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Exploring the glyphosate-degrading characteristics of a newly isolated, highly adapted indigenous bacterial strain, *Providencia rettgeri* GDB 1

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This study explored the characteristics of a newly isolated glyphosate (GLYP)-degrading bacterium *Providencia rettgeri* GDB 1, for GLYP bioremediation. Due to the serial selection pressure of high GLYP concentrations for enriched isolation, this highly tolerant GLYP biodegrader shows very promising capabilities for GLYP removal (approximately 71.4% degradation efficiency) compared to previously reported strains. High performance liquid chromatography analyses showed aminomethylphosphonic acid (AMPA) rather than sarcosine (SAR) to be the sole intermediate of GLYP decomposition via the AMPA formation pathway. Moreover, GLYP biodegradation was biochemically favorable in aerobic cultures due to its strong growth-associated characteristics. To the best of our knowledge, this is the first report to indicate that bacterial strains in the *Providencia* genus could demonstrate highly promising GLYP-degrading characteristics in environments with high GLYP contents.

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[**Key words:** *Providencia rettgeri*; Aminomethylphosphonic acid pathway; Glyphosate biodegradation; Growth association; Toxicity assessment]

Glyphosate as the active ingredient in the herbicide Roundup has been widely used around the globe to kill weeds in agricultural applications. However, since the World Health Organization (WHO) suspected that glyphosate might cause cancers in humans, environmental agencies in national governments (e.g., California EPA) consider glyphosate as a possible carcinogen. In fact, glyphosate (*N*-phosphonomethyl glycine, $C_3H_8NO_5P$, GLYP) has been one of the highly efficient, non-selective, and broad-spectrum systemic herbicides since its introduction in the 1970s (1–3). Due to its significant inhibitory potency on phosphate synthase that is essential for protein synthesis in weeds, GLYP was widely applied in plantations of rubber, mulberry, tea, and sugarcane, as well as in orchards (4). In agricultural application to the field, GLYP tends to adsorb on to soil minerals through its phosphonate group(s) (5). In fact, phosphonates are considered as a potential environmental hazard to the worldwide ecosystem due to their persistence, toxicity and abundant occurrence (6–9). Moreover, recent studies have indicated that extensive use of GLYP could result in considerable accumulation and residues in soil and water, likely stimulating further pollution with other contaminants due to its highly soluble phosphonate(s) (10). Particularly in the USA, excessive presence of GLYP found in crops has exponentially enhanced the incidence of autoimmune diseases (e.g., multiple sclerosis, autism, diabetes, coeliac disease and neuromyelitis optica). Of course, its long-term persistence could inevitably pose a serious threat to public health. The presence of GLYP above permitted levels has also been identified in many exported food items (e.g., potentially dangerous levels found in genetically-modified (GM) soy) (11,12). Thus, GLYP is attracting considerable attention as its effects are carcinogenic as well as life-threatening. GLYP can act as a non-coding amino acid analog of glycine and so, long-term exposure to GLYP may be responsible for many chronic diseases. Furthermore, gradually increased levels of GLYP in soil and water environments have raised global concern to explore effective methods for its environment-friendly remediation (13–15).

As aforementioned, selection of an ecologically promising and environmentally appropriate GLYP-degradation strategy for sustainable development is undisputedly the topmost concern. GLYP can be degraded/removed through either biological treatment or abiotic approaches (e.g., adsorption, thermolysis, and photodegradation) (16). For example, Manassero et al. (17) introduced a photocatalytic degradation system capable of completely decomposing GLYP into non-toxic products (e.g., carbon dioxide, inorganic ions and water). Xu et al. (18) mentioned that the reaction-determining step(s) of photocatalytic degradation were photocatalytic oxidation reactions triggered by the formation of highly reactive oxidant(s), hydroxyl radical(s). Barrett and Mcbride (19) indicated that GLYP could be effectively degraded in dilute aqueous suspensions of birnessite (a manganese oxide that is common in soils). However, as phosphonate-bearing GLYP is an organophosphorus compound with a carbon-to-phosphorus (C–P) bond, it shows extremely high stability and excessive resistance to

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physical and chemical degradation (5). Although abiotic GLYP removal methods may be well-characterized, unnatural methods of waste treatment still pose problems for further clean-up (e.g., secondary pollution). Evidently, use of indigenous GLYP-degrading microorganisms for bioremediation is the most ecologically appropriate and environmentally promising strategy for GLYP breakdown (20). For instance, some naturally-occurring microorganisms have been reported for the cost-effective bioremediation of GLYP-polluted environments. Zhan et al. (21) showed that *Achromobacter* sp. strain MPK 7A, *Comamonas odontotermitis* strain P2, *Ochrobactrum intermedium* strain Sq20 and *Pseudomonas* sp. strain 4ASW were all feasible GLYP-degrading microorganisms that were previously isolated from contaminated sites via serial enrichment cultures. Among GLYP-degrading microorganisms (e.g., bacteria, fungi, micromyces, and actinomycetes), bacteria play the most pivotal role, and optimal degradation conditions (e.g., pH, incubation temperature, GLYP concentration, inoculum size and incubation time) should be explored to obtain their maximal performance of biodegradation. Many GLYP-degrading bacteria have been reported previously; however, to prevent introduction of foreign bacteria for GLYP bioremediation, it is inevitable to isolate indigenous, highly tolerant, and functioning microbes for sustainable GLYP biodegradation. This study used extremely high levels of GLYP as selection pressure for soil contamination to isolate a highly effective GLYP biodegrader (i.e., *Providencia rettgeri* GDB 1) from highly tolerant bacterial populations. To the best of our knowledge, there are no reports in the public domain that reveal outstanding GLYP biodegradation characteristics in the *Providencia* genus.

Many GLYP-degradation pathways in bacteria have been extensively studied. Most studies indicate that GLYP biodegradation pathways include the aminomethylphosphonic acid (AMPA) formation pathway (AMPA pathway) and sarcosine (SAR) generating pathway (SAR pathway; Fig. 1). Regarding the AMPA pathway, GLYP oxidoreductase cleaves the C—N bond of GLYP to form intermediate AMPA. The action of C—P lyase on AMPA further generates methylamine which is further converted to formaldehyde and ammonia as raw chemicals for the carbon metabolic cycle. In another pathway, C—P lyase cleaves the C—P bond to degrade GLYP to sarcosine, which could then be oxidized into formaldehyde and glycine by sarcosine-oxidase. Table 1 summarizes these recently reported GLYP-degrading bacteria are summarized based on their metabolic pathways (22–27). In fact, Obojska et al. (22) indicated that the bacterium *Geobacillus caldoxylosilyticus* T20 could degrade GLYP through the generation of GLYP oxidoreductase to effectively cleave GLYP into AMPA. Kryuchkova et al. (24) reported the bacterial strain-*Enterobacter cloacae* K7 that degraded GLYP into nontoxic components (i.e., sarcosine and glycine) through the C—P lyase enzyme. In addition, a GLYP-degrading strain, *Ochrobactrum anthropi* GPK 3, could degrade GLYP into sarcosine as well as AMPA in parallel (26). Here, a newly isolated bacterial strain, *P. rettgeri*, was specifically isolated by serial selection of soils highly contaminated with GLYP (approximately 120,000 mg L^{-1}) and the possible routes and kinetics of GLYP biodegradation were investigated. Only AMPA, rather than SAR, was identified as the intermediate of GLYP decomposition via high performance liquid chromatography (HPLC) analysis. This suggests that the C—N cleavage pathway could be a metabolically appropriate route for GLYP biodegradation. The kinetic study also clearly indicated that the GLYP degradation rate was significantly influenced by many exogenous factors (e.g., the concentration of GLYP, dissolved oxygen and bacterial activity). This work provides an effective protocol to isolate indigenous bacterial strains for GLYP biodegradation. Importantly, it suggests a novel attempt of serial selection pressure (approximately 120,000 mg L^{-1} GLYP) in highly contaminated soils from highly populated Taiwan to isolate efficient bacterial strain(s) for GLYP degradation.

MATERIALS AND METHODS

Chemicals and reagents GLYP, AMPA, SAR, and 9-fluorenylmethyl chloroformate (FMOC) were purchased from Alfa Aesar (Heysham, UK). Acetic acid (CH_3COOH), ammonium acetate (CH_3COONa), and sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) were purchased from Showa Chemical Co. Ltd. (Tokyo, Japan). Bacto Agar, Mineral Salt Medium (MSM), and BactoLB broth were all purchased from Becton, Dickinson and Company (Sparks, MD, USA).

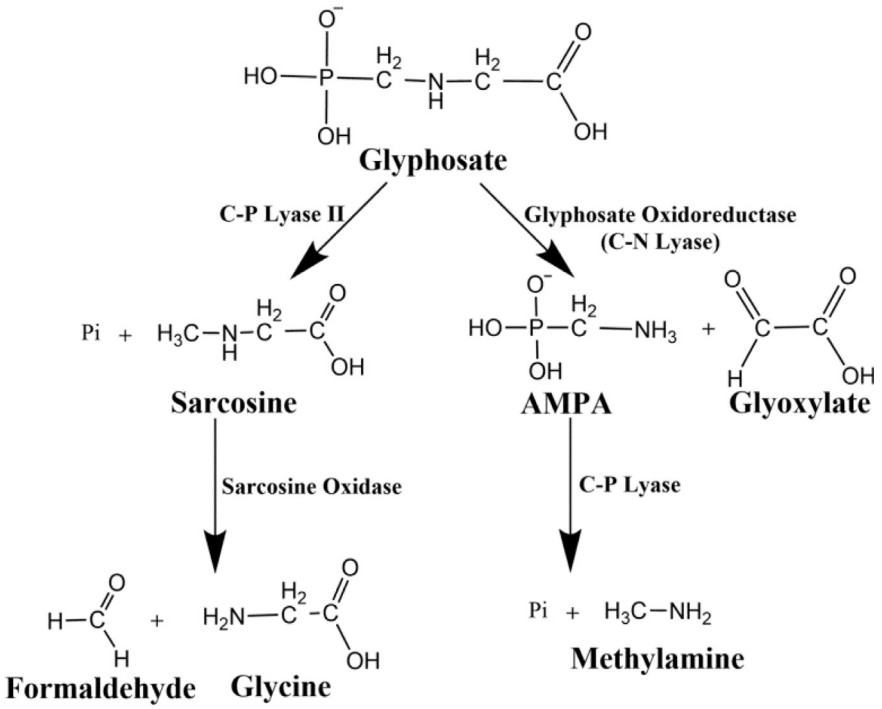

FIG. 1. Metabolic pathways of glyphosate-biodegradation.

TABLE 1. Comparative list of glyphosate-degrading bacteria (GDB).

Strain	Source	Gram status	Degradation pathway	Detected metabolites	Comments	References
Providencia rettgeri	Glyphosate-contaminated indigenous soil	−	AMPA pathway	AMPA	71.4 % degradation of glyphosate at GLYP of 10,000 mg L^{-1}	This work
Geobacillus caldoxylosilyticus T20	Central heating system water	+	AMPA pathway	AMPA and glyoxylate	Utilization of glyphosate as sole phosphorus source	Obojska et al. (22)
Ochrobactrum sp. GDOS	Soil	−	AMPA pathway	AMPA	Utilization of glyphosate as sole phosphate source and complete degradation (3 mM) within 60 h	Hadi et al. (23)
Enterobacter cloacae K7	Rhizoplane of various plants in Russia	−	Sarcosine pathway	Sarcosine and glycine	Utilization of glyphosate as sole phosphorus source and 40% degradation of glyphosate with initial 5 mM content	Kryuchkova et al. (24)
Ochrobactrum intermedium Sq20	Glyphosate-contaminated indigenous soil	−	Sarcosine pathway	Sarcosine and glycine	Utilization of glyphosate as sole carbon source	Firdous et al. (25)
Ochrobactrum anthropi GPK3	Glyphosate-contaminated soil	−	Simultaneous AMPA and sarcosine pathways	AMPA, glyoxylate, sarcosine and glycine	Utilization of glyphosate as sole phosphorus source	Sviridov et al. (26)
Bacillus cereus CB4	Glyphosate-polluted soil in the herbicide plant	+	Simultaneous AMPA and sarcosine pathways	AMPA, glyoxylate, sarcosine and glycine	Utilization of glyphosate as sole phosphorus source and 94.47% degradation in 5 days under the optimal capacity	Fan et al. (27)

Microorganism screening To obtain GLYP-degrading bacteria, soil samples were collected at a depth of 30 cm from the topsoil in a GLYP contaminated barren agricultural land near the Yilan river bank, under the Ximen Bridge, Yilan County, Taiwan. The site was chosen due to its persistent exposure to GLYP application. The GLYP-degrading bacteria were isolated from GLYP contaminated soil following the method of serial enrichment cultures under selective pressure of GLYP (28). To isolate indigenous GLYP-degrading bacteria, the collected soil was further contaminated with high concentrations of GLYP (125,000 mg L^{-1}) for 2 weeks. After serial selection, growth characteristics of bacterial populations tended to be stabilized and bacterial consortia were then used for further isolation. The contaminated soil was dispersed in MSM with a high concentration of GLYP (10,000 mg L^{-1}). This selection pressure of high GLYP concentration was to guarantee the stable bacterial capabilities for tolerating high GLYP concentrations. Thus, the most promising biodegrader(s) could then be obtained from this stable community for data reproducibility.

16S rRNA analysis The GLYP-degrading bacterial strain was subjected to partial genome sequencing for its taxonomical identification using 16S rRNA analysis (29,30). The bacterial strain was cultured in fresh Bacto-LB broth for 24 h and plated on 25 g L^{-1} Bacto Agar. The plates were incubated at 30°C in a static incubator for 24 h to allow bacterial growth. Single bacterial colonies obtained after serial streak isolation were subjected to 16S rRNA analysis for partial genome sequencing.

Kinetic assessment To achieve synchronized cell growth of the bacterial isolates for maximal tolerance to GLYP, a loopful of *P. rettgeri* seed taken from a well grown colony on an LB streak plate was first precultured in 100 mL of Bacto LB broth (pH 7.0) at 30°C, 125 rpm using a water bath shaker (Shinkwang, SKW-12). After cells were grown to the late exponential or early stationary phase (12 h), 1% (v/v) of the precultured *P. rettgeri* was inoculated into fresh LB medium supplemented with high concentrations of GLYP (5000 mg L^{-1} and 10,000 mg L^{-1}). Bacterial cultures were then sampled at various times to determine bacterial parameters and time courses for comparative assessment. Bacterial growth and GLYP biodegradation curves were also obtained simultaneously for kinetic evaluation (e.g., specific growth rate, specific degradation rate (SDR) and specific formation rate (SFR)).

CO_2 respirometric analysis The toxicity potential of GLYP on the reporter bacterium *P. rettgeri* via CO_2 respirometric activity that is associated with metabolically functioning cells, was investigated by supplementation with test biotoxicant(s) using the automated Columbus Micro-Oxymax Respirometer equipped with CO_2 sensors (Columbus Instruments, Columbus, OH, USA). The measurement relied on the circulation of air through closed-system testing bottles whereas the liquid in the closed-system bottles remain static (i.e., mobile liquid-flowing gas respirometer) (31). The respirometer was calibrated using standard CO_2 (g) at 0.5% prior to the experiments. Precultured *P. rettgeri* was inoculated into fresh LB medium supplemented with different concentrations of GLYP (2000 mg L^{-1}, 5000 mg L^{-1} and 10,000 mg L^{-1}) to assess its inhibitory potency in reporter bacterial cultures.

Sample derivatization For kinetic assessment, samples collected at designated times were first derivatized prior to HPLC analysis as described elsewhere (32). Then, 1 mL of aqueous sample was adjusted to pH 9.0 by adding 0.12 mL of 5% borate buffer, followed by the addition of 0.12 mL of 12,000 mg L^{-1} FMOC in acetonitrile. Derivatization was carried out at 22 ± 1°C for approximately 16 h. The reaction was then terminated by adding 0.015 mL of 6 mol L^{-1} HCl, resulting in a mixture at pH 1.5. After 1 h, derivatized samples were filtered through 0.45 μm syringe filters and 10 μL were injected into the chromatograph.

HPLC analysis Detection of products was performed on a Chromaster-5110 single pump, Chromaster-5260 auto sampler, and Chromaster-5420 UV-VIS Detector (Hitachi High-Tech Science Corporation, Tokyo, Japan) using an InertSustain C18 column (5 μm, 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan) under a gradient eluent mode. The gradient used was 5 mmol L^{-1} HAc/NH4Ac (pH 4.8)-acetonitrile, where the percentage of acetonitrile was changed over time as follows: 5–75% in 0–42 min, 100% in 42.1–45 min, and 5% in 45.1–55 min. The chromatographic separation was completed in 55 min for each sample. The separated components were detected at 290 nm using the Chromaster-5420 UV-VIS Detector. Pure GLYP, AMPA and SAR were also derivatized and analyzed as standards using the same aforementioned methods.

RESULTS AND DISCUSSION

Bacterial identification The phylogenetic analysis of GLYP degrading bacterial isolates (denoted as isolate GDB 1) was performed using BLASTN 2.7.1+. The phylogenetic tree as depicted in Fig. 2 indicates the association of related *Providencia* spp. to the isolate GDB 1 based on 16S rRNA sequences. The 16S rRNA sequence analysis of isolate GDB 1 indicated a 99% Bootstrap value similar to the 16S rRNA gene sequence of *P. rettgeri* (accession number AM040492). Therefore, the highly GLYP

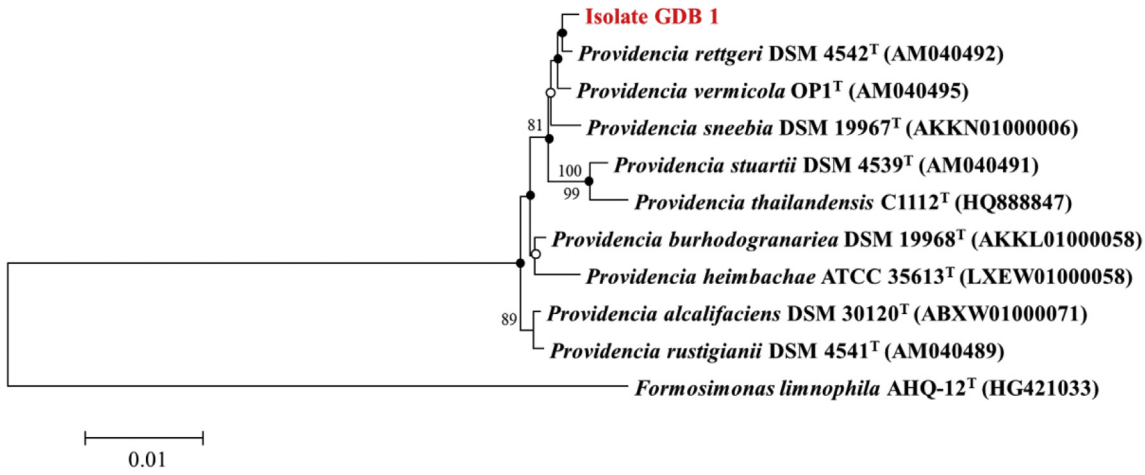

FIG. 2. Phylogenetic analysis of isolate GDB 1 based on 16S rRNA gene sequence analysis using the neighbor-joining method. Bootstrap values are indicated at the nodes. Only bootstrap values >50% are shown, where the scale bar represents 1% sequence dissimilarity (one substitution per 100 nt). Representative sequences in the dendrogram were obtained from GenBank (accession number in parentheses).

tolerant and GLYP-degrading bacterial strain (i.e., isolate GDB 1) was phylogenetically identified as *P. rettgeri*. It is a gram-negative bacterium belonging to family *Enterobacteriaceae* and genus *Providencia*. *P. rettgeri* are elongated rods characterized by petricious flagella, thereby making them motile. They are also opportunistic pathogens that can cause traveler's diarrhea and urinary tract infections (UTI). As reported previously, the maximal capability of GLYP tolerance and degradation was exhibited by *Bacillus* sp., followed by *Pseudomonas* sp. However, there are no reports of strains in the genus *Providencia* for degradation of the herbicide GLYP till date. In fact, bacterial strains of pollutant-degrading *Providencia* sp. have been explored to treat various environmental pollutants such as the pesticide paraquat dichloride (33), crude oil (34), oxalate (35) and numerous textile azo dyes (e.g., Reactive Blue 178 (36) and Disperse Red 78 (37)). To the best of our knowledge, this is the first report revealing the GLYP-degrading characteristics of bacterial strains in the genus *Providencia*. In addition, this isolate GDB 1 was serially selected through soils contaminated with 120,000 mg L^{-1} GLYP for 2 weeks. Moreover, stable growth profiles in repeated cultures were maintained in such environments for more than 2 months to guarantee long-term stability (data not shown). The isolate demonstrated high GLYP-degrading capabilities at GLYP concentrations of 5000–10,000 mg L^{-1} as mentioned afterwards.

Dose–response assessment Since GLYP biodegradation was strongly dependent on whether bioactivity could be fully expressed or not in hostile conditions, dose–response assessment of GLYP towards *P. rettgeri* was first inspected to determine the operation threshold/limits for degradation. As the cumulative amount of CO_2 present in the blank bottle was nearly negligible (approximately 1–2 mg) during 3 days of the test duration, different time-series profiles of CO_2 evolution were simply considered the result of different levels/doses of existing toxicant(s) in cultures. As shown in the time courses of CO_2 respirometric curves (Fig. 3A), transient dynamics of CO_2 accumulation with supplementation of different GLYP concentrations were almost identical to the blank. Also, the variation tendency of CO_2 accumulation corresponded to the bacterial growth phase as shown in Fig. 3B. This indicated that GLYP at 5000 and 10,000 mg L^{-1} exhibited negligible toxicity to *P. rettgeri* GDB 1. As *P. rettgeri* was originally enriched by selection pressure of high GLYP concentration up to 125,000 mg L^{-1} for 2 weeks, the isolate GDB 1 evolved with high tolerance towards GLYP. Compared to the blank, a slightly short time delay to trigger CO_2 accumulation at the beginning, suggested that the bacteria needed some lag time to adapt to the new condition of GLYP supplementation. GDB 1 may thus require some biomass metabolic alteration to overcome such adverse environments.

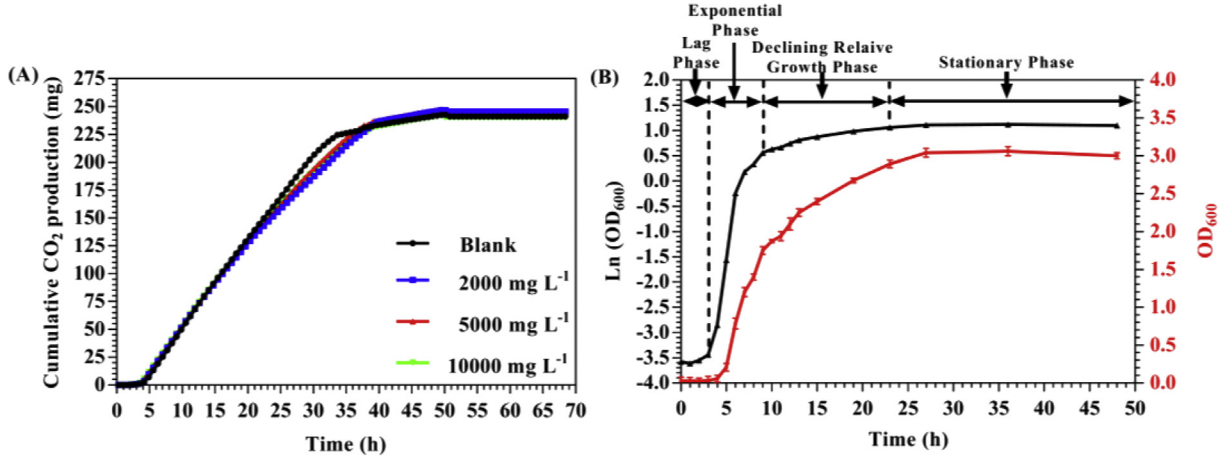

FIG. 3. (A) Time-series profiles of cumulative CO_2 evolution by *Providencia rettgeri* GDB 1 supplemented with different concentrations of glyphosate (GLYP). (B) Growth phase classification based on time courses of the GLYP-degrading bacterium GDB 1.

Degradation pathway identification As aforementioned, the biodegradation pathway of GLYP could usually be concluded as the AMPA pathway and SAR pathway. To directly identify the biodegradation route(s) of *P. rettgeri* towards GLYP, transient dynamics of possible compositions during GLYP biodegradation were determined. After derivatization with FMOC, the reaction products (i.e., GLYP-FMOC, AMPA-FMOC and SAR-FMOC) could clearly be distinguished in the HPLC results without any interference (Fig. 4A). They were eluted at different retention times (i.e., 16.3 min, 19.5 min and 21.6 min). The fluctuated peaks after approximately 25 min could be attributed to the residual FMOC that was not completely reacted, indicating that the added amount of FMOC in the derivatization reaction was in excess and more than sufficient for the complete reaction. Moreover, for quantitative analysis, standard calibration curves were generated with different concentrations of supplementation (r^2 >0.99; Fig. 4B, C). Biodegradation of GLYP (5000 mg L^{-1}) using *P. rettgeri* was first carried out in aerobic cultures (with shaking) and static incubation (without shaking). The transient dynamics of typical HPLC peaks (Fig. 4D) directly suggested that AMPA was likely the sole intermediate of GLYP degradation, apparently bypassing the sarcosine-forming pathway. This result clearly indicated that the AMPA pathway was the only metabolic pathway in *P. rettgeri* for GLYP biodegradation. Compared to aerobic cultures with shaking at 120 rpm, static incubation without shaking did not seem favorable for GLYP biodegradation (Fig. 5). The results apparently indicated that *P. rettgeri* grew at slower rates under anaerobic condition. The biomass of bacteria obtained from anaerobic growth was slightly more than half of that obtained in aerobic growth. Results of the biosorption experiment significantly indicated that approximately 370 ± 18 mg L^{-1} of GLYP could be absorbed by bacteria (data not shown). This further explained why 500 mg L^{-1} of GLYP was degraded

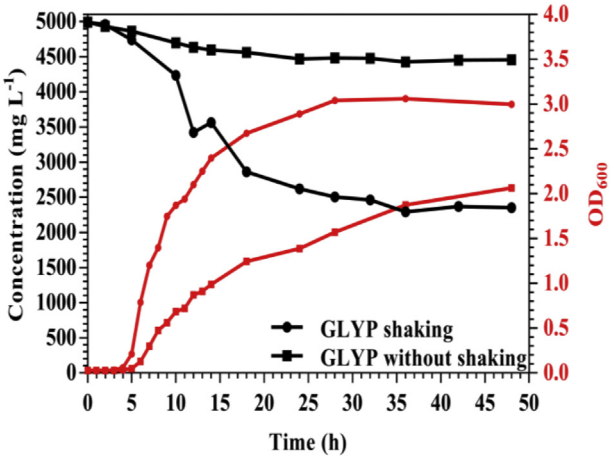

FIG. 5. Comparative time-series profiles of GLYP-degradation in aerobic culture (shaking) and static incubation (without shaking) with GLYP at 5000 mg L^{-1}.

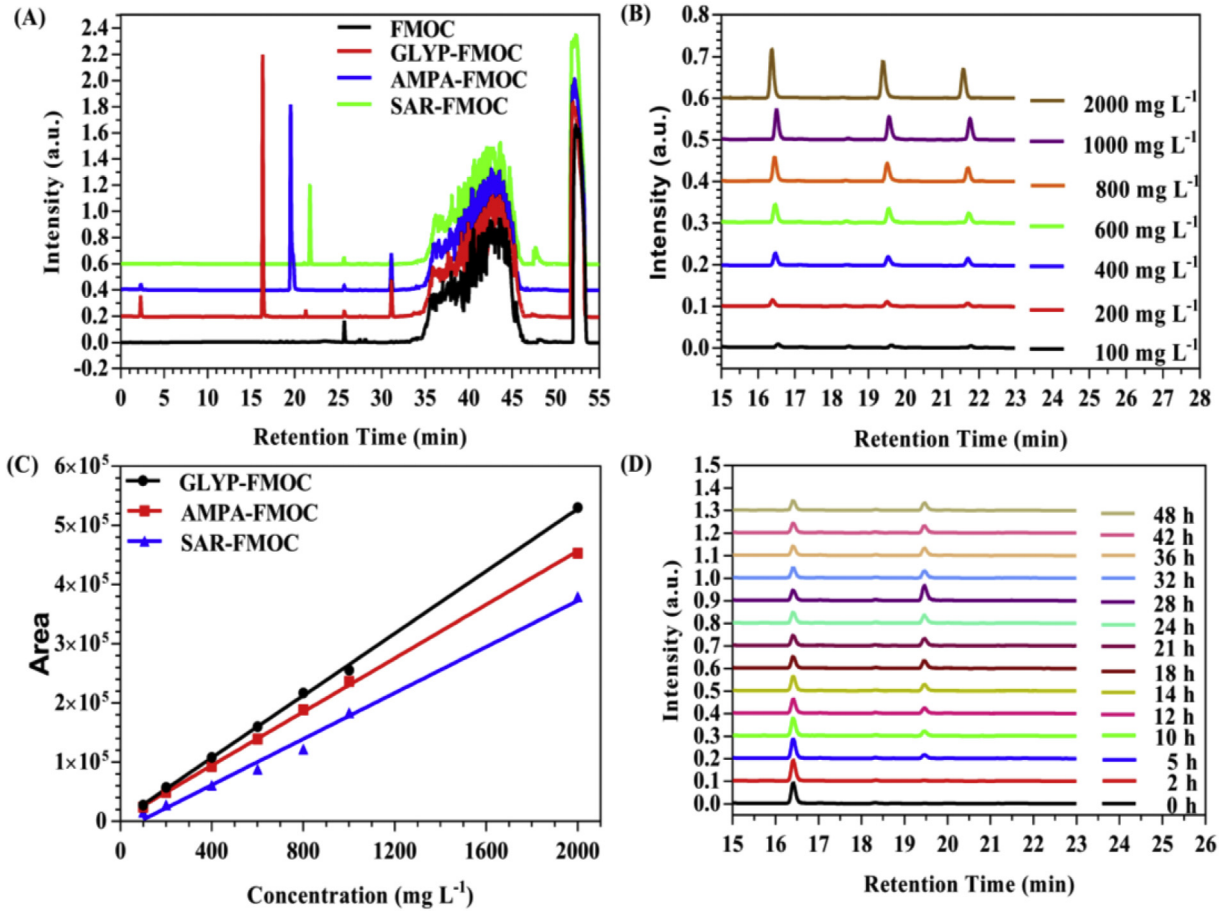

FIG. 4. (A) HPLC chromatograms of GLYP, AMPA and SAR after derivatization reaction with 9-fluorenylmethyl chloroformate (FMOC). (B) Typical intermediate peak intensity at different concentrations. (C) Standard calibration curves of GLYP, AMPA and SAR after the derivatization reaction. (D) Comparative time courses of peak intensities of GLYP (5000 mg L^{-1}) degradation and AMPA generation using *Providencia rettgeri* GDB 1 as the reporter biodegrader.

under the anaerobic conditions. Thus, it was suspected that approximately 10% of glyphosate was biosorbed, and not biodegraded under anaerobic conditions. First, GLYP was possibly attached onto bacterial cells. With the aid of oxygen provided by aeration, GLYP could be degraded effectively. In the absence of efficient aeration, GLYP may be biosorbed onto the bacterial cell surface. Thus, slightly decreasing profiles of GLYP with time are shown. Therefore, biodegradation process of *P. rettgeri* towards GLYP was aerobic. Talbot et al. (38) also studied the biodegradation of GLYP with *Pseudomonas* sp. SG-1 in an aerobic digester liquid via the AMPA pathway. In fact, as indicated in Table 1, it was very likely that the dominant aerobic metabolite intermediate of GLYP is AMPA; AMPA is then degraded to CO_2.

Kinetic analysis To assess the GLYP-degrading performance of *P. rettgeri*, a kinetic study of microbial degradation of GLYP at high concentrations (i.e., 5000 and 10,000 mg L^{-1}) was conducted. As indicated in Fig. 3B for the growth phase profiles and in Fig. 6, the exponential growth phase started at approximately 4 h, with a gradual decline in the growth phase at approximately 10–24 h. Then, the stationary phase was achieved after 24 h as indicated in Fig. 3A for CO_2 respirometric time courses. Transient dynamics of GLYP degradation and AMPA production indicated that both the maximal SDR and SFR occurred at the exponential growth phase, strongly suggesting the growth-associated characteristics of GLYP biodegradation. This point also explained why static incubation was not biologically favorable for treatment and indicated that GLYP degradation would be oxidation-oriented, which is not likely due to reduction (e.g., failure in electron-shuttle-supplemented test for possible stimulation to GLYP degradation; data not shown). Once bacterial growth reached the stationary phase at approximately 24 h, enzymatic activities for the AMPA pathway (e.g., glyphosate oxidoreductase (C–N lyase)) were gradually declined, further resulting in termination of GLYP biodegradation. Fan et al. (27) explored the effect of bacterial growth conditions on GLYP biodegradation (e.g., pH, temperature, inoculation amounts and growth phase). The results significantly indicated that the degradation rate reached a maximum at the end of the exponential growth phase, and remained nearly constant at the stationary phase. The results also indicated that GLYP breakdown in *Bacillus cereus* CB4 occurred enzymatically via C–P lyase and glyphosate oxidoreductase activities. According to Jayani et al. (39), the enzyme activity was continuously strengthened until the bacterial growth reached the late exponential growth phase. At the stationary phase, the enzyme activity would gradually attenuate over time. These results directly indicated that GLYP degradation was significantly influenced by the metabolically active status of bacterial cells. As indicated in the GLYP degradation performance, the degradation of GLYP supplemented at 10,000 mg L^{-1} could achieve the maximal efficiency of 71.38%, which is evidently better than that with 5000 mg L^{-1} (54.18%). Yu et al. (40) showed that the biodegradation rate of GLYP using *Bacillus subtilis* Bs-15 could achieve the maximum at approximately 65% after 72 h. Regarding the concentration of GLYP for treatment, a higher biodegradation rate also could be obtained at 10,000 mg L^{-1} compared to 5000 mg L^{-1}. This study further indicated that the newly isolated bacterial strain *P. rettgeri* GDB 1 showed outstanding degradation efficiency, compared to the bacterial performance for GLYP biodegradation reported previously. Moreover, with the GLYP-degrading bacterium GDB-1, a degradation rate of 71.4% could be achieved in 24 h and this promising potential for GLYP biodegradation could be likely achieved due to such a significantly high tolerance to GLYP at 125,000 mg L^{-1} as the pre-screening condition. This also suggested that with the strong selection pressure of high GLYP concentration, the highly tolerant and GLYP-degrading bacterium GDB 1 could be stably obtained without dispute.

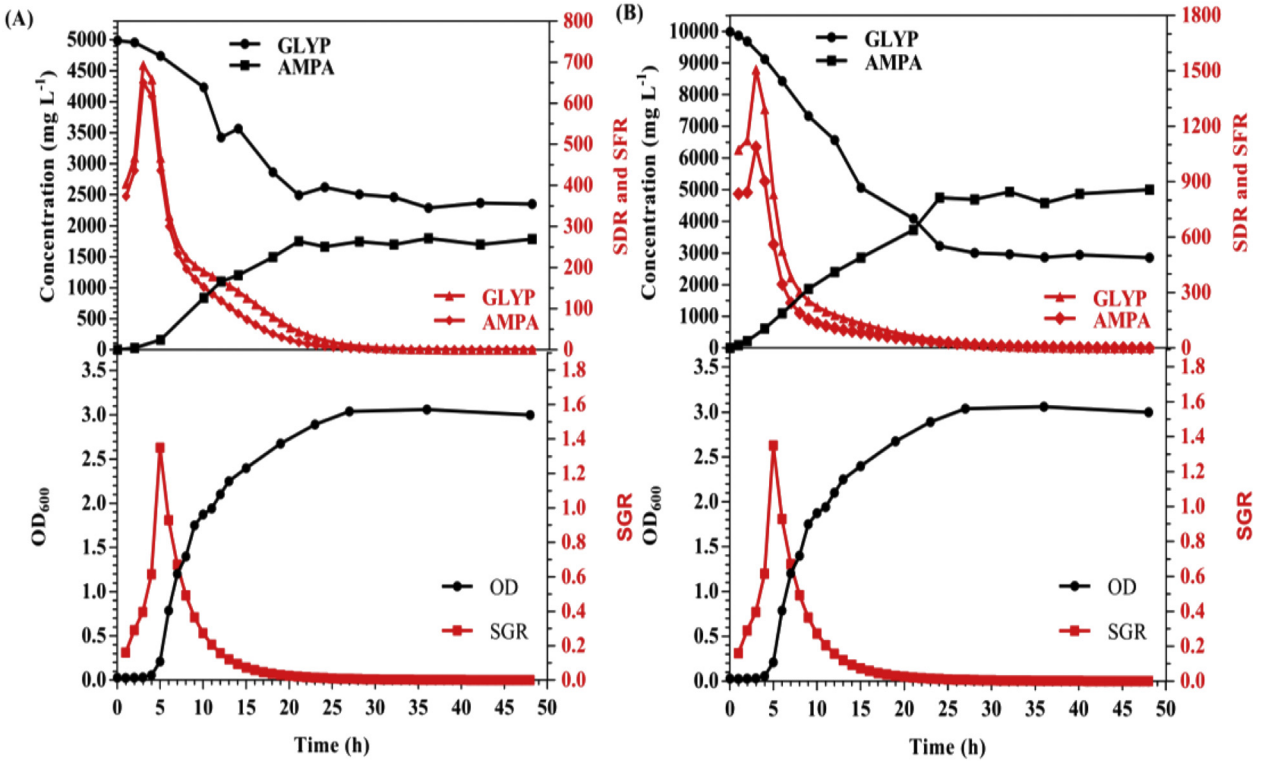

FIG. 6. Time courses of cell growth, GLYP degradation and AMPA production by *Providencia rettgeri* GDB 1 supplemented with GLYP at (A) 5000 mg L^{-1} and (B) 10,000 mg L^{-1}. SDR and SFR denote the specific degradation rate and specific formation rate, respectively (mg L^{-1} h^{-1} ODU^{-1}).

High tolerance mechanisms As glyphosate shows relatively lower toxicity compared to regularly-used herbicides and pesticides, our protocol for strain isolation intentionally set extremely high levels of glyphosate as the critical criteria to exclude non-biodegraders and to isolate effective GLYP-degrading bacteria. That is, GLYP tolerance was the most important characteristic for successful serial acclimation at high levels (125,000 mg L^{-1}) for effective biodegradation as aforementioned. Therefore, the proposed strategy behind this GLYP tolerance could be explained as follows (41–43): glyphosate was originally designed to kill weed plants by blocking the EPSPS enzyme (i.e., an inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimic acid-3-phosphate (EPSP)-synthase; or EPSPS enzyme). EPSPS is an enzyme involved in the biosynthesis of aromatic amino acids, vitamins and several secondary plant metabolites. The strategy was thus to intentionally isolate soil bacteria that could selectively switch on their genetic domains for the expression of GLYP-tolerant forms of EPSPS at high GLYP concentrations. Once such EPSPS could be successfully expressed or induced, strain GDB1 was capable of switching the corresponding enzymatic activities for GLYP biodegradation. Apparently, GLYP tolerance plays the most significant role as the necessary condition for glyphosate biodegradation. Thus, follow-up studies need to focus on the interactive mechanisms of GLYP tolerance and biodegradation to maximize the operation efficiency for *in-situ* or on-site GLYP biodegradation in sustainable agricultural applications.

Significance of study For environment friendliness, this study isolated a newly screened indigenous bacterial strain *P. rettgeri* GDB 1 for GLYP biodegradation. Due to the hostile selection pressure of high GLYP concentrations, isolate GDB 1 showed high tolerance (>120,000 mg L^{-1}) and degrading (71.4%) capabilities towards GLYP. The AMPA pathway rather than the SAR pathway was identified as the sole metabolic route towards GLYP degradation in aerobic conditions. To the best of our knowledge, this was the first report on a bacterial strain in genus *Providencia* (*P. rettgeri*) to reveal outstanding GLYP-degrading efficiency at high GLYP concentrations.

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