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Bioremediation potential of glyphosate-degrading *Pseudomonas* spp. strains isolated from contaminated soil

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Bacterial strains capable of utilizing glyphosate as the sole carbon source were isolated from contaminated soil by the enrichment culture method and identified based on partial 16S rRNA gene sequence analysis. *Pseudomonas* spp. strains GA07, GA09 and GC04 demonstrated the best degradation capabilities towards glyphosate and were used for the laboratory experiments of glyphosate bioremediation. Inoculating glyphosate-treated soil samples with these three strains resulted in a 2–3 times higher rate of glyphosate removal than that in non-inoculated soil. The degradation kinetics was found to follow a first-order model with regression values greater than 0.96. Cell numbers of the introduced bacteria decreased from 4.4×10^6 CFU/g to $3.4–6.7 \times 10^5$ CFU/g dry soil within 18 days of inoculation. Due to the intense degradation of glyphosate, the total dehydrogenase activity of the soil microbial community increased by 21.2–25.6%. Analysis of glyphosate degradation products in cell-free extracts showed that glyphosate breakdown in strain GA09 was catalyzed both by C-P lyase and glyphosate oxidoreductase. Strains GA07 and GC04 degraded glyphosate only via glyphosate oxidoreductase, but no further metabolite was detected. These results highlight the potential of the isolated bacteria to be used in the bioremediation of GP-contaminated soils.

Key Words: bioremediation; degradation; glyphosate; metabolite; *Pseudomonas*

Introduction

Glyphosate [N-(phosphonomethyl)glycine] (GP) is a broad-spectrum, non-selective herbicide widely used in agriculture for the control of weedy species. Its mode of action is to inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which involved in the biosynthesis of the aromatic amino acids in plants (Tomlin, 2006). The extensive use of GP leads to a potential risk of contamination. Due to its ability to accumulate in soils, GP causes continuous negative effects on the soil ecosystem by inhibiting the growth of many indigenous microorganisms (Carlise and Trevors, 1988). Although GP is thought to be non-toxic or relatively low toxic to mammals, because the shikimic acid pathway is specific to plants and microbes, some recent data has shown that GP possesses genotoxicity (Bolognesi et al., 2009) and cutaneous toxicity (Nagami et al., 2005). Benachour and Seralini (2009) reported that GP exposure caused the inhibition of the mitochondrial succinate dehydrogenase activity, resulting in the death of umbilical, embryonic, and placental cells of humans. Furthermore, GP is reported to be toxic to various kinds of aquatic organisms, such as *Daphnia magna* (Alberdi et al., 1996), *Cyprinus carpio* (Cattaneo et al., 2011), *Anguilla anguilla* (Guilherme et al., 2012) and *Jenynsia multidentata* (Sandrini et al., 2013).

The primary way of GP elimination in the environment is microbial degradation, which yields aminomethylphosphonic acid (AMPA) and several minor metabolites (Rueppel et al., 1977; Strange-Hansen et al., 2004). The half-life of GP in soil is varied, ranging from 2 to 197 days, and appears to depend on soil type, climate conditions and the level of microbial activity (Giesey et

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al., 2000; Sorensen et al., 2006). In consideration of GP's high resistance to chemical degradation and photodecomposition, bioremediation is regarded as the most efficient process of GP removal from the contaminated soils. Studies have described GP mineralization by several microorganisms from genera *Achromobacter* (Ermakova et al., 2010), *Arthrobacter* (Pipke et al., 1988), *Bacillus* (Fan et al., 2012), *Flavobacterium* (Balthazor and Hallas, 1986), *Ochrobactrum* (Ermakova et al., 2010), *Pseudomonas* (Jacob et al., 1988) and *Streptomycete* (Obojska et al., 1999). However, reports on the biodegradation behaviors of GP in contaminated soils are still limited.

The aim of the present work was to investigate the bioremediation of GP-contaminated soils by the isolated bacterial strains under laboratory conditions. GP metabolic pathways involved in these strains were also studied.

Materials and Methods

Chemicals and media. Glyphosate standard (99.5%) was obtained from Chengdu Ai Keda Chemical Technology Co., Ltd. (Chengdu, China). Solvents for HPLC were purchased from Sigma-Aldrich, USA. All other chemicals and solvents used were analytical grade. The mineral salt medium (MSM) containing (g/L) NH_3SO_4 2.0; K_2HPO_4 0.625; $NaH_2PO_4 \cdot 2H_2O$ 0.6; $MgSO_4 \cdot 7H_2O$ 0.2 and $CaCl_2 \cdot 6H_2O$ 0.15 (pH 7.0) were used for the isolation and biodegradation experiments. The medium was sterilized by autoclaving at 121°C for 30 min.

Enrichment and isolation. Soil samples were collected from a herbicide manufacturer in Pengshan, China, which had produced GP for many years. Approximately 5 g of each soil sample was added to a 250-mL Erlenmeyer flask containing 50 mL of sterilized MSM supplemented 0.5 g/L GP as the sole carbon source, and incubated at 33°C for 7 days under agitation (180 rpm). Then 5 mL of the fermented broth was transferred to 50 mL fresh MSM amended with 1 g/L GP. Four subcultures were performed before serial dilutions (10^{-2} to 10^{-6}) of final enrichment cultures were plated on MSM agar plates containing 0.5 g/L GP. Colonies were picked based on distinct colony morphology and then inoculated into liquid medium to test the use of GP.

GP degradation in liquid culture. The inoculum for all the biodegradation experiments was prepared by growing strains in 25 mL of MSM containing 0.5 g/L GP at 33°C and 180 rpm. Cells were harvested in the logarithmic growth phase by centrifugation at 5000 rpm for 10 min, washed twice with 25 mL of sterilized 0.2 M phosphate buffer (pH 7.0) and suspended in fresh MSM. GP degradation and the growth of the strains were monitored concomitantly in 250-mL Erlenmeyer flasks containing 50 mL sterile MSM spiked with 1 g/L GP. Colony forming units (CFU/mL) were quantified by the dilution plate count technique, and the initial cell density of the culture was 1.2–1.4×10^7 CFU/mL. The flasks were incubated at 33°C on a shaker at 180 rpm for up to 5 days and the following physiological characteristics were determined: specific growth rate μ (/h), lag phase duration and degradation efficiency Q (g GP/g dry biomass). The strains with high GP degradation efficiency were chosen for further studies. The biomass concentration in liquid media was monitored by measuring its absorbance at 600 nm wavelength with a spectrophotometer (Pgeneral, model TU-1800, Beijing, China). OD_{600} values were then converted to dry biomass (0.54 g/L per OD_{600} unit) using a calibration curve.

Taxonomic identification. The selected strains were identified by the morphology and 16S rDNA gene analysis. Cell morphology was observed with an electron microscope (Olympus, Japan). The 16S rDNA gene was amplified by PCR using the following primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR conditions consisted of a denaturation step at 95°C for 5 min, 24 amplification cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 10 min. The purified PCR products were sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The sequencing result was aligned against similar sequences obtained from the GenBank database. Neighbour-joining analysis with 1000 bootstrap replicates was then performed using the software program MEGA (version 5.0).

Soil bioremediation in laboratory-scale experiments. Soil samples used in bioremediation experiments were collected from a grass-covered field at Sichuan Academy of Agricultural Science (Chengdu, China). Soil properties were as follows: organic matter 1.6%, clay 37.9%, sand 19.5%, silt 25.1% and pH 6.9. These soils were never exposed to any pesticide before. Soil samples were sieved (2 mm) and placed into plastic cups (600 g of soils in each). GP was aseptically introduced to soil samples at a concentration of 50 mg/kg dry soil. Microbial suspensions (approximately 4.4×10^6 CFU/g dry soil) were then inoculated into soils by drip irrigation 72 h after the GP application. In addition, samples of non-inoculated soils were kept as controls. Soils were incubated at 20% of maximum water holding capacity in the dark at the room temperature (19–26°C). All experiments were carried out in triplicate. Soil samples were withdrawn at regular time intervals for the evaluation of GP content. GP remaining in the liquid phase was determined after the aqueous extraction, and then the residual GP (absorbed GP) was extracted by NaOH (Shushkova et al., 2010). Moreover, the total CFU of GP utilizing bacteria was determined by a serial dilution technique on MSM using 0.5 g/L GP as the sole carbon source. Cell numbers of the introduced strain were calculated as the difference between the total amount of GP-degrading cells and the amount of indigenous GP-degrading cells. The dehydrogenase (DH) activity of the soil microbial community was also analyzed by the method of Zvyagintsev (1991).

Identification of GP metabolites. Cells were harvested during exponential growth by centrifugation at 8000 rpm and 4°C for 15 min, washed twice with a phosphate buffer (0.05 M; pH 7.0), resuspended in the same buffer and disintegrated using an ultrasonic probe (45 cycles of 5 s on and 5 s off at 280 W). The homogenate was centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was filtered through 0.22 μm membrane filters. Subsequently, 0.3 mL of the filtrates were added to 2.7 mL of the phosphate buffer (0.05 M; pH 7.0) with 0.5 g/L GP. The mix-

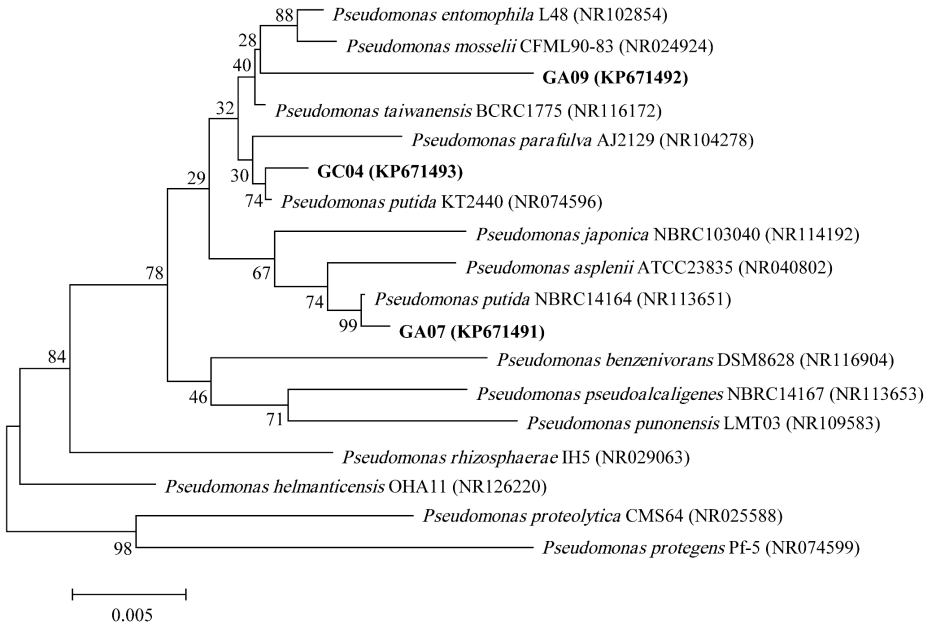

Fig. 1. Phylogenetic analysis of GA07, GA09, GC04 and related species by the Neighbor Joining method based on 16S rDNA gene sequences. Bootstrap values (%) are indicated at the nodes.

The bar represents sequence divergence. The accession numbers are shown in parentheses.

tures were incubated at 33°C for 2 h. After addition of 0.5 mL HCl (1 M), the reaction mixtures were used as samples for the analysis of GP metabolites. GP in the culture liquid and soil extracts was detected by HPLC (LC-20A Shimadzu, Japan) with UV detection at 240 nm using an Intersil ODS-SP C18 reversed-phase column (5 μm × 4.6 mm × 150 mm) and 10 mM phosphoric acid as the mobile phase at a flow rate of 0.8 mL/min. AMPA, glycine and sarcosine were determined following the procedures of Zelenkova and Vinokurova (2008). Glyoxylate was determined by the method of Qureshi et al. (1982). Formaldehyde was measured by the Hantzsch method (Nash, 1953).

Data analysis. The degradation rate constant (k) is calculated using the first-order kinetic model (Eq. (1)):

$$C_t = C_0 \times e^{-kt}, \quad (1)$$

where C_0 is the initial GP concentration, C_t is the remaining GP at time t and t is the degradation period in days.

The half-life (DT_{50}) of GP was calculated using the algorithm expressed in Eq. (2):

$$DT_{50} = \frac{\ln 2}{k}, \quad (2)$$

where k is the degradation rate constant.

Results and Discussion

Isolation and selection of GP-degrading bacteria

23 pure bacterial strains capable of utilizing GP as sole carbon and energy source were isolated from enrichment culture. As the results of the biodegradation activity test, strains GA07, GA09 and GC04 were chosen for further studies as demonstrating relatively high degrading efficiency (5.36, 4.38 and 5.93 g GP/g dry biomass) and specific growth rate (0.26, 0.31 and 0.24/h) (Table 1). Among the three strains, GC04 possessed the greatest degrading activity towards GP. These strains were cultivated in liquid MSM at a low concentration of GP (0.5 g/L) for 7 days and then transferred into fresh MSM amended with 0.5 g/L GP. Several re-inoculations were done to adapt the strains to growth on GP.

Table 1. Physiological characteristics of GP-degrading strains.

Microorganisms	μ (/h)	Lag phase (h)	Q (g GP/g dry biomass)
GA07	0.26 ± 0.02	18	5.36 ± 0.14
GA09	0.31 ± 0.02	18	4.38 ± 0.33
GC04	0.24 ± 0.01	14	5.93 ± 0.38

"±" indicates the standard error of the mean of triplicate.

Identification of the bacterial isolates

Strains GA07, GA09 and GC04 were gram-negative, rod-shaped and aerobic. The sequences (about 1400 bp) of the partial 16S rRNA gene of the three strains were submitted to GenBank and compared with the sequences of previously reported strains. Phylogenetic comparison of the nucleotide sequences indicated that they showed a high similarity with the species of genus *Pseudomonas*. Strains GC04 and GA07 clustered strongly (99% bootstrap support) with *Pseudomonas putida* KT2440 and *Pseudomonas putida* NCBC14167, respectively. Of the named species, GA09 was most closely related to *Pseudomonas taiwanensis* BCRC17751 (97.8% sequence similarity). On the basis of the BLASTn results, a phylogenetic tree (Fig. 1) showing the relationship between these three strains

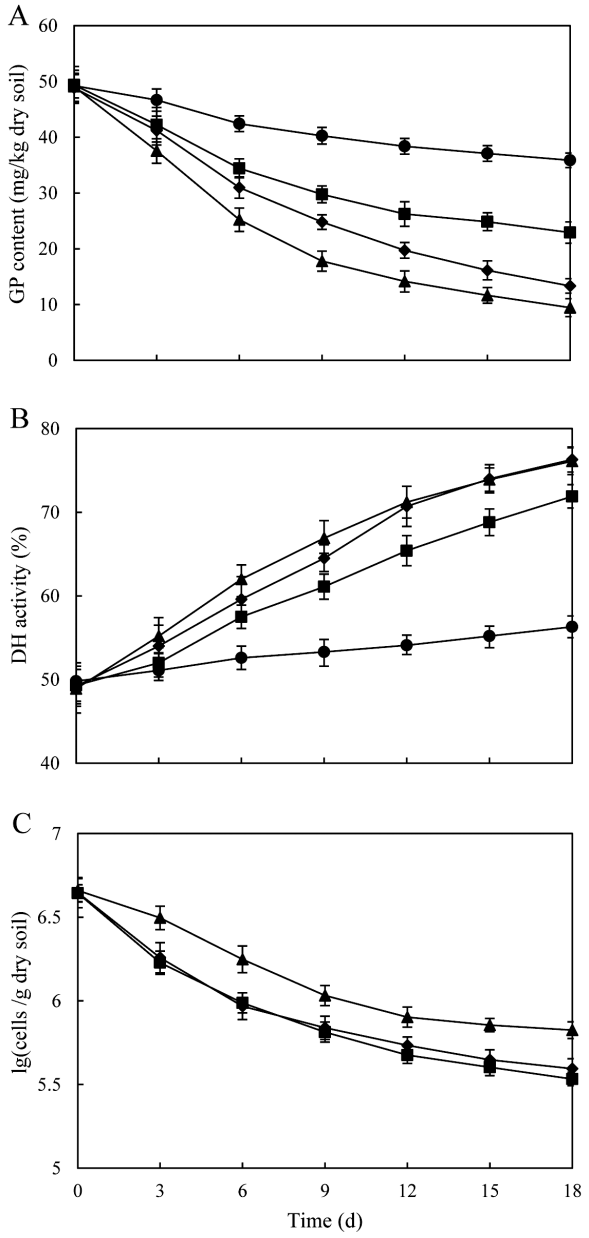

Fig. 2. Dynamics of GP content (a), DH activity (b) and cell numbers of the introduced strains (c) during the bioremediation of GP-contaminated soils in laboratory-scale experiments.

●, control; ◆, non-sterilized soil introduced with strain GA07; ■, non-sterilized soil introduced with strain GA09; ▲ non-sterilized soil introduced with strain GC04. The vertical bars indicate the standard error of the mean of triplicate.

and the closely related *Pseudomonas* spp. strains was constructed.

Bacteria from genus *Pseudomonas* are known as very metabolically active bacteria that can utilize a wide range of xenobiotic compounds. *Pseudomonas pseudomallei* 22 (Penaloza-Vazquez et al., 1995) degraded 85% of GP at concentrations of 0.17 g/L in a liquid medium within 80 h. Jacob et al. (1988) isolated a *Pseudomonas* sp. strain LBr which completely metabolized 3.21 g/L GP with a degrading efficiency of about 2 g GP/g dry biomass. Compared with these previously reported *Pseudomonas* strains, the 3 bacteria isolated in the present study exhibit markedly higher GP-degrading capabilities.

Table 2. Kinetic parameters of GP degradation in soil samples.

Soil treatments	k (/day)	DT_{50} (day)	R^2
nSS	0.0180 ± 0.0011	38.65 ± 2.36	0.9701
nSS + GA07	0.0740 ± 0.0069	9.45 ± 0.88	0.9972
nSS + GA09	0.0434 ± 0.0034	16.07 ± 1.26	0.9637
nSS + GC04	0.0936 ± 0.0060	7.43 ± 0.47	0.9834

nSS, non-sterilized soil; +, introduced with; k, degradation rate constants; DT_{50}, half-lives of GP; R^2, correlation coefficient. "±" indicates the standard error of the mean of triplicate.

Soil bioremediation in the laboratory

The efficiency of GP biodegradation by the introduced *Pseudomonas* spp. strains in soil samples was tested. The degradation dynamics of GP under various conditions are shown in Fig. 2A. After 18 days of incubation, indigenous microorganisms (control) degraded GP by 27.2% with a rate constant (k) of 0.0180/day and a half-life (DT_{50}) of 38.65 days (Table 2), following first-order kinetics. When strains GA07, GA09 and GC04 were inoculated into the soils, the removal rate of GP reached 72.7%, 53.6% and 80.8%, respectively, indicating that these strains could enhance the degradation of the pesticide in cooperation with the indigenous microorganisms. Kinetic data showed that the GP disappearance process was characterized by rate constants (k) of 0.0740, 0.0434 and 0.0946/day for strains GA07, GA09 and GC04, respectively. The DT_{50} calculated from the linear equation was 9.45, 16.07 and 7.43 days for strains GA07, GA09 and GC04, respectively. The most effective degradation occurred during the first 6 days in the experiment as 30.3–48.7% of the pesticide was utilized. In the following period, the GP biodegradation efficiency was observed to decrease noticeably.

As indicated by other experiments, GP biodegradation in soils was described as two-phase dynamics (Getenga and Kengara, 2004; Sorensen et al., 2006). The water-extractable GP was consumed during the initial fast phase, and the majority of the GP associated with the soil matrix (adsorbed GP) degraded considerably more slowly in the second phase. The bioavailability of GP depends on its degree of sorption and the intensity of mineralization. In this study, about 81.9% of GP was adsorbed by the soil matrix within 3 days after GP application. At the end of the experiment, water-extractable GP (18.1% of total content) was utilized in all the experimental variants. Thus, the introduced bacteria GA07, GA09 and GC04 utilized a much larger amount of adsorbed GP (54.6%, 35.5% and 62.7%, respectively) as compared with indigenous microorganisms (9.1%).

As the addition of GP, the development of indigenous microbial community was inhibited, causing a significant reduction of the DH activity by 50.7%. Due to the herbicide elimination by the introduced strains, total DH activity reached 71.9–76.3% by the end of the experiment (Fig. 2B). In contrast, this value in control remained almost unchanged during the same period. Similar results were observed in other GP degraders (Ermakova et al., 2010).

The CFU count indicated a gradual decrease in the introduced bacterial population during the bioremediation of GP-treated soil (Fig. 2C). A 76.5–85.2% reduction of

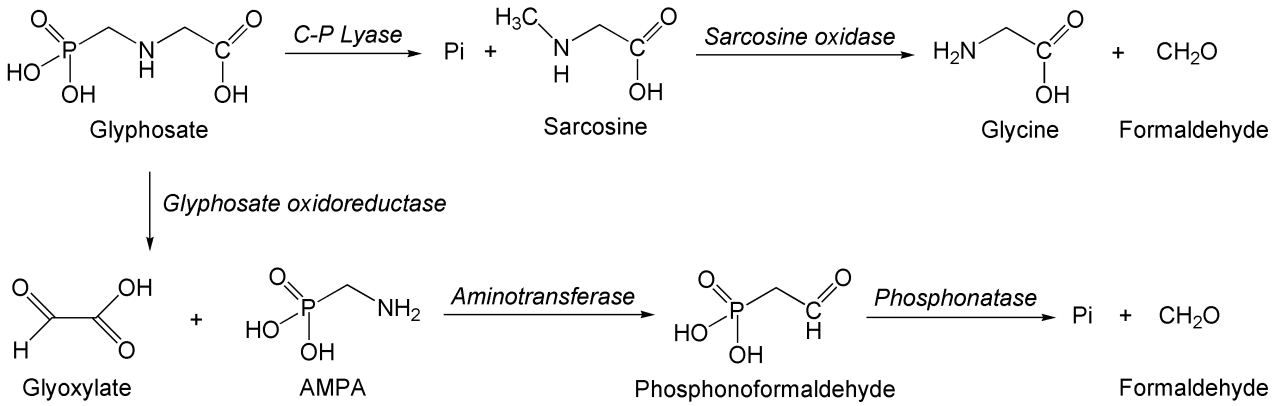

Fig. 3. Proposed biodegradation pathway of GP.

cell numbers was obtained within the first 9 days of incubation, followed by a slight change thereafter. The highest CFU count was observed in the treatment of strain GC04 (6.7×10^5 CFU/g dry soil) followed by strains GA07 (3.9×10^5 CFU/g dry soil) and GA09 (3.4×10^5 CFU/g dry soil) at the end of the experiment. To achieve a successful bioremediation of GP-contaminated soil, the survival of the introduced microorganisms is a deciding factor. The reduction in the level of invading bacteria in soil may be explained as a result from the synergetic action of various factors, such as soil conditions, competition and predation by soil protozoa (Zhang et al., 2012). Cell numbers of *Pseudomonas* spp. strains maintained at 3.4–6.7 × 10^5 CFU/g dry soil during 18 days of experiment without the addition of any nutrient, indicating that these bacteria could persist in the soil in relatively high populations over extended time periods.

GP metabolism

According to earlier reports, two pathways have been proposed for GP degradation (Fig. 3) (Liu et al., 1991; Sviridov et al., 2011). GP is cleaved into AMPA and glyoxylate by the presence of glyphosate oxidoreductase, whereas, in the other pathway, degradation of GP is catalyzed by C-P lyase with the formation of sarcosine, which eventually forms formaldehyde and glycine in a reaction catalyzed by sarcosine oxidase. In order to elucidate the GP degradation pathway in *Pseudomonas* spp. GA07, GA09 and GC04, the degradation products of GP were analyzed. As summarized in Table 3, the accumulation of AMPA and glyoxylate was observed during the experiments with cell-free extracts of all the three strains, which pointed out the presence of glyphosate oxidoreductase activity. Meanwhile, trace amounts of glycine and formaldehyde were detected only in a cell-free extract of strain GA09, indicating the involvement of both pathways in this strain. A combination of AMPA and sarcosine pathway was first found in *Pseudomonas* sp. strain LBr (Jacob et al., 1988), which metabolize about 5% of GP to glycine via sarcosine. Two bacterial strains, *Ochrobactrum anthropi* GPK 3 (Sviridov et al., 2011) and *Bacillus cereus* CB4 (Fan et al., 2012), were also reported capable of degrading GP via two concurrent pathways. Furthermore, sarcosine was not detected within the cells of GA09 after the completion of the reaction, despite the existence of GP residual. One possible explanation for this phenomenon was that C-P lyase involved in sarcosine pathway was inhibited by AMPA formed via glyphosate oxidoreductase during the cultivation on GP (Sviridov et al., 2011), and the generated small amounts of sarcosine were completely converted into glycine and formaldehyde.

Table 3. Intracellular concentrations of GP metabolites in cell-free extracts of GP-degrading strains.

Metabolites	Concentration (mg/L)		
	GA07	GA09	GC04
GP	4.41 ± 0.24	29.21 ± 2.01	5.06 ± 0.19
AMPA	44.26 ± 4.75	89.09 ± 5.64	35.74 ± 2.41
Glyoxylate	8.05 ± 0.34	42.34 ± 2.72	9.76 ± 0.30
Sarcosine	0	0	0
Glycine	0	7.02 ± 0.77	0
Formaldehyde	0	4.11 ± 0.85	0

"±" indicates the standard error of the mean of triplicate.

It was evident from the results that strains GA07 and GC04 degraded GP via glyphosate oxidoreductase, but the further possible metabolite, formaldehyde, was not detected in the experiments. AMPA catabolism in the phosphonatase pathway requires the presence of an aminotransferases that catalyzes AMPA transformation, and a phosphonatases that cleaves phosphonoformaldehyde to form Pi and formaldehyde (Sviridov et al., 2011; Talbot et al., 1984). In this study, the absence of formaldehyde in cell-free extracts of strains GA07 and GC04 implied the lack of the phosphonatase pathway on GP. Further studies on the two enzymes are needed to clarify this issue.

Conclusions

GP-utilizing bacterial strains, *Pseudomonas* spp. GA07, GA09 and GC04, were isolated from polluted soil by the enrichment culture method. Studies on bioremediation in the open microcosm showed that the 3 isolates exhibited efficient degradation of GP and contributed to improve the quality of contaminated soil. Cell numbers of the introduced microorganisms in non-sterilized soil remained at a relatively high level during 18 days of experiment,

which indicated their strong adaptabilities. Further, the biodegradation products of GP were traced from the HPLC analysis, and the distribution of different GP catabolism pathways in these strains was proposed. The results of the present study can be used for further developing bioremediation strategies towards GP.

Acknowledgments

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