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Cloning of a Gene from *Pseudomonas* sp. Strain PG2982 Conferring Increased Glyphosate Resistance

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A plasmid carrying a 2.4-kilobase-pair fragment of DNA from *Pseudomonas* sp. strain PG2982 has been isolated which was able to increase the glyphosate resistance of *Escherichia coli* cells. The increase in resistance was dependent on the presence of a plasmid-encoded protein with a molecular weight of approximately 33,000, the product of a translational fusion between a gene on the vector, pACYC184, and the insert DNA. An overlapping region of the PG2982 chromosome carrying the entire gene (designated *igrA*) was cloned, and a plasmid (pPG18) carrying the gene was also able to increase glyphosate resistance in *E. coli*. A protein with a molecular weight of approximately 40,000 was encoded by the PG2982 DNA contained in pPG18. This plasmid was not able to complement a mutation in the gene for 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*) in *E. coli*, and modification of glyphosate by *E. coli* cells containing the plasmid could not be demonstrated. The nucleotide sequence of the PG2982 DNA contained an open reading frame able to encode a protein with a calculated molecular weight of 39,396.

Glyphosate [*N*-(phosphonomethyl)glycine] is the active ingredient in the herbicide Roundup, produced by the Monsanto Chemical Co., St. Louis, Mo. Glyphosate is able to inhibit the growth of a wide variety of procaryotic and eucaryotic organisms primarily through its ability to inhibit strongly the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; E C 2.5.1.19) (31), the product of the *aroA* gene in *Escherichia coli*. This enzyme catalyzes the conversion of shikimate-3-phosphate to 5-enolpyruvylshikimate-3-phosphate in the prechorismate aromatic amino acid biosynthetic pathway (13).

Glyphosate resistance has been the subject of several investigations (1, 6, 16, 23, 24), and recent efforts to genetically engineer plants displaying herbicide selectivity have involved the construction of plants resistant to glyphosate (5, 10, 25, 29). Some of these studies have involved the expression, in plants, of genes encoding a glyphosate-resistant EPSPS. While these plants did show an increase in resistance to glyphosate, they usually did not grow as well as plants which were not exposed to the herbicide (5, 10). This was probably due to a decrease in the ability of the mutant enzyme to carry out catalysis. Alternatively, it has been suggested (5) that other targets of glyphosate exist and were affected in these plants. A study by Comai et al. (6) has also suggested that multiple targets of glyphosate may exist. After ethyl methanesulfonate mutagenesis of *Salmonella typhimurium*, only about 1% of the glyphosate-resistant mutants they isolated were also *aroA* mutants.

Pseudomonas sp. strain PG2982, isolated by Moore et al. (19), is able to grow in concentrations of glyphosate exceeding 100 mM (H. D. Braymer, unpublished observations), while growth of *E. coli* is severely inhibited by a concentration of 5 mM (24) and very little growth of *Corydalis sempervirens* (a higher plant) occurs in the presence of 0.5 mM glyphosate (29). PG2982 also has the ability to utilize glyphosate (19), as well as several other phosphonate compounds (27), as a sole phosphorus source.

In this report, we describe the cloning of a novel gene from PG2982 which was able to increase the glyphosate resistance of *E. coli* cells.

MATERIALS AND METHODS

Bacteria and plasmids. *Pseudomonas* sp. strain PG2982 has been described by Moore et al. (19). *E. coli* strains used in this study included K802 (*metB1 hsdR2 mcrB1 mcrA*) (33), LC3 [*hsdR*::Tn*5*(Tc^r) *aroA*] (6), CSR603 (*recA uvrA6 phr-1*) (21), and NM522 [Δ(*lac-pro*) F′*lacZ*ΔM15 *lacI*^{q} *hsd*Δ*5*] (12). Plasmids pACYC184 and pUC19 have been described before (3, 34). Phage vectors M13mp18, M13mp19, and λEMBL3 have also been described (12, 32, 34).

Media and reagents. Minimal medium (9) without phosphorus, containing 1% gluconate, 100 μg of thiamine per ml, 1% methionine, and the appropriate antibiotic, was used to test for the ability to utilize glyphosate as a sole phosphorus source. M63M broth, used to test for glyphosate resistance, was M63 minimal broth with glucose as the carbon source, described by Silhavy et al. (28), plus methionine (1%). *E. coli* strains were maintained on L agar plus the appropriate antibiotic. L agar was Luria broth (LB; 18) with 1.5% agar. Antibiotic concentrations were as follows: ampicillin (Ap), 50 μg/ml; chloramphenicol (Cm), 50 μg/ml (200 μg/ml for plasmid amplification); tetracycline (Tc), 10 μg/ml.

Glyphosate (free acid form, 99.7% purity) and [3-^{14}C] glyphosate were supplied by the Monsanto Chemical Co., St. Louis, Mo.

DNA manipulations. Small-scale plasmid preparations were done by the method of Birnboim and Doly (2). Large-scale cesium chloride plasmid preparations were done by the method of Guerry et al. (15) after amplification with chloramphenicol (4) when possible. The methods of Silhavy et al. (28) were used for the preparation of lambda DNA and chromosomal DNA from *Pseudomonas* sp. strain PG2982. The methods of Maniatis et al. (17) were used for all other basic DNA manipulations, including hybridizations and lambda library construction.

Maxicell preparations. The maxicell procedure of Silhavy

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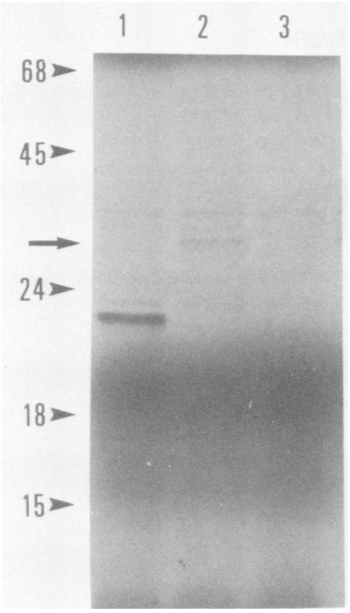

FIG. 1. Autoradiograph of sodium dodecyl sulfate-polyacrylamide gel showing plasmid-encoded proteins labeled with L-[^{35}S] methionine, using the maxicell procedure. Lanes: 1, pACYC184; 2, pPG2.4; 3, pPG4.2. The position of the 33-kDa hybrid protein is marked by the arrow. The sizes (kilodaltons) and positions of molecular weight marker proteins are indicated.

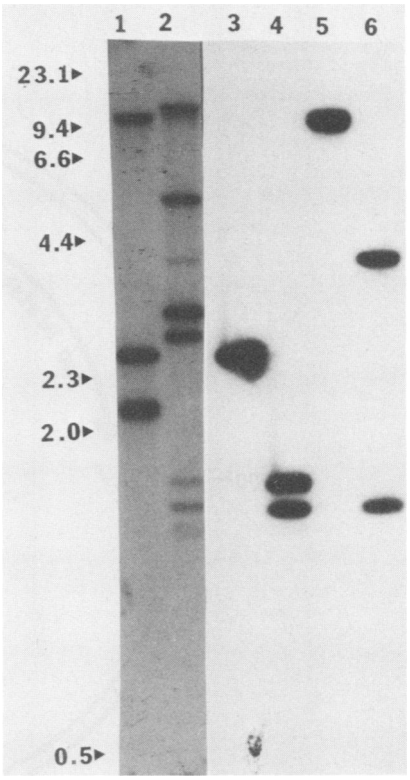

FIG. 2. Hybridization of ^{32}P-labeled pPG124 to chromosomal DNA isolated from PG2982 and phage DNA prepared from λPG2 and λPG6. Lanes: 1, PG2982 DNA digested with *Eco*RI; 2, PG2982 DNA digested with *Sal*I; 3, λPG2 DNA digested with *Eco*RI; 4, λPG2 DNA digested with *Sal*I; 5, λPG6 DNA digested with *Eco*RI; 6, λPG6 DNA digested with *Sal*I.

et al. (28) was performed for ^{35}S labeling of plasmid-encoded proteins, using *E. coli* CSR603 (21).

Glyphosate breakdown assay. Breakdown of [3-^{14}C]glyphosate by *Pseudomonas* sp. strain PG2982 results in the evolution of $^{14}CO_2$ and loss of radioactivity from the culture (26). To assay for the breakdown of glyphosate by *E. coli* cultures containing DNA from PG2982, cells were grown to mid-log phase in M63M broth plus appropriate antibiotics. They were concentrated 20-fold in the same medium, and [3-^{14}C]glyphosate was added to a final concentration of 1 mM. Cells were then incubated for 2 days at room temperature with constant shaking. Samples were removed periodically, and radioactivity was measured by liquid scintillation on a Beckman LS6800 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

TLC. After incubation with [3-^{14}C]glyphosate, supernatants were spotted onto cellulose thin-layer chromatography (TLC) plates (Brinkmann Instruments, Inc., Westbury, N.Y.). The plates were chromatographed in solvent system I of Sprankle et al. (30) consisting of ethanol-water-15 N NH_4OH-trichloroacetic acid-17 N acetic acid (55:35:2.5:3.5g:2, vol/vol/vol/wt/vol/). To localize ^{14}C-labeled compounds on the TLC plates, consecutive 1-cm lengths of the TLC plate lanes were scraped into vials and counted by liquid scintillation. Control lanes, spotted with glyphosate only, were cut from the plate, and glyphosate was visualized by spraying the plate uniformly with a 1% ninhydrin solution in ethanol and heating at 60°C for 10 min. The position of the glyphosate spot on the plate was then correlated with peaks of radioactivity from the labeled samples.

Glyphosate uptake and resistance assays. [3-^{14}C]glyphosate uptake assays were performed by the method of Fitzgibbon and Braymer (11). Briefly, bacteria, grown to mid-log phase in M63M broth plus antibiotics, were suspended in the same medium to an optical density at 600 nm (OD_{600}) of 1.0 and [3-^{14}C]glyphosate was added to 1 mM. Samples (100 μl) were drawn periodically between 30 s and 10 min after addition of glyphosate. They were immediately filtered through 0.45-μm-pore-size Metricel membrane filters (Gelman Sciences Inc., Ann Arbor, Mich.), washed with 5 ml of M63M broth at room temperature, and placed in vials; radioactivity was measured by liquid scintillation.

To test for glyphosate resistance, overnight cultures, grown in M63M broth with the appropriate antibiotics, were used to inoculate 20 ml of M63M broth plus antibiotics in 50-ml flasks. When the cultures reached an OD_{600} of between 0.250 and 0.400, glyphosate was added to the culture (to 1 or 2 mM) and OD_{600} was monitored during subsequent growth.

DNA sequencing. For sequencing, DNA from pPG18 was subcloned into M13mp18 or M13mp19 (34). Single-stranded template DNA was prepared from M13 lysates by the method of Gough and Murray (14). Sequencing was done by the method of Sanger et al. (22) or using the recommended procedures with the Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Both strands of the DNA were sequenced for the entire coding region of the gene and approximately 300 nucleotides flanking the coding region.

Analysis of the sequence data was performed with the University of Wisconsin Genetics Computer Group software described by Devereux et al. (8). The GenBank accession number for the DNA sequence of the *igrA* gene is M 37389.

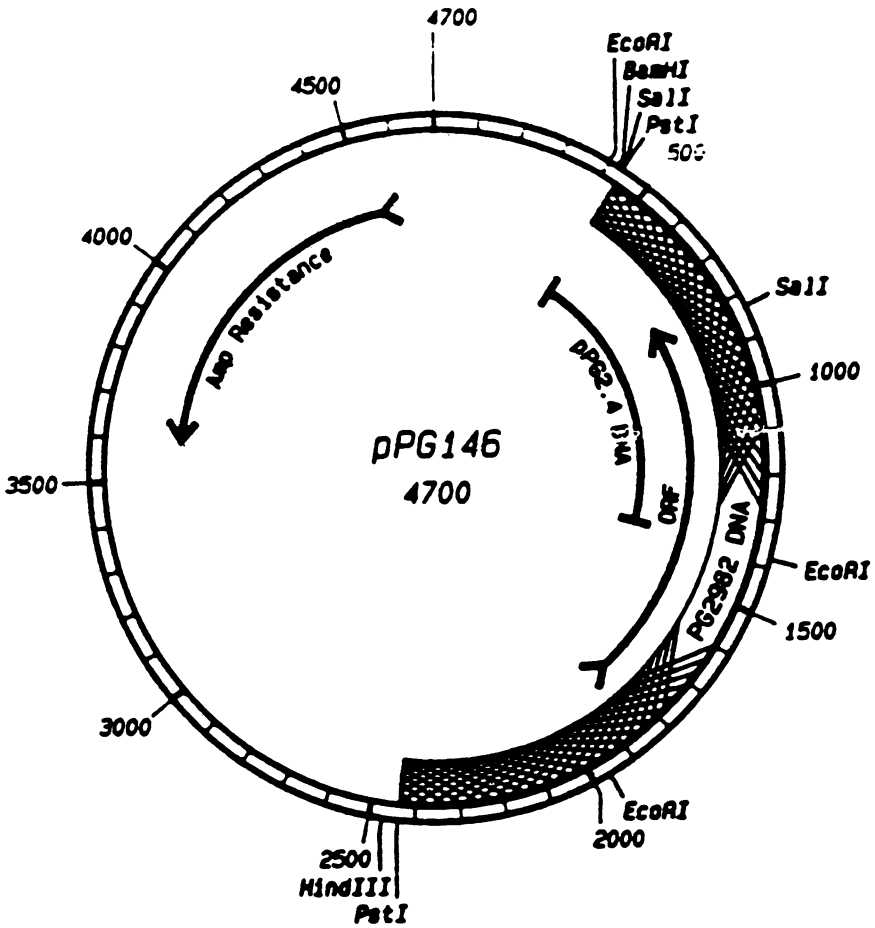

FIG. 3. Map of pPG146. The position of the open reading frame is indicated. Also shown is the portion of pPG146 DNA which is also contained in pPG2.4 (*Eco*RI-*Pst*I restriction fragment).

RESULTS

Chromosomal DNA isolated from *Pseudomonas* sp. strain PG2982 was partially digested with *Eco*RI, and the resulting restriction fragments were ligated into the *Eco*RI site of pACYC184. Ligations were used to transform *E. coli* K802. After transformation and outgrowth, the transformation mixtures were washed twice in M63M broth and placed into four 50-ml flasks each containing 20 ml of M63M broth plus 2 mM glyphosate. After 24 h, growth was apparent in one of the flasks. This culture was streaked onto an L-agar plate containing tetracycline, and small-scale plasmid preparations were done with six of the isolated colonies from the plate. Of the six plasmids recovered, three contained the same *Eco*RI insert of approximately 2.4 kbp. This plasmid was designated pPG2.4. To be sure that a chromosomal mutation was not responsible for the observed increase in glyphosate resistance, pPG2.4 was used to retransform strain K802 to tetracycline resistance. In the presence of 2 mM glyphosate (added at an OD_{600} of 0.250), this transformant had a generation time of 5 h, while the control culture, containing pACYC184, had a generation time of 12 h. (In the absence of glyphosate, each had a generation time of 2 h.)

The ability of this plasmid to increase glyphosate resistance was found to be dependent on the orientation of the *Eco*RI insert within the vector. Plasmid pPG4.2, containing the same insert in the opposite orientation (isolated by digesting pPG2.4 with *Eco*RI, religating, and screening plasmids from Tc^r Cm^s colonies by restriction analysis), was not able to confer an increase in glyphosate resistance. Maxicell analysis of plasmid-encoded proteins demonstrated the production of a 33,000-Da protein from pPG2.4 but not from pPG4.2 (Fig. 1). Therefore, a translational fusion between the Cm^r gene of pACYC184 and the PG2982 DNA was thought to be responsible for the glyphosate-resistant phenotype of cells containing pPG2.4.

To isolate this entire gene from *Pseudomonas* sp. strain PG2982, a lambda library was constructed in the vector λEMBL3. The library was screened for hybridization to the ^{32}P-labeled plasmid pPG124 (pUC19 plasmid vector containing the *Eco*RI insert from pPG2.4). Positive plaques were purified and confirmed by rehybridization. One lambda clone which hybridized very strongly to pPG124 was found (λPG2). Another, λPG6, did not hybridize as well as λPG2 but the hybridization signal was quite strong. In addition, three other phages showed slight hybridization to the probe. Hybridization of the same probe to *Eco*RI-digested DNA from PG2982, λPG2, and λPG6 demonstrated that λPG2 contained the 2.4-kbp *Eco*RI restriction fragment originally found in pPG2.4. λPG6 contained an *Eco*RI restriction fragment of approximately 10.0 kbp which also hybridized to the probe (Fig. 2).

λPG2 contained a *Pst*I fragment of approximately 2,000 bp which included about 950 bp of DNA that was also contained in pPG2.4 (Fig. 3, *Eco*RI-*Pst*I restriction fragment) in addi-

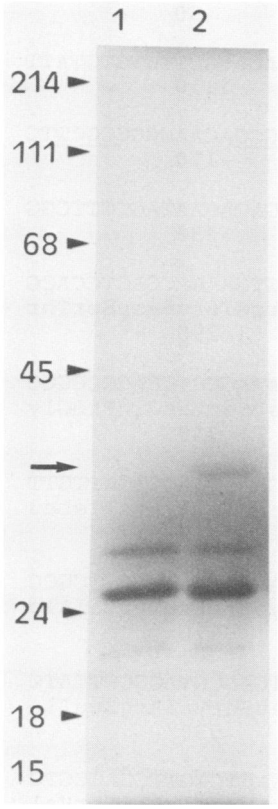

FIG. 4. Autoradiograph of sodium dodecyl sulfatepolyacrylamide gel showing plasmid-encoded proteins labeled with L-[^{35}S] methionine, using the maxicell procedure. Lanes: 1, pUC19; 2, pPG146. Arrow marks the position of the 40-kDa protein produced by pPG146. The sized (kilodaltons) and positions of molecular weight marker proteins are indicated.

tion to 1,050 bp upstream of this region. This fragment was ligated into the *Pst*I site of vector pUC19, and the resulting plasmid was designated pPG146 (Fig. 3). Maxicell analysis of pPG146 demonstrated production of a protein of about 40 kDa in addition to the β-lactamase polypeptides produced by the vector (Fig. 4). The gene was then removed from pPG146 by digestion with *Bam*HI and *Hin*dIII and ligated into pACYC184 which had been digested with the same enzymes. The resulting plasmid (pPG18) was able to confer a significant increase in glyphosate resistance; in resistance assays the generation time of K802 (pPG18) was 9 h as opposed to 12 h for K802(pACYC184). (Glyphosate was added to 1 mM at an OD_{600} of 0.400.) We have designated this gene *igrA* for increased glyphosate resistance.

Both plasmids (pPG2.4 and pPG18) were tested for the ability to complement the *aroA* mutation of *E. coli* LC3. Neither plasmid was able to complement the mutant. (The *E. coli aroA* gene would not hybridize to chromosomal DNA from PG2982 and would not hybridize to the plasmids isolated in this study [data not shown].)

Increased glyphosate resistance could also have resulted if the *igrA* gene product was responsible for the ability to break down or modify glyphosate. However, *E. coli* cells carrying either pPG2.4 or pPG18 were unable to grow in minimal medium with glyphosate as the sole phosphorus source (after 4 days at 37°C with shaking), while control cultures, containing 2 mM inorganic phosphate in place of glyphosate, showed significant growth in <24 h. Glyphosate breakdown assays, performed on cells containing pPG2.4, showed that even after 48 h at room temperature with constant shaking, [3-^{14}C]glyphosate was not lost from the culture in the form of $^{14}CO_2$ and the label was not found in anything other than glyphosate after TLC analysis of the culture supernatant (data not shown).

It was also possible that the *igrA* gene product was responsible for exclusion of glyphosate from the cell. However, [3-^{14}C]glyphosate uptake assays indicated that uptake was similar in cells containing pPG2.4 and pACYC184. In both cultures, uptake reached a maximum of approximately 10 nmol/ml of cells during the first 3 min of the assay and thereafter remained very close to this value.

The nucleotide sequence of *igrA* is shown in Fig. 5. It contains an open reading frame from nucleotide 217 to nucleotide 1272. A search of the DNA upstream of the possible translational initiation site has revealed a region which resembles known promoter sequences of *Pseudomonas* genes (7). In addition, a sequence containing 7 bp in common with the *E. coli* consensus promoter sequence (20) has been located in this region (Fig. 5). The reading frame would encode a protein with a calculated molecular weight of 39,396. This is very close to the molecular weight (40,000) of the protein estimated by its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Nucleotide sequence comparisons have not shown any clear similarity to known genes in the GenBank data base, including the *E. coli* and *S. typhimurium* genes for EPSPS. Translation of the putative open reading frame and subsequent amino acid sequence comparisons have also shown no significant similarity to known proteins.

DISCUSSION

A gene has been isolated from *Pseudomonas* sp. strain PG2982 which has the ability to confer an increase in glyphosate resistance when cloned in *E. coli*. PG2982 is able to grow in concentrations of glyphosate exceeding 100 mM (Braymer, unpublished observations); its EPSPS is probably resistant to glyphosate. Expression of the gene encoding this enzyme would likely increase the glyphosate resistance of *E. coli* cells. However, comparisons of the *igrA* gene with the *aroA* genes of *E. coli* and *S. typhimurium* revealed no significant similarity, or regions of similarity, between these sequences. Also, the *igrA* gene was not able to complement the *E. coli aroA* mutation of strain LC3. (We have, as yet, been unable to isolate an *aroA* complementing plasmid or phage from the PG2982 genomic library.) Therefore, if *igrA* is the gene for EPSPS, the protein is not capable of functioning properly in the heterologous host. Efforts to construct an EPSPS mutant of PG2982 have not been successful; therefore, we have not been able to test the ability of this gene to complement such a mutation in the homologous system. Proof that this gene does, or does not, encode the PG2982 EPSPS must await construction of the PG2982 mutant or purification of the 40-kDa protein and testing of its EPSPS activity.

PG2982 is also able to utilize glyphosate and other phosphonate compounds as a sole phosphorus source (19, 27). Expression of genes responsible for glyphosate catabolism could increase the glyphosate resistance of *E. coli*. It seems unlikely that this would occur in a medium such as M63M, containing 50 mM inorganic phosphate, since inorganic phosphate prevents the breakdown of glyphosate by PG2982 (K. Moore, M.S. thesis, Louisiana State University Baton Rouge, 1983). However, if any of the plasmids were respon-

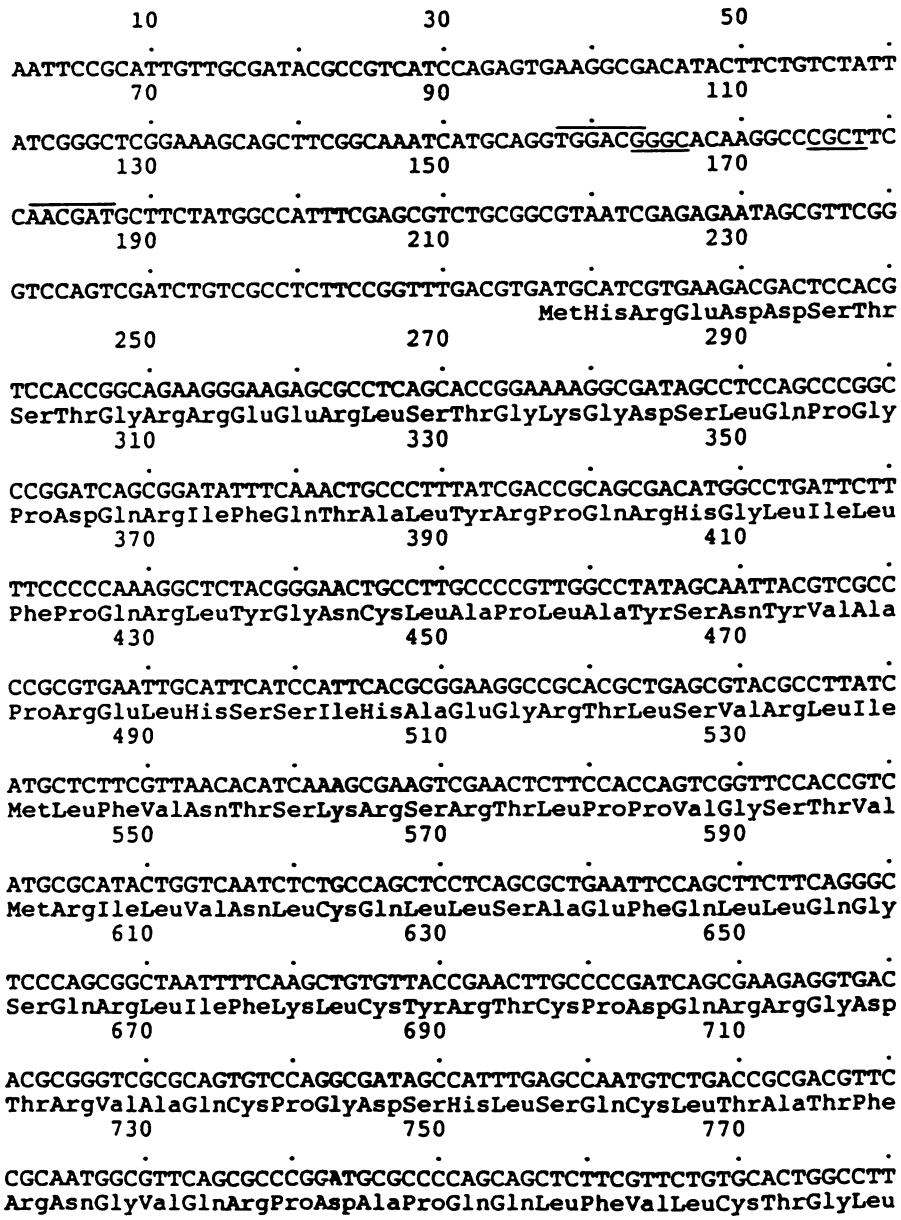

FIG. 5. Nucleotide sequence of the *igrA* gene. The possible *Pseudomonas* promoter found upstream of the coding region is underlined, and the region bearing some similarity to the *E. coli* consensus promoter is overlined. The open reading frame would encode a protein with a calculated molecular weight of 39,396.

sible for the production of enzymes involved in catabolism, host cells would probably be able to utilize glyphosate as a phosphorus source. Our results indicate that such utilization did not occur, and it does not seem as though glyphosate was modified by these cells. It is, however, possible that glyphosate was intracellularly modified to a form which remained within the cell or was not easily distinguished from glyphosate in our TLC system.

It does not seem likely that the *igrA* gene product was responsible for exclusion of glyphosate from the cell since our assays indicated that uptake is similar in cells containing pPG2.4 or pACYC184.

While the ability to break down glyphosate and the expression of a glyphosate-resistant EPSPS probably contribute to the extraordinary glyphosate resistance of PG2982, other factors may also be involved. Mutations in unidentified genes other than *aroA* are known to increase glyphosate resistance in *S. typhimurium* (6). In *Pseudomonas* sp. strain PG2982, one or more of these gene products may be naturally resistant to glyphosate. When cloned in *E. coli*, the gene(s) confers an increase in glyphosate resistance.

Finally, other fragments of DNA from PG2982 were found to hybridize to the pPG2.4 insert (Fig. 2; see text). Preliminary resistance assays indicate that DNA from λPG6 may be able to confer a similar phenotype. In addition to genes encoding EPSPS and proteins involved in glyphosate breakdown, there may be other (possibly related) genes whose

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       790                  810                  830
        .                    .                    .                    .                    .                    .
CAGGGAGCCGCCCTGATTCGCCCGCGCCCCTTCGGGGATTCCGTTCAAATAGCGGGAGGT
GlnGlyAlaAlaLeuIleArgProArgProPheGlyAspSerValGlnIleAlaGlyGly
       850                  870                  890
        .                    .                    .                    .                    .                    .
CAGCAATCCTTGAGCCAGAGGCGAAAAGACAATGCAGCCGGTTCCGATCTCGCCAAGGGT
GlnGlnSerLeuSerGlnArgArgLysAspAsnAlaAlaGlySerAspLeuAlaLysGly
       910                  930                  950
        .                    .                    .                    .                    .                    .
ATCGAGCAGGCCATTTTCGATCCAGCGATTGAACATGTTGTAATTGGGCTGATGAATGAA
IleGluGlnAlaIlePheAspProAlaIleGluHisValValIleGlyLeuMetAsnGlu
       970                  990                 1010
        .                    .                    .                    .                    .                    .
AAGCGGAACCCGCTCTTCCTTCAGGATCGCGGCAGCCTTCTGCGTCAGTTCCGGCGAATA
LysArgAsnProLeuPheLeuGlnAspArgGlySerLeuLeuArgGlnPheArgArgIle
      1030                 1050                 1070
        .                    .                    .                    .                    .                    .
GCTGGAAATCCCCACATAAAGCGCCTTGCCCTGACGGTGCAGATGCGCGAGCGCTCCCAT
AlaGlyAsnProHisIleLysArgLeuAlaLeuThrValGlnMetArgGluArgSerHis
      1090                 1110                 1130
        .                    .                    .                    .                    .                    .
CGTCTCTTCCAGCGGCGTGGTGGCGTCCACACGGTGCGAATAGAAGATGTCGACATAGTC
ArgLeuPheGlnArgArgGlyGlyValHisThrValArgIleGluAspValAspIleVal
      1150                 1170                 1190
        .                    .                    .                    .                    .                    .
GAGCCCCATCCGCTTCAGCGACCTGGTCGAGGCTGGCGATCAGGTATTTGCGACCTGCCG
GluProHisProLeuGlnArgProGlyArgGlyTrpArgSerGlyIleCysAspLeuPro
      1210                 1230                 1250
        .                    .                    .                    .                    .                    .
CCAATGCCTCCGTACGGCCCCGGCCACATGTCCCAACCGGCTTTTGAAGAGATGATCATT
ProMetProProTyrGlyProGlyHisMetSerGlnProAlaPheGluGluMetIleIle
      1270                 1290                 1310
        .                    .                    .                    .                    .                    .
TCATCGCGATAGGGCCGAAAATCACTGGCCATCATGCGGCCGAAGTTTTCTTCCGCTGAG
SerSerArg
      1330                 1350                 1370
        .                    .                    .                    .                    .                    .
CCGGGAGGCGGTCCATAATTGTTTGCGAGGTCGAAGTGGGTCACGCCGAGATGCAACGCC
      1390                 1410                 1430
        .                    .                    .                    .                    .                    .
CGCGCAGAATCGCCGGACCGTTTTCATAGACATCGCGACCGCCGAAATTGTGCCAGAGAC
      1450                 1470                 1490
        .                    .                    .                    .                    .                    .
CAAGCGAAATAGCTGGCAGAATCGATAGCCGCTTCGACCAGTGCGGCGATAGATCATATC
      1510
        .                    .
CGACTGATAACGGTCGGAGCTGCAG
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FIG. 5—*Continued.*

products all contribute to glyphosate resistance in *Pseudomonas* sp. strain PG2982.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Amrhein, N., D. Johanning, J. Schab, and A. Schulz.** 1983. Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture. FEBS Lett. **157**:191–196.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1140–1156.
4. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667–676.
5. **Comai, L., D. Facciotti, W. R. Hiatt, G. Thompson, R. E. Rose, and D. M. Stalker.** 1985. Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. Nature (London) **317**:741–744.
6. **Comai, L., L. C. Sen, and D. M. Stalker.** 1983. An altered *aroA* gene product confers resistance to the herbicide glyphosate. Science **221**:370–371.
7. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Alginate biosynthesis: a model system for gene regulation and function in *Pseudomonas*. Biotechnology **5**:469–477.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
9. **Dworkin, M., and J. W. Foster.** 1958. Experiments with some microorganisms which utilize ethane and hydrogen. J. Bacte-

riol. **75**:592–595.
10. **Filliatti, J. J., J. Kiser, R. Rose, and L. Comai.** 1987. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Biotechnology **5**:726–730.
11. **Fitzgibbon, J., and H. D. Braymer.** 1988. Phosphate starvation induces uptake of glyphosate by *Pseudomonas* sp. strain PG2982. Appl. Environ. Microbiol. **54**:1886–1888.
12. **Frischauf, A., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
13. **Gibson, F., and J. Pittard.** 1968. Pathways of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. Bacteriol. Rev. **32**:465–492.
14. **Gough, J. A., and N. E. Murray.** 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. **166**:1–19.
15. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
16. **Kishore, G. M., L. Brundage, K. Kolk, S. R. Padgette, D. Rochester, Q. H. Huynh, and G. della-Cioppa.** 1986. Isolation, purification and characterization of a glyphosate tolerant mutant *E. coli* EPSP synthase. Fed. Proc. Am. Soc. Exp. Biol. **45**:1506.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Moore, J. K., H. D. Braymer, and A. D. Larson.** 1983. Isolation of a *Pseudomonas* sp. which utilizes the phosphonate herbicide glyphosate. Appl. Environ. Microbiol. **46**:316–320.
20. **Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure.** 1984. *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. Nucleic Acids Res. **12**:789–800.
21. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**: 692–693.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
23. **Schulz, A., A. Kruper, and N. Amrhein.** 1985. Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiol. Lett. **28**:297–301.
24. **Schulz, A., D. Sost, and N. Amrhein.** 1984. Insensitivity of 5-enolpyruvylshikimic acid-3-phosphate synthase to glyphosate confers resistance to this herbicide in a strain of *Aerobacter aerogenes*. Arch. Microbiol. **137**:121–123.
25. **Shah, D. M., R. B. Horsch, H. J. Klee, G. M. Kishore, J. A. Winter, N. E. Tumer, C. M. Hironaka, P. R. Sanders, C. S. Gasser, S. Aykent, N. R. Siegel, S. G. Rogers, and R. T. Fraley.** 1986. Engineering herbicide tolerance in transgenic plants. Science **233**:478–481.
26. **Shinabarger, D. L., and H. D. Braymer.** 1986. Glyphosate catabolism by *Pseudomonas* sp. strain PG2982. Appl. Environ. Microbiol. **168**:702–707.
27. **Shinabarger, D. L., E. K. Schmitt, H. D. Braymer, and A. D. Larson.** 1984. Phosphonate utilization by the glyphosate-degrading *Pseudomonas* sp. strain PG2982. Appl. Environ. Microbiol. **48**:1049–1050.
28 **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Smart, C. C., D. Johanning, G. Muller, and N. Amrhein.** 1985. Selective overproduction of 5-enolpyruvylshikimic acid-3-phosphate synthase in a plant cell culture which tolerates high doses of the herbicide glyphosate. J. Biol. Chem. **260**:16338–16346.
30. **Sprankle, P., C. L. Sandberg, W. F. Meggitt, and D. Penner.** 1978. Separation of glyphosate and possible metabolites by thin layer chromatography. Weed Sci. **26**:673–674.
31. **Steinrucken, H. C., and N. Amrhein.** 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. **94**: 1207–1212.
32. **Viera, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
33. **Wood, W. B.** 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.
34. **Yanisch-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.