Iranian J Biotech. 2020 July;18(3): e2597 DOI: 10.30498/IJB.2020.204133.2597

Research Article

Novel *aroA* of Glyphosate-Tolerant Bacterium *Pseudomonas* sp. Strain HA-09 Isolated from Roundup-Contaminated Garden Soils in Iran

Hadi Ghaderitabar[1], Amir Mousavi[1*], Ali Hatef Salmanian[2], Faranak Hadi[3]

[1] Department of Plant Molecular Biotechnology, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.
[2] Department of Plant Bioproducts, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.
[3] Department of Biology, Faculty of Science, Lorestan University, Khoramabad, Iran.

**Corresponding author:* Amir Mousavi, Department of Plant Molecular Biotechnology, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.Tel: +98-21-44787310, Fax: +98-21-44787399 ; E-mail: m-amir@nigeb.ac.ir

Background: Glyphosate is a non-selective systemic herbicide with a broad spectrum of weed control that inhibits a key enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, in the shikimate pathway.

Objectives: Isolation and analysis of the *epsps (aroA)* gene responsible for glyphosate-tolerance in bacteria from Roundup-contaminated soils was the aim of this study.

Materials and Methods: Sampling was done from the soil of the gardens which were heavily contaminated by Roundup herbicide and then bacterial screening was performed in the presence of high concentrations of glyphosate. The genus of bacterium was identified via molecular methods such as 16S rRNA sequencing. The *aroA* gene of this bacterium ($aroA_{HA\text{-}09}$) was isolated using the primers designed-upon specific regions of *aroA* genes available in NCBI GenBank database. The PCR product was cloned, sequenced and subcloned into pET28a as an expression vector and transferred into *E. coli* strain BL21(DE3). The cells were inoculated in liquid M9 minimal medium containing IPTG and different concentrations of glyphosate.

Results: The genus of bacterium was identified as *Pseudomonas* sp. strain HA-09. The isolated $aroA_{HA\text{-}09}$ gene from this bacterium was approximately 2.2 kb in size. Bioassay of *E. coli* expressing this gene showed high tolerance to glyphosate (up to 300 mM).

Conclusions: The $aroA_{HA\text{-}09}$ gene could be considered as a novel and efficient candidate for development of glyphosate-tolerant crop plants.

Keywords: aroA gene, EPSP synthase, Glyphosate-tolerant, *Pseudomonas sp.*

1. Background

Genetic manipulation of crop plants to obtain resistance to the broad-spectrum and nonselective herbicide glyphosate (N-phosphonomethyl glycine) is one of the most effective and economic approach for weed elimination (1-3). Glyphosate-based herbicides, such as Roundup, inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) in the shikimate pathway (1). EPSPS (AroA) is the sixth enzyme in the shikimate pathway, which is essential for the synthesis of aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan) in plants, fungi and microorganisms (4-6). AroA catalyzes the transition of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) to produce 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (**Fig. 1**). It seems there is a lot of competition between PEP binding sites and glyphosate. Therefore, glyphosate competes with PEP for binding to the active site of AroA (7, 8).

Two classes of AroA sharing less than 30% analogous amino acid sequence have been identified (9). Class

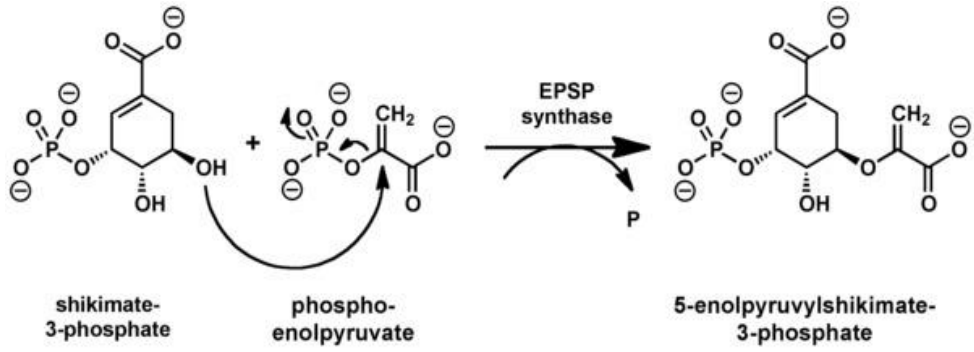

Figure 1. The sixth enzymatic step of the shikimate pathway

I enzymes found in all plants and bacteria such as *Escherichia coli* and *Salmonella typhimurium* show that low micromolar concentrations of glyphosate can inhibit the activity of the enzyme (10). Nevertheless, the glyphosate-tolerant EPSPS can be generated with one or more amino acid alteration in EPSPS sequence (11). This method was first reported in EPSPS of *S. typhimurium* with a single substitution of P101S (12). Another mutation was a G96A substitution in *E. coli* EPSPS sequence (13, 14). Class II enzymes found in bacteria such as *Pseudomonas* sp. strain PG2982, *Agrobacterium tumefaciens* strain CP4, *Streptococcus pneumonia*, *Staphylococcus aureus*, *Ochrobactrum anthropi*, *Bacillus cereus* and other bacteria species have shown good tolerance in the presence of high concentrations of glyphosate (15-24) and also have high affinity for PEP (25). Among different types of AroA enzymes which have been reported from various bacteria, only CP4 EPSP synthase derived from *A. tumefaciens* strain CP4 has been successfully used in glyphosate-tolerant commercial plants (9).

2. Objectives

The main goal of this study was to isolate bacterial strains present in Roundup-contaminated garden soils in different areas of Iran and to clone and characterize respective *aroA* gene(s) followed by expression and glyphosate resistance evaluation in *E. coli*.

3. Materials and Methods

3.1. Sample Collection from Glyphosate-Contaminated Soils

Soil samples were collected from gardens in different areas of Iran with 5-7 years history of continuous Roundup treatment (26). In each site, samples were taken from different points at 0–20 cm depth, completely mixed and stored at 4 °C in a refrigerator until use.

3.2. Isolation of Glyphosate-Tolerant Bacteria

For the inoculum, 5 g of each soil sample was suspended in 10 mL sterile water and after centrifugation, 1 mL of each sample was inoculated into 250 mL M9 minimal medium Erlenmeyer flasks and incubated at 30 °C. After two days, 200 µL of the content of each flask was spread onto M9 plates containing 60 mM glyphosate (glyphosate was purchased from Sigma-Aldrich). At the end, after three days of incubation at 30 °C, each colony was transferred to a new M9 medium plate containing 200 mM glyphosate. Among them, one colony grew well in this concentration of glyphosate that was selected for further verification.

3.3. Identification of Bacterium

In order to identify selected bacterial isolate using 16S rRNA gene amplification, the chromosomal DNA was isolated using a bacterial DNA extraction kit (Roche Applied Science, Germany). Then amplification by PCR was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACT-3') (27) under standard conditions in a 50 μL volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTP mixture, 1 μM primers, 1 μL of *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and 2 ng of template DNA. Thermocycling conditions were followed by initial denaturation at 94 °C for 4 min and 30 amplification cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, primer extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. After separation of PCR products on 1% agarose gel, desired DNA fragment was purified from the gel using agarose gel extraction kit (Roche Applied Science, Germany) and sequenced. For identifying the genus of bacterium, the obtained 16S rRNA gene sequence was compared to the NCBI (National Center for Biotechnology Information) GenBank database using nucleotide BLAST software (28).

3.4. Isolation and Cloning of aroA Gene from Bacterium
According to the similarity of the isolated bacterium (*Pseudomonas* sp. strain HA-09) with other species such as *P. fluorescens* F113, *P. fluorescens* Pf-5, *P. fluorescens* Pf0-1 and *P. brassicacearum* subsp. *brassicacearum* NFM421 resulting from the nucleotide BLAST in NCBI, the *aroA* gene sequences of these selected bacteria (CP003150.1 locus-tag PSF113_1640; CP000076 locus-tag Pfl_4309; CP000094 locus-tag Pfl01_4074; CP002585.1 locus-tag PSEBR_a1552) were aligned using ClustalW. Forward and reverse primers were then designed based on the full length of their *aroA* gene sequences. PCR was performed with new forward (5'-ATGATCGGTCGCCTGGTGG-3') and reverse (5'-TCACGACTGTGCCTCTTGTG-3') primers in 50 µL volume reaction mixture as previously mentioned. PCR amplicon was gel-purified, cloned into the pJET vector (Thermo Scientific CloneJET PCR Cloning Kit) and sequenced.
The sequence of the partial 16S rRNA gene of *Pseudomonas* sp. strain HA-09 and its *aroA* gene ($aroA_{HA-09}$) were deposited in EBI-EMBL database under the accession numbers LT844660 and LT853254, respectively.

3.5. Construction of Bacterial Expression Vector
After checking the restriction sites in $aroA_{HA-09}$ gene sequence, forward and reverse primers were designed with *Eco*RI and *Hind*III at 5′ and 3′ termini, respectively. Then the inserted gene was amplified by PCR, digested with *Eco*RI and *Hind*III, purified and cloned into the corresponding restriction sites of pET28a (Novagen). The plasmid pET28a-$aroA_{HA-09}$ was confirmed by DNA sequencing and transformed into competent *E. coli* strain BL21(DE3).

3.6. Expression and Purification of EPSPS
Initially and in order to optimize effecting factors such as temperature, pH adjustment, induction time and IPTG concentration, the *E. coli* BL21(DE3) harboring plasmid pET28a-$aroA_{HA-09}$ was grown into 15 Erlenmeyer flasks containing 250 mL M9 minimal medium and incubated at 37 °C. After optimization of the expression condition, the cells were grown to $OD_{600\ =}$ 0.3, and to express $aroA_{HA-09}$ gene, 0.5 mmol/L IPTG was added to the growing culture. After 8 h (before adding glyphosate), 1 mL of each bacterial culture was taken for SDS-PAGE expression analysis. The purification of the expressed EPSPS was performed according to the manufacturer's instructions of purification kit (QIAGEN). Transformed bacteria were cultured in liquid M9 minimal medium and incubated at 37 °C. Cells were harvested and then disrupted by adding lysis buffer containing lysozyme and nuclease and incubated on ice for 20 min. The lysate was centrifuged at 12000 rpm for 25 min in order to collect the supernatant. The cleared lysate containing the 6xHis-tagged protein was loaded onto the pre-equilibrated Ni-NTA spin column and centrifuged at 1500 rpm for 5 min. Then, after washing twice with wash buffer (containing 20 mM imidazole) and centrifugation at 2700 rpm for 2 min, protein was eluted twice with elution buffer containing 250 mM imidazole and centrifuged at 2900 rpm for 2 min. The protein concentration was measured by the Bradford assay method (29) and the purity of protein was determined by SDS–PAGE in a 12% polyacrylamide gel.

3.7. Cell Growth in the Presence of Glyphosate
For investigation and comparison of cell growth, 8h after IPTG inoculation, glyphosate was added into 15 Erlenmeyer flasks (in three repetitions) in concentrations of 60, 120, 180, 240 and 300 mM. The wild type *E. coli* BL21(DE3) was also inoculated in M9 minimal medium and considered as a negative control for each concentration of glyphosate. The optical density was measured at 600 nm at approximately 6 h intervals (6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 h).

4. Results

4.1. Isolation of Glyphosate-Tolerant Pseudomonas sp. Strain HA-09
The main objective of this research was to obtain high glyphosate resistant bacterial strains from indigenous soil. Among isolated bacterial strains from high-contaminated soils, 29 colonies were able to grow onto M9 plates containing 60 mM glyphosate; one of the isolates grew well in the presence of 200 mM glyphosate. The 16S rRNA gene sequence of this isolate was analyzed confirming that the isolated strain (HA-09) belongs to the genus *Pseudomonas.* Alignment of the sequenced amplicon using nucleotide BLAST software showed 99% identity to the 16S rRNA genes of *P. brassicacearum, P. fluorescens* and *P. thivervalensis* (**Fig. 2**).

4.2. Isolation and Cloning of the $aroA_{HA-09}$ Gene
Primers were designed based on the conserved region of the full length sequence of the most similar *aroA* genes with $aroA_{HA-09}$. Amplification of a complete novel *aroA* gene from *Pseudomonas* sp. strain HA-09 was then performed using degenerate primers and the PCR product, approximately 2.2 kb in size, was cloned and confirmed by sequencing. The resulting

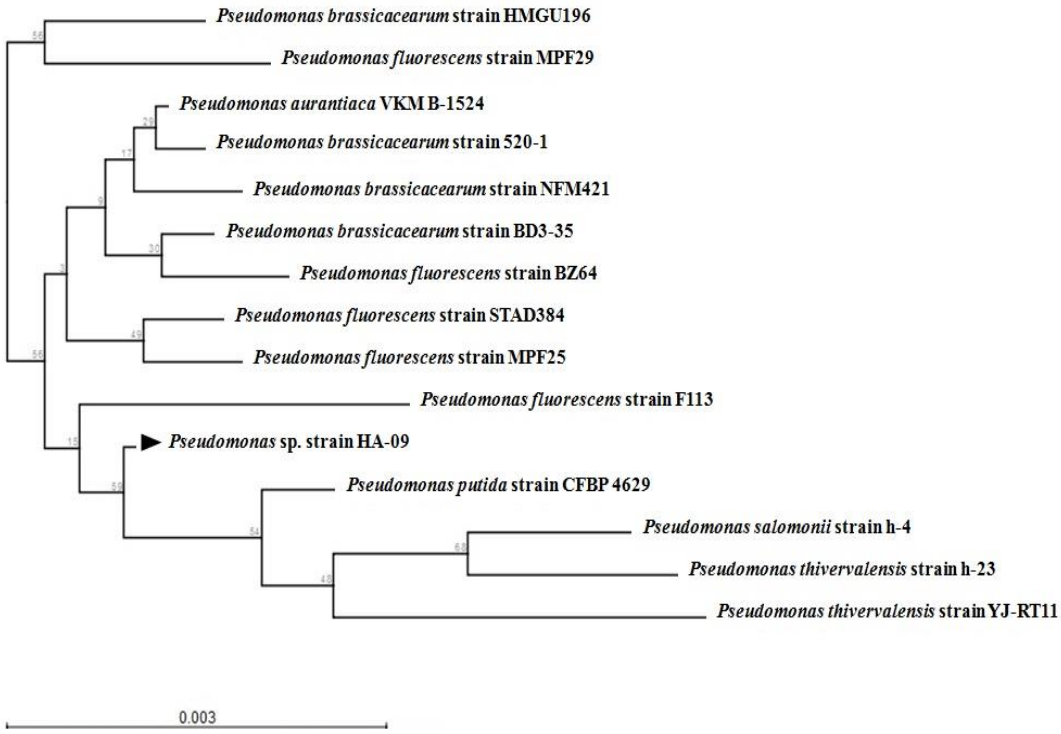

Figure 2. Phylogenetic tree based on partial 16S rRNA sequence, showing the relationships of *Pseudomonas* species and phylogenetic position of strain HA-09. Bootstrap values below 50% are not displayed.

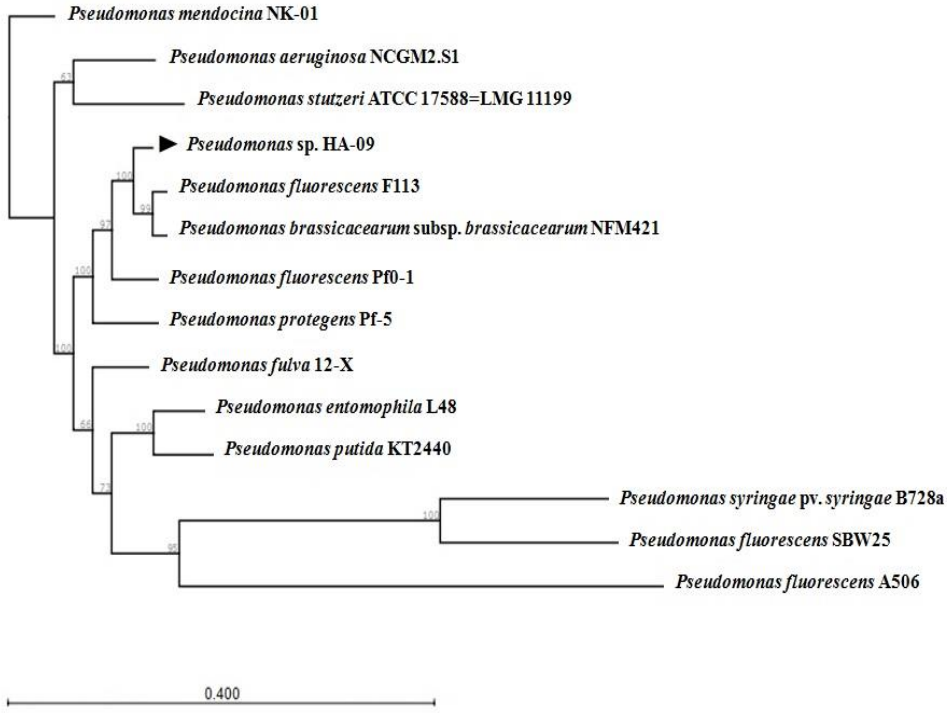

Figure 3. Phylogenetic tree based on the full-length sequence of *aroA* genes within *Pseudomonas* species.

sequence showed high percent of similarity with identified bacterial genes from *P. fluorescens* F113 and *P. brassicacearum* subsp. *brassicacearum* NFM421 (**Fig. 3**). The nucleic acid and deduced amino acid sequences of $aroA_{HA\text{-}09}$ were analyzed by the command line version of NCBI BLAST showing 95% nucleotide

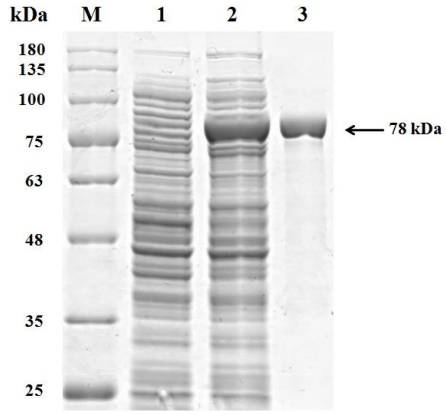

Figure 4. SDS-PAGE analysis of total lysate and purified 6xHis-AroA$_{HA-09}$ protein. M, Pre-stained protein ladder (PR901641, Sinaclon, Iran); 1 and 2, total bacterial proteins from IPTG-induced *E. coli* BL21(DE3) harboring pET28a and pET28a-*aroA*$_{HA-09}$ vectors, respectively; 3, purified 6xHis-AroA$_{HA-09}$ fractions from soluble proteins.

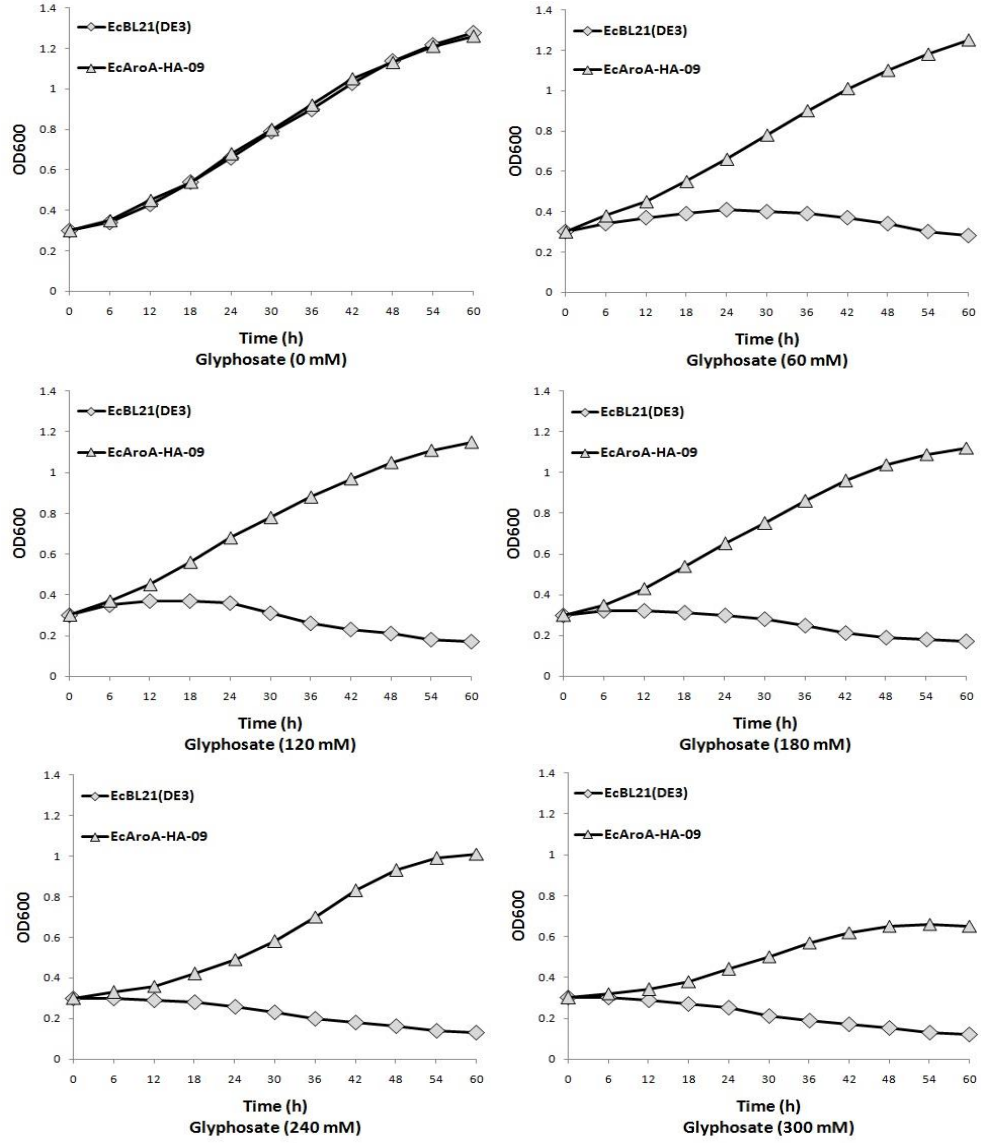

Figure 5. Growth of native *E. coli* BL21(DE3) abbreviated as EcBL21(DE3) compared to transformed *E. coli* BL21(DE3) harboring AroA$_{Pseudomonas\ sp.\ HA-09}$ abbreviated as EcAroA-HA-09 in liquid M9 minimal medium supplemented with glyphosate at different concentrations indicated in the figure.

sequence identity and 98% amino acid sequence identity to *aroA* genes of the above-mentioned three bacteria.

4.3. Expression and Purification of $AroA_{HA-09}$

The full length fragment of the $aroA_{HA-09}$ gene was subsequently cloned into pET28a expression vector. The *E. coli* BL21(DE3) harboring pET28a-$aroA_{HA-09}$ was harvested and disrupted by lysis buffer containing lysozyme after which a high percent of the target fusion protein was detected in the supernatant at 37 °C. The fusion protein was easily purified from the soluble fractions of the cell lysate using pre-equilibrated Ni-NTA spin column. To determine the optimal imidazole concentration needed to elute the protein, different concentrations of imidazole (up to 500 mM) were tested and finally, elution buffer containing 250 mM imidazole was used to elute the 6xHis-$AroA_{HA-09}$. SDS-PAGE analysis indicated distinct band at 78 kDa, which corresponds well with calculated molecular weight (**Fig. 4**).

4.4. Bioassay for Glyphosate Sensitivity

Growth curves of *E. coli* BL21(DE3) harboring expression vector are shown in **Figure 5**. The cells were inoculated and grown in liquid M9 minimal medium containing varying concentrations of glyphosate. All cultures were induced with 0.5 mmol/L IPTG. Transformed *E. coli* BL21(DE3) grew well in the presence of 300 mM glyphosate, whereas wild type *E. coli* BL21(DE3) was strongly inhibited in the presence of 60 mM glyphosate suggesting that $AroA_{Pseudomonas\ sp.\ HA-09}$ was more tolerant to glyphosate exposure than $AroA_{E.\ coli\ BL21(DE3).}$

5. Discussion

For the benefit of engineering glyphosate-tolerant crops, a novel *aroA* gene can be obtained from microorganisms that play key roles in glyphosate tolerance in many contaminated soils. Indiscriminate application of glyphosate-based herbicides such as Roundup, in addition to soil pollution, could lead to genetic mutations of soil microorganisms. These changes, regardless of the problems caused by pollution, can be used as an opportunity to extract novel genes and proteins for environmental and industrial utilizations. Since the 1980s, researchers have tried to identify glyphosate-insensitive bacteria from polluted soils. One of these bacteria is *Agrobacterium tumefaciens* strain CP4, which glyphosate-tolerant *epsps* (*cp4 epsps*) has been isolated from and commercially used in genetically modified crops such as soybean, corn, cotton, and canola (16, 30).

Site-directed mutagenesis in glyphosate-sensitive *epsps* genes has also been performed and demonstrated by many researchers partially conferring resistance to glyphosate (11, 31-35). In our previous report, point mutations were introduced in *E. coli epsps* gene (Gly96 to Ala and Ala183 to Thr) showing resistance to concentrations of glyphosate only up to 20 mM (33, 36), whereas in this study, we identified a novel glyphosate-tolerant *epsps* ($aroA_{Pseudomonas\ sp.\ HA-09}$) from the soil bacterium *Pseudomonas* sp. strain HA-09 and evaluated its expression in *E. coli* in the presence of higher glyphosate concentrations (up to 300 mM). This threshold of tolerance created by $AroA_{Pseudomonas\ sp.\ HA-09}$ enzyme has a significant importance in comparison with other similar studies in the past (up to 200 mM) (15, 19-22). Therefore, despite environmental problems, contaminated soils can be a good source for achieving glyphosate-insensitive EPSPS (AroA) enzyme.

6. Conclusion

The results suggest that the $AroA_{HA-09}$ from *Pseudomonas* sp. strain HA-09 shows significant resistance in the presence of high glyphosate concentrations. According to this, the $aroA_{HA-09}$ could be considered as a novel and efficient candidate gene in commercial development of transgenic glyphosate-tolerant plants in the future.

Acknowledgment

This project was financially supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB), project no. 516, Tehran, Iran. The authors wish to thank Sepideh Niazi for her technical assistance throughout the study and the students of horticultural science, Islamic Azad University (Science and Research Branch) for collecting the soil samples.

References

1. Steinrücken H, Amrhein N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochem Bioph Res Co*. 1980;**94**(4):1207-12. doi: org/10.1016/0006-291X(80)90547-1.
2. Duke SO, Powles SB. Glyphosate: a once-in-a-century herbicide. *Pest Manag Sci*. 2008;**64**(4):319-25. doi: org/10.1002/ps.1518.
3. Green JM. Current state of herbicides in herbicide-resistant crops. *Pest Manag Sci*. 2014;**70**(9):1351-7. doi: org/10.1002/ps.5378.
4. Herrmann KM, Weaver LM. The shikimate pathway. *Annu*

Rev Plant Biol. 1999;**50**(1):473-503. doi: org/10.1146/annurev.arplant.50.1.473.
5. Bentley R, Haslam E. The shikimate pathway—a metabolic tree with many branche. *Crit Rev Biochem Mol Biol*. 1990;**25**(5):307-84. doi: org/10.3109/10409239009090615.
6. Haslam E. Shikimic acid: metabolism and metabolites: *Wiley Chichester*, UK; 1993. doi: org/10.1002/ange.19951070532.
7. Gruys KJ, Walker MC, Sikorski JA. Substrate synergism and the steady-state kinetic reaction mechanism for EPSP synthase from *Escherichia coli*. *Biochem*. 1992;**31**(24):5534-44. doi: org/10.1021/bi00139a016.
8. Gruys KJ, Marzabadi MR, Pansegrau PD, Sikorski JA. Steady-state kinetic evaluation of the reverse reaction for *Escherichia coli* 5-enolpyruvoylshikimate-3-phosphate synthase. *Arch Biochem Biophys*. 1993;**304**(2):345-51. doi: org/10.1006/abbi.1993.1360.
9. Funke T, Han H, Healy-Fried ML, Fischer M, Schönbrunn E. Molecular basis for the herbicide resistance of Roundup Ready crops. *Proc Natl Acad Sci.*. 2006;**103**(35):13010-5. doi: org/10.1073/pnas.0603638103.
10. Franz JE, Mao MK, Sikorski JA. Glyphosate: a unique global herbicide: *American Chemical Society*; 1997. doi: org/10.1017/S0890037X0004433X.
11. Comai L, Sen LC, Stalker DM. An altered *aroA* gene product confers resistance to the herbicide glyphosate. *Science*. 1983;**221**(4608):370-1. doi: 10.1126/science.221.4608.370.
12. Comai L, Facciotti D, Hiatt W, Thompson G, Rose R, Stalker D. Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. *Nature*. 1985;**317**(6039):741-4. doi: org/10.1038/317741a0.
13. Kishore G, Brundage L, Kolk K, Padgette S, Rochester D, Huynh Q, et al., editors. Isolation, purification and characterization of a glyphosate-resistant mutant *E. coli* EPSP synthase1986: *Proc Fed Am Soc Exp Biol*.
14. Padgette S, Re DB, Gasser C, Eichholtz DA, Frazier R, Hironaka CM, et al. Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. *J Biol Chem*. 1991;**266**(33):22364-9.
15. Fitzgibbon J, Braymer H. Cloning of a gene from *Pseudomonas* sp. strain PG2982 conferring increased glyphosate resistance. *Appl Environ Microbiol*. 1990;**56**(11):3382-8.
16. Barry G, Kishore G, Padgette S. DNA encoding class II 5-enolpyruvyl shikimate-3-phosphate synthase—for producing plants and bacteria tolerant to glyphosate herbicides. *World Patent WO*. 1992;**9**(204,449):Q03321.
17. Du W, Wallis NG, Mazzulla MJ, Chalker AF, Zhang L, Liu WS, et al. Characterization of *Streptococcus pneumoniae* 5-enolpyruvylshikimate 3-phosphate synthase and its activation by univalent cations. *FEBS J*. 2000;**267**(1):222-7. doi: org/10.1046/j.1432-1327.2000.00994.x.
18. Priestman MA, Funke T, Singh IM, Crupper SS, Schönbrunn E. 5-Enolpyruvylshikimate-3-phosphate synthase from *Staphylococcus aureus* is insensitive to glyphosate. *FEBS lett*. 2005;**579**(3):728-32. doi: org/10.1016/j.febslet.2004.12.057.
19. Sun Y-C, Chen Y-C, Tian Z-X, Li F-M, Wang X-Y, Zhang J, et al. Novel AroA with high tolerance to glyphosate, encoded by a gene of *Pseudomonas putida* 4G-1 isolated from an extremely polluted environment in China. *Appl Environ Microbiol*. 2005;**71**(8):4771-6. doi: 10.1128/AEM.71.8.4771-4776.2005.
20. Tian Y-S, Xiong A-S, Xu J, Zhao W, Gao F, Fu X-Y, et al. Isolation from *Ochrobactrum anthropi* of a novel class II 5-enopyruvylshikimate-3-phosphate synthase with high tolerance to glyphosate. *Appl Environ Microbiol*.. 2010;**76**(17):6001-5. doi: 10.1128/AEM.00770-10.
21. Tian Y-S, Xu J, Han J, Zhao W, Fu X-Y, Peng R-H, et al. Complementary screening, identification and application of a novel class II 5-enopyruvylshikimate-3-phosphate synthase from *Bacillus cereus*. *World J Microb Biotechnol.* 2013;**29**(3):549-57. doi: 10.1007/s11274-012-1209-9.
22. Tian Y-S, Xu J, Xiong A-S, Zhao W, Gao F, Fu X-Y, et al. Functional characterization of Class II 5-enopyruvylshikimate-3-phosphate synthase from *Halothermothrix orenii* H168 in *Escherichia coli* and transgenic *Arabidopsis*. *Appl Microbiol Biotechnol.* 2012;**93**(1):241-50. doi: 10.1007/s00253-011-3443-8.
23. Wang L, Peng R, Tian Y, Han J, Zhao W, Wang B, et al. Characterization of a class II 5-enopyruvylshikimate-3-phosphate synthase with high tolerance to glyphosate from *Sinorhizobium fredii*. *World J Microb Biot*. 2014;**30**(11):2967-73. doi: 10.1007/s11274-014-1724-y.
24. Zhang Y, Yi L, Lin Y, Zhang L, Shao Z, Liu Z. Characterization and site-directed mutagenesis of a novel class II 5-enopyruvylshikimate-3-phosphate (EPSP) synthase from the deep-sea bacterium *Alcanivorax* sp. L27. *Enzyme Microb Technol.* 2014;**63**:64-70. doi: org/10.1016/j.enzmictec.2014.02.010.
25. Jin D, Lu W, Ping S, Zhang W, Chen J, Dun B, et al. Identification of a new gene encoding EPSPS with high glyphosate resistance from the metagenomic library. *Curr Microbiol*. 2007;**55**(4):350-5. doi: org/10.1007/s00284-007-0268-x.
26. Hadi F, Mousavi A, Noghabi KA, Tabar HG, Salmanian AH. New bacterial strain of the genus *Ochrobactrum* with glyphosate-degrading activity. *J Environ Sci Heal B*. 2013;**48**(3):208-13. doi: org/10.1080/03601234.2013.730319.
27. Lane D. 16S/23S rRNA sequencing. In 'Nucleic acid techniques in bacterial systematics'.(Eds E Stackebrandt, M Goodfellow) pp. 115–175. *John Wiley and Sons*: Chichester, UK; 1991.
28. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;**215**(3):403-10. doi: org/10.1016/S0022-2836(05)80360-2.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;**72**(1-2):248-54. doi: org/10.1006/abio.1976.9999.
30. Padgette SR, Kolacz K, Delannay X, Re D, LaVallee B, Tinius C, et al. Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci*. 1995;**35**(5):1451-61. doi: 10.2135/cropsci1995.0011183X003500050032x.
31. Eschenburg S, Healy ML, Priestman MA, Lushington GH, Schönbrunn E. How the mutation glycine96 to alanine confers glyphosate insensitivity to 5-enolpyruvyl shikimate-3-phosphate synthase from *Escherichia coli*. *Planta*. 2002;**216**(1):129-35. doi: 10.1007/s00425-002-0908-0.
32. Zhou M, Xu H, Wei X, Ye Z, Wei L, Gong W, et al. Identification of a glyphosate-resistant mutant of rice 5-enolpyruvylshikimate 3-phosphate synthase using a directed evolution strategy. *Plant Physiol*. 2006;**140**(1):184-95. doi: org/10.1104/pp.105.068577.
33. Kahrizi D, Salmanian AH, Afshari A, Moieni A, Mousavi A.

Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. *Plant Cell Rep*. 2007;**26**(1):95-104. doi: org/10.1007/s00299-006-0208-4.

34. Funke T, Yang Y, Han H, Healy-Fried M, Olesen S, Becker A, et al. Structural basis of glyphosate resistance resulting from the double mutation Thr97→ Ile and Pro101→ Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. *J Biol Chem*. 2009;**284**(15):9854-60. doi: 10.1074/jbc.M809771200.
35. Tian YS, Xu J, Peng RH, Xiong AS, Xu H, Zhao W, et al. Mutation by DNA shuffling of 5-enolpyruvylshikimate-3-phosphate synthase from *Malus domestica* for improved glyphosate resistance. *Plant Biotechnol J*. 2013;**11**(7):829-38. doi: org/10.1111/pbi.12074.
36. Salmanian AH, Zakikhan K, Afshari A, Moshashaie M. Site-directed mutagenesis, expression and biological activity of *E. coli* 5-enolpyruvylshikimate 3-phosphate synthase gene. *Iran J Biotechnol*. 2006;**4**(4):224-9.