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Cloning and Sequencing of the Genes Involved in Glyphosate Utilization by *Pseudomonas pseudomallei*

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Thirty-four strains of *Pseudomonas pseudomallei* isolated from soil were selected for their ability to degrade the phosphonate herbicide glyphosate. All strains tested were able to grow on glyphosate as the only phosphorus source without the addition of aromatic amino acids. One of these strains, *P. pseudomallei* 22, showed 50% glyphosate degradation in 40 h in glyphosate medium. From a genomic library of this strain constructed in pUC19, we have isolated a plasmid carrying a 3.0-kb DNA fragment which confers to *E. coli* the ability to use glyphosate as a phosphorus source. This 3.0-kb DNA fragment from *P. pseudomallei* contained two open reading frames (*glpA* and *glpB*) which are involved in glyphosate tolerance and in the modification of glyphosate to a substrate of the *Escherichia coli* carbon-phosphorus lyase. *glpA* exhibited significant homology with the *E. coli* hygromycin phosphotransferase gene. It was also found that the hygromycin phosphotransferase genes from both *P. pseudomallei* and *E. coli* confer tolerance to glyphosate.

Glyphosate (*N*-phosphonomethyl)glycine is an effective nonselective herbicide that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), of the shikimic acid pathway (19). Disruption of this pathway prevents synthesis of aromatic amino acids, their secondary metabolites, and the products derived from chorismate (15, 18). Glyphosate is a competitive inhibitor of EPSPS with respect to phosphoenolpyruvate (4) and has been shown to block import of the EPSPS preprotein into chloroplast (6), the subcellular location of the shikimic acid pathway. One of the many attractive aspects of the use of this herbicide is its rapid and complete degradation by soil organisms (9). However, only a few bacteria that can utilize phosphonates, which include glyphosate, as their sole source of phosphorus for growth have been isolated (3, 14). Two pathways for the breakdown of glyphosate have been identified in *Pseudomonas* spp. One involves cleavage of the molecule to form aminomethylphosphonic acid (AMPA), which is further broken down by subsequent steps (2, 10). The second pathway is via initial cleavage of the C-P bond to produce sarcosine by a C-P lyase activity (12, 14). In this study, *Pseudomonas pseudomallei* glyphosate-degrading strains were isolated from soil. The kinetic parameters associated with glyphosate uptake were studied in *P. pseudomallei* 22, and this strain selected for cloning and sequencing of the genes involved in glyphosate utilization.

MATERIALS AND METHODS

Strains. The wild-type strain of *P. pseudomallei* was isolated from soil with a history of repeated applications of glyphosate. *Escherichia coli* strains used in this study were JM103 (*aroA*), AB1321, K38, BW14893 (lyase mutant defective in phosphonate transport), and BW16711 (lyase mutant defective in catalysis genes), kindly donated by Barry L. Wanner (Purdue University, West Lafayette, Ind.).

Media and chemicals. Glyphosate free acid form (99.7% active ingredient) was a gift from PYOSA (Monterrey, Mexico). All other chemicals were of analytical grade. Bacteria were isolated in glucose–10 mM morpholinepropanesulfonic acid (MOPS) medium (13) containing (250 mg of 1.5 to mM) glyphosate per liter as the sole phosphorus source instead of P_i. This medium was designated MG.

Isolation procedure. Erlenmeyer flasks (250 ml) containing 25 ml of MG broth were inoculated at a rate of 1% (vol/vol) with samples from soil (on a weight/volume basis). The flasks were shaked at 200 rpm for 5 days at 28°C. After six transfers in MG broth, cultures were streaked for bacterial isolation on MG plates. The bacteria were identified by the procedure and methods outlined in the *Bergey's Manual of Systematic Bacteriology* (12a).

The reported intermediate metabolites of glyphosate breakdown, sarcosine and AMPA, were tested as an alternative carbon-nitrogen or phosphorus sources, using MG medium with incubation at 28°C. Glyphosate levels in the experiments with glyphosate as the only phosphorus source were measured by the method of Ames (1) and by the ninhydrin reagent. Samples were analyzed in triplicate.

Cloning and genetic manipulations. Chromosomal DNA was partially digested with *Sau*3A, and DNA fragments of 5 to 10 kb were purified and ligated into the *Bam*HI site of pUC19. *E. coli* JM103 competent cells were transformed by using the ligated DNA (21). The transformants were selected on MG medium supplemented with ampicillin at 100 μg/ml and incubated at 37°C for 5 days. Recombinant clones with the ability to utilize glyphosate as a P source were transferred to fresh MG-ampicillin broth to verify the glyphosate degradation phenotype. To determine the minimal size of the DNA required for the cellular expression of glyphosate utilization, subclones were constructed from the original plasmid (pGlp-2.0). The methods of Sambrook et al. (21) were used for Southern blot analysis of genomic DNA from *P. pseudomallei.*

DNA sequencing. Overlapping DNA fragments from pGlp-2.0 were subcloned into Bluescript plasmids $pBSK^+$ and $pBSK^-$, and the recombinants were transfected into *E. coli* JM103 to produce single-stranded templates (21). Nucleotide sequences of the clones were determined by the dideoxy-chain termination method of Sanger et al. (22) by using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio). Sequence analysis was carried out for both strands by computer analysis (Gene Works; IntelliGenetics, Inc.).

Analysis of protein expressed from cloned genes. The polypeptide chains encoded by the 3.0-kb *Hin*dIII fragment inserted in pGlp-2.0 were analyzed by using a T7 RNA polymerase expression system. Strain K38 was first transformed by the T7 RNA polymerase-containing plasmid pGP1-2 (23). The kanamycin-resistant strain was further transformed with the pT7 recombinant plasmids under T7 RNA polymerase promoter ϕ10 control. The transformants were selected simultaneously by incubation in the presence of kanamycin and ampicillin. [^{35}S]methionine-labeled cells were prepared, and the labeled proteins were analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE). Protein gels were stained with Coomassie blue and then dried by a gel dryer under vacuum. Autoradiography was performed with Du Pont Cronex 4 film.

Phosphotransferase assay. Cell extracts from *E. coli* JM103 harboring plasmids pGlp-2.0, pUC19, and pHph+1 (Boehringer Mannheim) were tested with hygromycin B or glyphosate for aminoglycoside-phosphorylating ability with [γ-^{32}P]ATP as described by Haas and Dowding (8). Enzyme activities were assayed by means of the phosphocellulose paper binding assay, which measures

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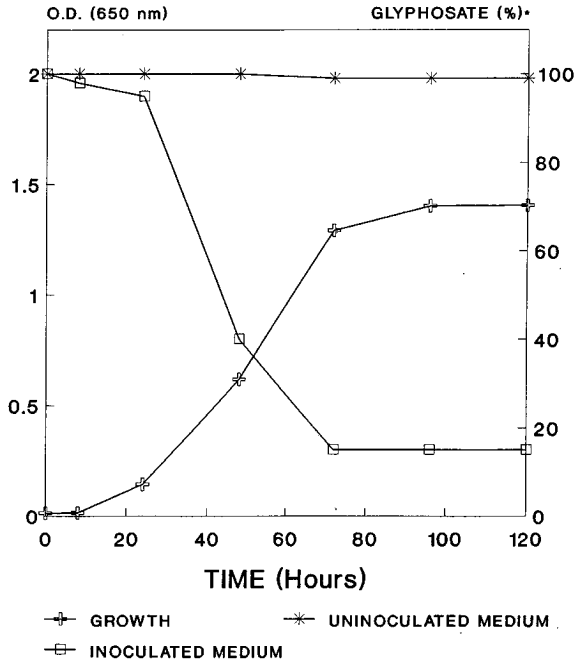

FIG. 1. Metabolism of glyphosate with an initial concentration of 170 mg/liter (100%) and growth of *P. pseudomallei* 22 in MG medium with glyphosate as the only phosphorus source. O.D., optical density.

transfer of radiolabel from a suitable cofactor to the substrate. Quantification of enzyme activities and dot blot analysis were performed.

Nucleotide sequence accession number. The GenBank accession number for the sequence of the glyphosate utilization genes is X74325.

RESULTS

Isolation of *P. pseudomallei* 22. Using a phosphate-free mineral medium with glyphosate as the phosphorus source, a total of 34 cultures of *P. pseudomallei* that could metabolize the herbicide were isolated from soil and identified.

An initial experiment was performed to determine the amount of glyphosate necessary to saturate the MG medium with phosphorus. The addition of glyphosate above 250 mg/liter did not result in additional growth in a medium with 10 mM D-glucose. The strains were able to grow in concentrations of herbicide as high as 10.2 g/liter. Concentrations of glyphosate below 85 mg/liter limited growth. Only traces of P_i were detected by the assay of Ames (1) in analytical-grade glyphosate (99.7% active ingredient).

P. pseudomallei 22 was tested for its ability to utilize AMPA and sarcosine, since both compounds had been reported as intermediate metabolites in the breakdown pathway of glyphosate. *P. pseudomallei* 22 grew well in mineral medium containing equimolar amounts of AMPA, glyphosate, or P_i. No growth was recorded with glyphosate, AMPA, or sarcosine as a carbon source.

P. pseudomallei 22 metabolized 50% glyphosate in MG broth medium within the first 40 h of growth, with a generation time of 4 h at 28°C (Fig. 1). Therefore, the studies of glyphosate utilization and cloning of the genes for its metabolism were performed with this organism.

Cloning of the genes involved in glyphosate utilization. To isolate the genes involved in glyphosate utilization from *P. pseudomallei* 22, a genomic library was constructed from this strain in the vector pUC19. The library was screened by complementation of *E. coli* JM103 for growth on glyphosate as a phosphorus source. From approximately 7.1×10^4 ampicillin-resistant *E. coli* transformants, one colony was observed to have the ability to grow in MG medium. Control *E. coli* transformants harboring plasmid pUC18 did not grow in this medium. This transformant contained a plasmid with an insert of 3,069 bp and was designated pGlp-2.0. Southern blot analysis of genomic DNA from *P. pseudomallei* 22 digested with *Hin*dIII and probed with ^{32}P-labeled pGlp-2.0 showed that pGlp-2.0 contained an approximately 3.0-kb *Hin*dIII fragment originally cloned from *P. pseudomallei* 22 (data not shown). *E. coli* cells carrying pGlp-2.0 were able to grow in minimal medium with glyphosate (250 mg/liter) as the sole phosphorus source (after 48 h at 37°C with shaking), while control cultures were unable to grow under this condition. To eliminate the possibility that an *aroA* gene was present on the *Hin*dIII fragment, pGlp-2.0 was tested for the ability to complement the *aroA* mutation of *E. coli* AB1321. This plasmid was unable to complement this mutant.

Characterization of genes involved in glyphosate utilization. The 3.0-kb *Hin*dIII DNA fragment conferring glyphosate utilization activity isolated from *P. pseudomallei* 22 was sequenced completely on both strands. The nucleotide sequence is shown in Fig. 2. Two open reading frames (*glpA* and *glpB*) were located on one DNA strand. *glpA* is 1,260 nucleotides in length and could code for a protein as large as 420 amino acids (46.2 kDa); *glpB* is 927 nucleotides in length and could code for a protein of 309 amino acids (33.9 kDa).

To determine the minimum amount of DNA required for the glyphosate utilization, several subclones were constructed. Subclones were made in which terminal fragments with a length of 609 bp (*Xba*I-*Xba*I; pGlp-2.1) and 612 bp (*Nhe*I-*Hin*dIII; pGlp-2.2) were removed from the 3.0-kb insert from either end. Only pGlp-2.1 could support the growth of *E. coli* JM103 on MG medium. This result indicated that both *glpA* and *glpB* were intact in pGlp-2.1 and that *glpB* was essential for glyphosate utilization in pGlp-2.2 (Fig. 3).

To test the activities, *glpA* and *glpB* were independently cloned (Fig. 3). The activity of *glpA* was tested by subcloning the *Xba*I-*Nhe*I fragment from pGlp-2.0. *glpA* could not confer the glyphosate assimilation phenotype in *E. coli* even when aromatic amino acids were added to MG medium (pGlp-2.3). Similarly, *glpB* was subcloned as a *Eco*RI-*Hin*dIII fragment (pGlp-2.4). It was observed that JM103 strains containing pGlp-2.4 were able to use glyphosate as a phosphorus source but only in the presence of aromatic amino acids.

In *E. coli*, phosphonates are broken down solely by the C-P lyase pathway, and the products of the 14 genes involved in a 10.9-kb operon have been extensively studied and mutagenized (16, 17). We transformed a lyase mutant (*phnC*? *DEFGHIJKLMNOP*) defective in phosphonates transport system and a lyase mutant (*phnHIJKLMNOP*) in which most of the catalysis genes coding for the C-P lyase are deleted with the different subclones constructed (Fig. 3) and found no complementation of the respective mutants when glyphosate or AMPA was used as the phosphorus source.

Gene expression. To determine whether the open reading frames encoded by the 3.0-kb *Hin*dIII DNA fragment were translated, the T7 RNA polymerase-dependent in vivo transcription-translation system was used. Cells harboring plasmid pGP1-2 were cotransformed with the pT7 recombinant plasmids carrying the 3.0-kb *Hin*dIII-*Hin*dIII DNA fragment (pT7-71) and 2.4-kb *Xba*I-*Hin*dIII DNA fragment (pT7-72), both subcloned from pGlp-2.0. Plasmid pT7-7 was used as a control. The [^{35}S]methionine-labeled polypeptides were analyzed by SDS-PAGE. Two proteins of 46 and 33 kDa were seen for pT7-71- and pT7-72-transformed cells (data not shown). The

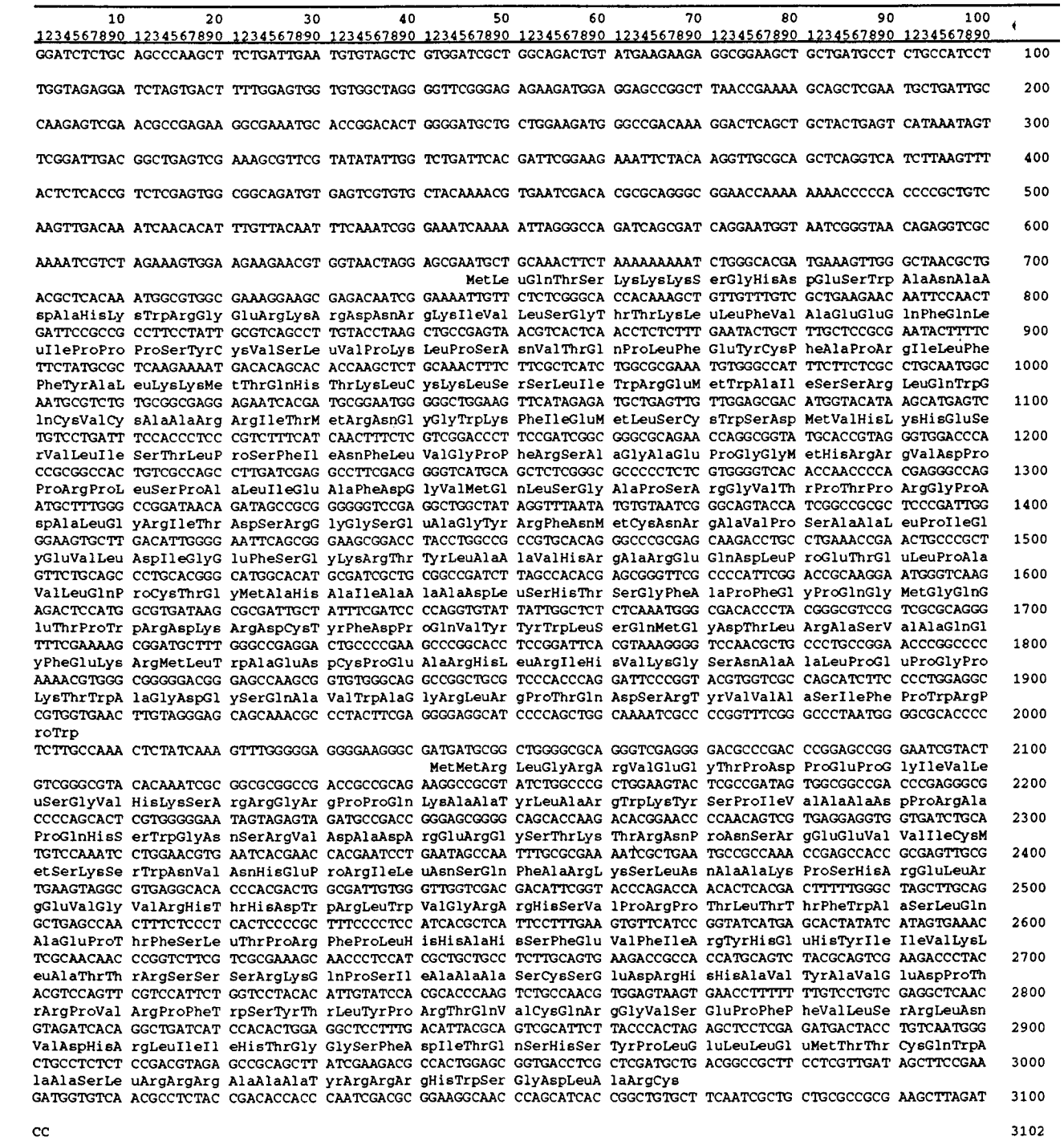

FIG. 2. Nucleotide sequence of the glyphosate utilization genes and deduced amino acid sequence.

two polypeptides matched in size the predicted products of *glpA* and *glpB*, respectively. Thus, the number of polypeptide chains produced is in agreement with the open reading frames predicted by DNA sequence.

Homology searches. The deduced amino acid sequences of *glpA* and *glpB* were compared for similarity to known proteins in the New Swiss-Prot database (Gene Works; IntelliGenetics). It was found that *glpA* product has 42% identity to *E. coli* hygromycin B phosphotransferase (7, 11). This homology is exactly the kind of homology that we would expect for the enzyme which is responsible for glyphosate tolerance activity.

Fitzgibbon and Braymer (5) reported the cloning from *Pseudomonas* sp. strain PG2982 of a DNA sequence which is able to increase glyphosate resistance (*igrA* gene). We compared the sequences of the *glpA* DNA and its translated polypeptide with sequences of the *igrA* gene and its protein. No homology was found in either case. The same results were obtained with the DNA and protein sequences from *glpB*.

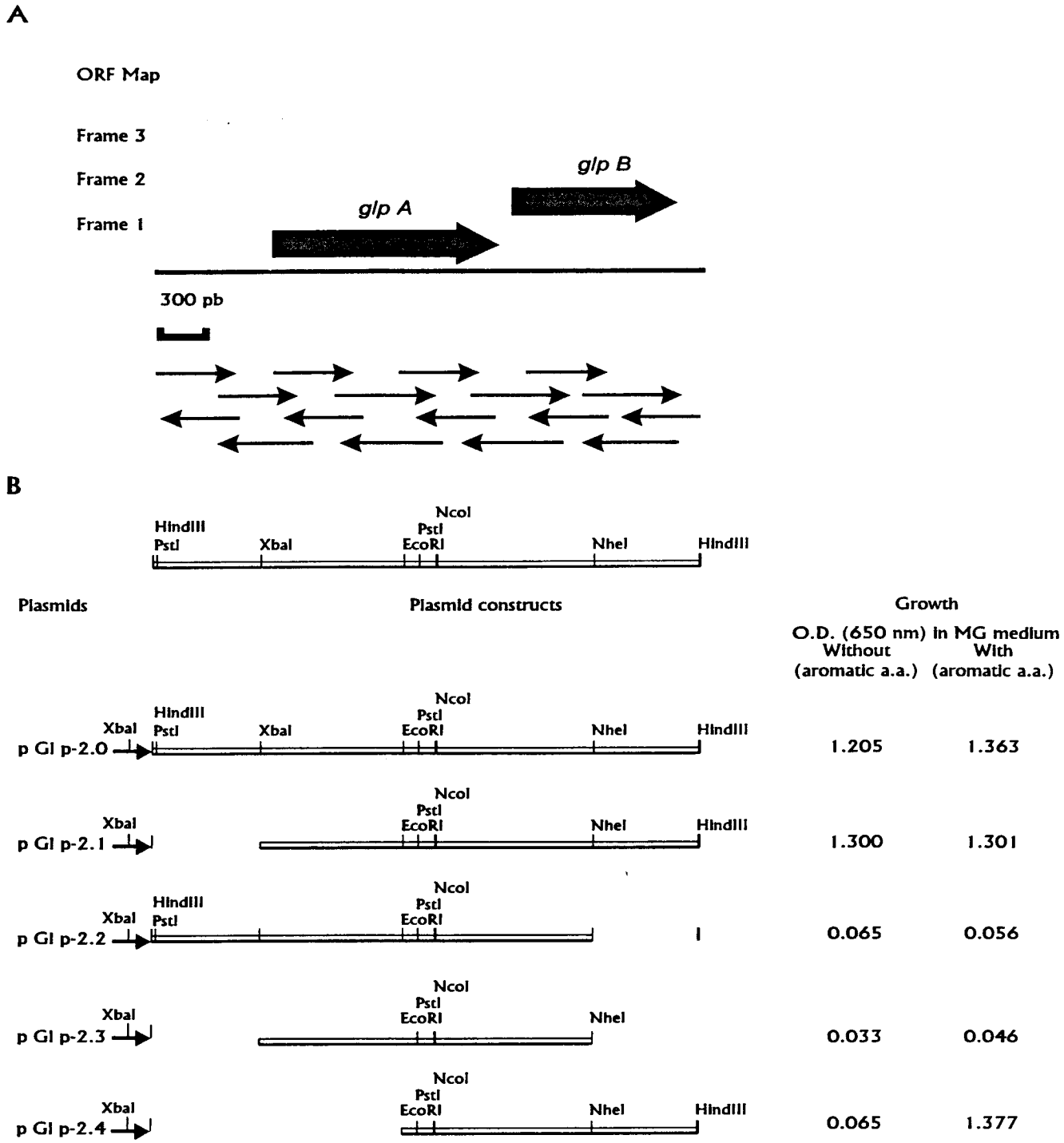

FIG. 3. Schematic representation of the 3.0-kb DNA fragment from *P. pseudomallei* containing glyphosate resistance genes. (A) The open reading frames (ORFs) are shown by thick arrows, with transcriptional direction indicated. The sequencing strategy is shown; each arrow represents the direction and distance of the sequencing. (B) The positions of restriction sites are indicated; *Xba*I belongs to pUC19 polylinker. The plasmid constructs and growth phenotypes (right) are shown. Growth was determined by optical density (O.D.) at 650 nm in test tubes at 37°C for 24 h (average of four replicates). The aromatic amino acids (a.a.) were added as follows: Trp, 0.1 mM; Tyr, 0.1 mM; and Phe, 0.3 mM. The double bar represents the cloned sequence, and the blank space indicates deleted regions.

glpA had 42% identity with the *E. coli* hygromycin B phosphotransferase gene (*hph*), as determined from a homology search (7, 11). To analyze whether *glpA* encodes a functional hygromycin B phosphotransferase, *P. pseudomallei* 22 was grown in M9 medium containing different hygromycin B concentrations. The strain tested was able to grow in concentrations of hygromycin B exceeding 150 μg/ml, while growth of *E. coli* JM103 was inhibited by a concentration of 50 μg/ml. It was also found that *E. coli* strains harboring pGlp-2.0 and pGlp-2.1 were able to grow in hygromycin B at 100 μg/ml. In addition,

TABLE 1. Phosphotransferase assay[a]

Plasmid	Substrate	cpm/μg of protein[b]
pUC19	Glyphosate	64.0
pHph+1	Glyphosate	16,793.0
pGlp-2.0	Glyphosate	13,788.0
pUC19	Hygromycin B	70.0
pHph+1	Hygromycin B	37,401.0
pGlp-2.0	Hygromycin B	14,403.0

[a] Cell extracts from *E. coli* harboring different plasmids were tested for the ability to phosphorylate hygromycin B or glyphosate with ATP as described by Haas and Dowding (8).

[b] The values shown are corrected by reference to values for controls without active enzyme.

E. coli cells containing the *hph* gene (plasmid pHph+1; Boehringer Mannheim) were able to grow in M9 medium with glyphosate at 1 g/liter, whereas *E. coli* control cells not harboring the *hph* gene were inhibited at 0.25 g of glyphosate per liter. *E. coli* cells containing the *hph* gene failed to utilize glyphosate as a phosphorus source. These results suggest that tolerance to glyphosate can be achieved by the presence of a phosphotransferase gene. To test this activity, a phosphotransferase assay was performed and the aminoglycoside-phosphorylating activity was tested by using glyphosate or hygromycin B with [γ-^{32}P]ATP (Table 1 and Fig. 4). The results from the phosphotransferase assays demonstrated that glyphosate was phosphorylated by the *E. coli* or *P. pseudomallei* phosphotransferase. However, the phosphotransferase from *P. pseudomallei* showed a lower activity to phosphorylate hygromycin B compared with the phosphotransferase from *E. coli*.

In the plasmid isolated from *E. coli*, the *hph* gene was found downstream of the apramycin acetyltransferase gene (11). Thus, we tested the ability of *E. coli* cells harboring plasmid pGlp-2.0 to grow in M9 medium with apramycin, kanamycin, neomycin, and streptomycin at 100 μg/ml. Results showed that *E. coli* cells harboring plasmid pGlp-2.0 were inhibited at 25 μg/ml by the tested antibiotics. The predicted protein product of *glpB* showed no similarities with known proteins.

DISCUSSION

Strains of *P. pseudomallei* that could metabolize glyphosate as well as AMPA as sole sources of phosphorus were isolated from soil. However, these cultures could not utilize either glyphosate or AMPA as a carbon source. This finding is consistent with previous studies in which it has been noticed that few phosphonic acids can be used as sole carbon sources by microorganisms (14, 24). In *P. pseudomallei*, the initial attack on the glyphosate molecule seems not to be at the C-P bond, since this bacterium cannot utilize sarcosine or glyphosate as a sole source of either carbon or nitrogen. If glyphosate is first converted into sarcosine by a C-P lyase activity as previously reported (14, 24), *P. pseudomallei* would be able to utilize this compound as either a carbon or nitrogen source. Instead, glyphosate may undergo a stepwise degradation from the carboxyl end of the molecule, with AMPA as a metabolic intermediate, and therefore these organisms are able to grow with AMPA or glyphosate as a phosphorus source. Similar results were reported for *Pseudomonas* sp. strain LBr (10). This strain was found to degrade glyphosate by way of AMPA, and it has characteristics similar to those of *P. pseudomallei* 22, such as the ability to grow in high levels of glyphosate. Balthazor and Hallas (3) found that *Flavobacterium* sp. degraded glyphosate in the presence of P_i with AMPA as a metabolic intermediate. *P. pseudomallei* 22 was able to grow in the presence of both compounds.

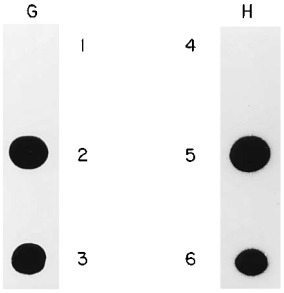

FIG. 4. Autoradiogram of a dot blot showing the phosphotransferase assay. Cell extracts from *E. coli* JM103 harboring plasmids pGlp-2.0 (dots 3 and 6), pHph+1 (dots 2 and 5), and pUC19 (dots 1 and 4) were analyzed for phosphotransferase-phosphorylating activity on Whatman P-81 phosphocellulose paper (8), using glyphosate (G) or hygromycin B (H) as the substrate.

We report the DNA sequence for *P. pseudomallei glp* genes which permit *E. coli* cells to grow on glyphosate as a sole phosphorus source. This utilization involves the activities of two enzymes: a phosphotransferase and a unknown protein that probably converts glyphosate by cleavage of the N-C bond to AMPA a substrate of the *E. coli* C-P lyase.

The phosphotransferase (encoded by *glpA*) was able to use the aminoglycoside hygromycin B antibiotic as a substrate, and the enzyme hygromycin B phosphotransferase from *E. coli* phosphorylated glyphosate. Therefore, *glpA* confers increased tolerance to glyphosate in *E. coli* cells, and its product has significant homology to phosphotransferases, whereas *E. coli* cells harboring plasmid pGlp 2.4 carrying *glpB* were able to use glyphosate as a phosphorus source. These results might suggest that *glpB* encodes a protein with the ability to break down the N-C bond of glyphosate to AMPA. There are no reports of sequences with enzymatic activities similar to the glyphosate activity of this *glpB* gene.

However, in *Pseudomonas* sp. strain PG2982, which can grow in glyphosate as a phosphorus source, Fitzgibbon and Braymer (5) also reported the presence of a plasmid-encoded protein with the ability to increase glyphosate resistance (*igrA* gene) in *E. coli*. Therefore, we compared the sequences and found no homology between DNA and protein sequences with *glpA* and *glpB*. These results suggest that the mechanisms of tolerance in the two bacteria are different. The addition of aromatic amino acids in the family *Rhizobiaceae* (14) was found necessary to degrade glyphosate because of the lack of an alternative tolerance mechanism. Additional data supporting the tolerance mechanism were found when all of the strains tested in this work were able to grow with glyphosate even in the presence of an inorganic source of phosphorus. Therefore, our strain should have a mechanism which confers tolerance to glyphosate by first phosphorylating (*glpA*) the herbicide and then cleaving at the N-C bond (*glpB*) to probably form AMPA, a substrate metabolized by the *E. coli* C-P lyase, thus supporting very high levels of glyphosate (10.2 g/liter).

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