered metabolic imbalances (Gérard \textit{et al.}, 1999; Schultz, 2003; Bearson \textit{et al.}, 2009).

**Upregulated genes in response to glyphosate treatment**

The glyphosate-adapted strain displayed 21 genes with increased transcription levels after glyphosate treatment (Table 2). They were all part of protective mechanisms induced by \textit{Enterobacter} to alleviate the damage induced by glyphosate. Among them, the periplasmic protein (\textit{osmY}) gene is hyperosmotically induced (Oh \textit{et al.}, 2000). Formate acetyltransferase 1 (\textit{pfIB}) can reassert the redox balance and provide energy for cell growth (Sun \textit{et al.}, 2000). Len \textit{et al.} (2004) reported an increase in elongation factor G (\textit{fusA}) expression during the acid-tolerant growth of \textit{Streptococcus}, which works by accelerating the rates of mRNA–tRNA translocation, stabilizing the conformation of the ribosome.

Proteins, including arginine decarboxylase (\textit{speA}), alkyl hydroperoxide reductase subunit C (\textit{ahpC}), RNA polymerase sigma factor \textit{RpoD} (\textit{rpoD}), ncRNA (\textit{rprA}), and sets of ribosomal proteins protect against multiple stresses. The \textit{speA} gene is involved in biofilm formation in microorganisms, and protects against environmental challenges (Burrell \textit{et al.}, 2010). It is upregulated under conditions of high salt, osmotic stress (Jantaro \textit{et al.}, 2006), reactive oxygen species (Pan \textit{et al.}, 2006) and acidic environments (Bearson \textit{et al.}, 2009). Gene \textit{ahpC} can scavenge peroxide (Wasim \textit{et al.}, 2009), neutralize its cellular toxicity (Charoenlap \textit{et al.}, 2012), and affect the morphology, surface properties, biofilms, aggregation and flocculation of microbes, all of which are important for adaptive responses (Wasim \textit{et al.}, 2009). The gene protects bacteria from heat, salt, pesticides (carbofuran), herbicides (paraquat), methyl tert-butylether, cadmium, copper and UV-B (Mishra \textit{et al.}, 2009). The \textit{rpoD} gene is involved in transcription, translation processes and biofilm formation, which are important for cell survival and proliferation under universal stress (Penaloza-Vázquez \textit{et al.}, 2010; Bai \textit{et al.}, 2012). The \textit{rprA} gene increases its mRNA levels to increase the transcription of \textit{rpos}, which is a stress response factor (McCullen \textit{et al.}, 2010). Ribosomal proteins assemble precursors, elongation factors and transcriptional modulators, and participate in fidelity decoding, which are important for ribosome function, and their expression is increased in stressful environments (Schnier, 1987; Mikulik \textit{et al.}, 2001).

Thymidine phosphorylase (\textit{deoA}), glucuronate isomerase (\textit{uxaC}) and miscellaneous RNA (\textit{spf}) are not known to be related to bacterial stress responses. Further research will be required to explain the findings that their expression was affected by exposure to glyphosate.

**Relationship among DEGs in response to glyphosate**

Genes found to have changed expression levels in response to glyphosate in this study were analyzed using KEGG (Fig. 4). Four lines of protection are induced to protect bacteria against glyphosate by these genes. On the cell membrane, periplasmic proteins provide physical defense as the first protection barrier. The second barrier is cell metabolism, where EPSPS and many associated proteins provide resistance to glyphosate. Under high concentrations of glyphosate, \textit{deoA}, \textit{uxaC}, \textit{pfIB} and \textit{speA}, which are directly or indirectly associated with carbon fixation, glycolysis, pentose or propanoate metabolism, were induced and the shikimate pathway then activated. However, \textit{sdhA} and \textit{argF} are downregulated to accumulate thymidine phosphorylase, which enhances the synthesis of aromatic amino acids. All of these DEGs not only integratively promote the shikimate pathway but also protect the bacteria...
from stresses such as acid, osmosis and oxidation caused by high concentrations of glyphosate. The third barrier is the combination of the four genes, \textit{rprA}, \textit{rpoD}, \textit{spf} and \textit{rbbL}, which work to regulate the other functional genes to help the cell survive in challenging surroundings. The last form of protection is from ribosome synthesis and is the foundation for bacterial survival under stress; as glyphosate is cytotoxic, enhanced ribosome synthesis is a plausible response. The downregulation of the \textit{rrfA–H} genes is the response to the destruction of ribosomes by glyphosate, but the ribosomal protein and \textit{fusA} are stimulated to protect against this over time.

In an experiment measuring global transcription, few changes in gene expression were detected. This could be several reasons for this. First, the \textit{Enterobacter} strain NRS-1 was selected in the presence of high glyphosate concentrations and adapted to growth in 60 g l\(^{-1}\) glyphosate; this markedly reduced the cellular damage caused by glyphosate. Secondly, hybridization-based microarrays have certain limitations and cannot provide a comprehensive picture of what exactly is happening in cells (Croucher & Thomson, 2010). Thirdly, there is not a specific genechip product for the transcription analysis of NRS-1 and therefore the \textit{E. coli} Genome 2.0 Array was used to detect DEGs due to the relatively close relationship between \textit{E. coli} and the NRS-1 strain. However, their genetic differences can also reduce the ability to identify more strongly regulated genes due to the poor match of gene hybridization. Nonetheless, the DEGs found in this study suggest that other pathways, in addition to the shikimate pathway, are modified directly or indirectly by glyphosate.

Bacterial adaptation can be achieved through several mechanisms. For example, gene duplication events can relieve the fitness cost; resistance mutations occur in target enzymes that abrogate drug binding without compromising enzymatic functions; intracellular drug concentrations are reduced by reducing influx or increasing efflux; lyases in cells can protect bacteria by degrading the herbicide; and plasmid-borne resistant genes are also crucial for adaptive evolution (Wong & Kassen, 2011). The bacteria may evolve by any or all of the mechanisms described above to survive in high concentrations of glyphosate. Many complex systems will act together to induce resistance to glyphosate, and many supporting and complementary mechanisms may simultaneously contribute to survival under the heavy selection pressure of high glyphosate concentration; however, to balance the change, NRS-1 certainly incurs a fitness cost.

**Conclusion**

The soil-borne \textit{Enterobacter} strain, NRS-1, successfully grew in medium supplemented with 60 g L\(^{-1}\) glyphosate. Compared with the strain grown in plain medium, several changes in gene expression were observed directly or indirectly in response to glyphosate; many pathways are involved that are very important for bacterial fitness. Collectively, the glyphosate-resistant \textit{epsps} and the DEGs induced by osmotic, acidic and oxidative stresses may confer resistance to glyphosate, either alone or in combination with each other.

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**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Transmission electron micrograph analysis of the sample.

Fig. S2. Phylogenetic neighbor-joining analysis; (a) neighbor-joining tree of 16S rDNA. (b) neighbor-joining tree of rpoB. ● indicated the bacteria strain is NSR-1 which is the object in the experiment.

Table S1. Other downregulated and upregulated genes on the GeneChip.