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Glutathione-*S*-Transferase Activity and Metabolism of Glutathione Conjugates by Rhizosphere Bacteria

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Glutathione-*S*-transferase (GST) activity was determined in 36 species of rhizosphere bacteria with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and in 18 strains with the herbicide alachlor. Highest levels of CDNB-GST activity (60 to 222 nmol · h^{-1} · mg^{-1}) were found in gram-negative bacteria: *Enterobacter cloacae*, *Citrobacter diversus*, *Klebsiella planticola*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Xanthomonas campestris*. There was very low CDNB-GST activity in the gram-positive strains. Rapid metabolism of CDNB-glutathione conjugates, attributable to high levels of γ-glutamyltranspeptidase, also occurred in the gram-negative bacteria, especially pseudomonads. Alachlor-GST activity detected in cell extracts and whole-cell suspensions of some strains of the families *Enterobacteriaceae* and *Pseudomonaceae* was 50- to 100-fold lower than CDNB-GST activity (0.5 to 2.5 nmol · h^{-1} · mg^{-1}) and was, for the most part, constitutive. The glutathione-alachlor conjugate was rarely detected. Cysteineglycine and/or cysteine conjugates were the major products of alachlor-GST metabolism. Whole-cell suspensions of certain *Pseudomonas* spp. dechlorinated from 20 to 75% of 100 μM alachlor in 24 h. Results indicate that rhizosphere bacteria, especially fluorescent pseudomonads, may play an important role in the degradation of xenobiotics such as alachlor via GST-mediated reactions.

Glutathione-*S*-transferase (GST) catalyzes the nucleophilic conjugation of glutathione (GSH) with many diverse electrophilic substrates (9). Glutathione conjugation is a major mechanism of detoxification in mammals (9) and detoxification of at least six major families of herbicides in plants (17). Although the role of GST in detoxification-degradation of xenobiotics by terrestrial microorganisms has been postulated (3, 7, 20), there is need to elucidate the role of this enzyme in biodegradation of xenobiotic compounds.

Lau et al. (22) surveyed GST distribution in a wide range of type cultures of various microorganisms. GST activity occurred in 50% of the bacterial strains tested with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Lower GST activity occurred in bacteria compared with fungi, algae, and protozoa (22). Bacterial GST has been extensively studied in *Escherichia coli*, *Proteus mirabilis*, and other enteric bacteria (4, 31). The finding that the dichloromethane dehalogenase gene from *Methylobacterium* sp. belongs to the GST supergene family suggests that GST-like enzymes may be widely distributed in bacterial xenobiotic degradation pathways (20).

The role of GSH conjugation in soil metabolism of chloroacetamide herbicides was first reported for propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] (19) and later demonstrated for acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide] (7). Initial degradation of the GSH conjugate proceeds via γ-glutamyltranspeptidase to the cysteineglycine (CysGly) conjugate and then hydrolysis by a carboxypeptidase to the cysteine (Cys) conjugate. The resulting thiol-mediated dechlorination produced metabolites corresponding to the oxanilic, sulfonic, and sulfinylacetic acids (7, 19).

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In the terrestrial environment, the rhizosphere provides a habitat supporting a diverse microbial community (28). The rhizosphere microbial populations are manyfold higher than in root-free soil because root exudates provide substrates for a wide range of microorganisms. Because of the increased population density and potentially greater community diversity, xenobiotic degradation may be enhanced in rhizospheres. Accelerated degradation of organophosphorous insecticides and trichloroethylene in plant rhizospheres has been reported (12, 27, 32). Information on GST distribution among terrestrial bacterial isolates is limited. Considering the potential ecological implications of GST for xenobiotic detoxification-degradation, we studied the occurrence of GST in a diverse collection of rhizosphere bacteria, initially with the standard substrate CDNB. Many strains in this collection exhibited biological disease control and/or plant growth-promoting activity, and several *Rhizobium* and *Bradyrhizobium* strains were included. Investigations of the role of bacterial GST in the degradation of the herbicide alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide] and preliminary studies of metabolism of CDNB- and alachlor-glutathione conjugates were undertaken.

MATERIALS AND METHODS

Bacterial strains and preparation of cell extracts (CFEs). Bacterial strains used in this study and their sources are summarized in Table 1. Taxonomic identification was supplied by the source. Stock cultures were maintained in glycerol broth suspensions at −5°C. *Rhizobium* and *Bradyrhizobium* cultures were grown on yeast extract-mannitol broth. All other cultures were grown on tryptic soy broth (TSB).

CFEs were prepared from 48-h-old TSB cultures for nonrhizobial strains. *Rhizobium* cultures and *Bradyrhizobium* cultures were prepared from 96- and 192-h yeast extract-mannitol broth cultures, respectively. Cells were harvested by centrifugation and washed twice in buffer K (0.1 M potassium phosphate buffer [pH 6.8] with 1.0 mM EDTA). The final pellet was resuspended in 3 to 4 volumes of buffer K per volume of cells. Cells were disrupted by sonication with a V1A sonic probe (Teckmar Co., Cincinnati, Ohio) in three 20-s bursts at 50% power at 0°C. Disrupted cells were centrifuged for 5 min at 14,000 × *g* at 10°C. The

TABLE 1. CDNB-GST activity in CFEs of bacterial strains

Strain	Source[a]	Sp act[b] (nmol $h^{-1} \cdot mg^{-1}$)	
		Expt 1	Expt 2
Alcaligenes spp.			
UA2-55	E. A. Milus, University of Arkansas	<12	<12
UA3-25	E. A. Milus, University of Arkansas	<12	<12
Arthrobacter spp.			
UA2-44	E. A. Milus, University of Arkansas	36 ± 24	54 ± 12
UA3-37	E. A. Milus, University of Arkansas	36 ± 6	30 ± 6
UA3-17	E. A. Milus, University of Arkansas	36 ± 24	24 ± 6
Bacillus cereus			
UW85	J. Handelsman, University of Wisconsin	18 ± 6	30 ± 12
Bacillus subtilis			
GB07	Gustafson, Inc., Plano, Tex.	36 ± 12	30 ± 12
Bacillus thuringiensis			
HD-2	NRRL, Peoria, Ill.	30 ± 12	18 ± 12
UZ404	R. Zablotowicz	<12	24 ± 12
Bradyrhizobium japonicum			
Tal435	Niftal, Paia, Hawaii	<12	NT
Tal427	Niftal, Paia, Hawaii	18 ± 6	NT
Citrobacter diversus			
JM92	J. McInroy, Auburn University	90 ± 6	108 ± 12
Enterobacter cloacae			
EC39979	C. Howell, USDA-ARS, College Station, Tex.	222 ± 18	168 ± 24
ECH1	C. Howell, USDA-ARS, College Station, Tex.	192 ± 6	174 ± 12
ECMS	R. Zablotowicz	180 ± 24	138 ± 12
Enterobacter asburiae			
JM895	J. McInroy, Auburn University	18 ± 6	NT
Erwinia carotovora			
JM705	J. McInroy, Auburn University	18 ± 6	NT
Klebsiella pneumoniae			
KP399	W. Hickey, University of Wisconsin	54 ± 12	NT
JM443	J. McInroy, Auburn University	12 ± 6	NT
Klebsiella planticola			
JM676	J. McInroy, Auburn University	48 ± 6	126 ± 12
Pseudomonas aureofaciens			
30-84	D. Weller, USDA-ARS, Pullman, Wash.	54 ± 6	NT
Pseudomonas cepacia			
AMMD	J. Parke, University of Wisconsin	132 ± 18	96 ± 12
PCGA	P. Haratel, University of Georgia	<12	<12
Pseudomonas chlororaphis			
ATTC9446	D. Weller, USDA-ARS, Pullman, Wash.	66 ± 6	NT
Pseudomonas fluorescens			
BD4-13	C. Hagedorn, VPI	48 ± 6	66 ± 6
PRA25	J. Parke, University of Wisconsin	210 ± 12	132 ± 12
UA5-40	E. L. Milus, University of Arkansas	90 ± 6	78 ± 6
PF5	C. Howell, USDA-ARS, College Station, Tex.	48 ± 18	NT
Pseudomonas putida			
M-17	M. Mount, University of Massachusetts	66 ± 6	90 ± 30
Rhizobium meliloti			
102F52	S. Smith, Lipha Tech., Milwaukee, Wis.	42 ± 18	NT
102F62	S. Smith, Lipha Tech., Milwaukee, Wis.	18 ± 6	NT
102F72	S. Smith, Lipha Tech., Milwaukee, Wis.	72 ± 24	NT
Rhizobium leguminosarum			
Tal421	Niftal, Paia, Hawaii	18 ± 6	NT
Salmonella choleraesuis			
JM411	J. McInroy, Auburn University	<12	NT
Serratia plymuthica			
SP	S. Nemec, USDA-ARS	72 ± 12	90 ± 12
Xanthomonas campestris			
XC	D. Boyette, USDA-ARS, Stoneville, Miss.	108 ± 36	66 ± 12

[a] NRRL, Northern Regional Research Laboratory; VPI, Virginia Polytechnic Institute.
[b] Mean and standard deviation of three replicates. NT, not tested.

CFEs were maintained at 5°C till assayed (typically within 2 to 8 h). Less than a 10% loss of CDNB-GST activity was observed over a 24-h storage period at 5°C for several strains (data not shown). Protein was determined according to the method of Bradford (2) with bovine serum albumin as standard. A unit of enzyme activity for CFEs is defined as the production of 1 nmol of product h^{-1}. For whole-cell extracts, it is defined as 1 nmol of product 24 h^{-1}. Specific activity is defined as 1 unit $\cdot$ mg^{-1}. Protein yield in the CFEs ranged from 4 to 25 mg ml^{-1}.

Chemicals, conjugate synthesis, and thin-layer chromatography (TLC) methods. CDNB, GSH, CysGly, Cys · HCl, L-γ-glutamyl-*p*-nitroanilide (GPNA), and

equine GST were purchased from Sigma Chemical Co. (St. Louis, Mo.). Alachlor (99% purity) was purchased from Chem Service (Chester, Pa.). Radiolabelled [U-ring-^{14}C]alachlor (11.5 mCi mmol^{-1}) was a gift from Monsanto Agricultural Corp. (St. Louis, Mo.). Additional [U-ring-^{14}C]alachlor (27 mCi mmol^{-1}) was purchased from Sigma Chemical Co. Purified CDNB-SG was provided by Gerald Lamoureux, U.S. Department of Agriculture Agricultural Research Service (USDA-ARS), Fargo, N.D. The alachlor-glutathione conjugate (AL-SG) and two GSH fragment conjugates, AL-CysGly and AL-Cys, were synthesized by alkaline-mediated (Na_2CO_3) conjugation described elsewhere (8). [U-ring-^{14}C]alachlor (110 nmol, 3.1 μCi) was diluted with 1,890 nmol of unlabelled alachlor in 5 ml of 20% methanol. A 1.5 M excess of the peptide was added with 50 mg of Na_2CO_3 to catalyze conjugation during incubation at 40°C, until greater than 90% of the alachlor was conjugated as determined by TLC. Then, the reaction mixture was acidified and unreacted alachlor was extracted with hexane. Similar syntheses were used for 2,4-dinitrophenol (DNP)-CysGly and DNP-Cys, with nonlabelled compounds. DNP-SG was enzymatically synthesized by reacting 75 μmol each of CDNB and GSH in 200 ml of buffer K with 1 mg of equine liver GST (57 μmol of DNP-SG formed min^{-1} · mg^{-1}) for 4 h and then diluting in buffer K to 0.25 mM DNP-SG.

All TLC was performed on Whatman LK60F (Whatman Inc., Clifton, N.J.) silica gel plates (250 μm with preadsorbent). Samples were applied with a multispotter (Analytical Instruments, Inc., Baltimore, Md.) at 50°C. TLC plates were placed in twin trough chambers (Camag Scientific Inc., Wilmington, N.C.) to facilitate solvent vapor equilibration and developed 10 cm above the preadsorbent zone. Radioactivity distribution in chromatograms was analyzed with an imaging scanner (Bioscan 200; Bioscan Inc., Washington, D.C.). Three solvent systems were used: A = CH_3CN:H_2O:NH_4OH (44:9:1) (24), B = butanol:H_2O:acetic acid (12:3:5), and C = butanone:H_2O:acetic acid (10:1:1) (19, 24). Typical R_fs were as follows: alachlor, 0.84, 0.88, and 0.93; AL-SG, 0.18, 0.12, and 0.03; AL-CysGly, 0.42, 0.31, and 0.09; and AL-Cys, 0.43, 0.42, and 0.15, in systems A, B, and C, respectively.

CDNB-GST assays. CDNB-GST assays were conducted in 1-cm cuvettes as described previously (9, 22). The reaction mixture consisted of 1.0 μmol of CDNB, 1.0 μmol of GSH, and CFE (200 μl, 1 to 5 mg of protein) in a final volume of 1.05 ml. The reaction was initiated with GSH addition, vortexing, and a 15-s equilibration prior to recording absorbance changes. CDNB-GSH conjugation (formation of DNP-glutathione conjugate [DNP-SG] via nucleophilic displacement of Cl with the GSH-thiol) was monitored spectrophotometrically at λ = 340 nm for 2.0 min (kinetic mode, 3 to 6 scans min^{-1}) with a Gilford Response (Gilford Instrument Co., Oberlin, Ohio) or Shimadzu UV160U (Shimadzu Corp., Columbia, Md.) spectrophotometer equipped with a 6-cell automatic cell positioner. DNP-SG concentration was calculated with an extinction coefficient of 9.6 mM^{-1} · cm^{-1} (9). Enzyme preparations for each strain were assayed in triplicate, and assays of most strains were repeated with enzyme preparations from new batches of bacterial cells. Control reactions (without enzyme) were included to determine nonenzymatic CDNB-GSH conjugation. Kinetics of CDNB-GST activity for two strains of *Enterobacter cloacae* (ECH1 and EC39979), *Pseudomonas cepacia* (AMMD), and *Pseudomonas fluorescens* (PRA25) were conducted as described above at GSH concentrations of 2 μM to 4 mM. Values for kinetic parameters of V_{max} and K_m were determined according to the Michaelis-Menten equation with nonlinear regression (SAS version 6.0; SAS Institute, Cary, N.C.). Linearity of CDNB-GST activity was evaluated for a subset of strains, and spectral changes at 340 and 410 nm were recorded over 10 min.

Alachlor-GST assays. Initial alachlor-GST activity was assessed with [U-ring-^{14}C]alachlor in a phase-partitioning assay modified from an atrazine-GST assay (18) with hexane in place of methylene chloride. Substrate was prepared in buffer K–100 μM alachlor–0.01 μCi of [^{14}C]alachlor ml^{-1}–5% methanol. Assays were conducted in polypropylene microcentrifuge tubes containing a final alachlor concentration of 50 μM–2 mM GSH–CFE (1 to 5 mg of protein)–buffer K to a final volume of 550 μl. All assays were conducted with four replicates and with controls (buffer instead of CFE). The reaction was initiated by GSH addition, followed by vortexing and incubation for 1 h at 25°C. Termination was by addition of 550 μl of hexane and vortexing for 30 s. Phase separation was enhanced by centrifugation (5 min, 14,000 × *g*). An aliquot of the aqueous fraction (200 μl) was mixed with 15 ml of Ecolume scintillation cocktail, and radioactivity was determined by liquid scintillation counting (MinaxiB Tricarb 4000 series; Packard Instruments, Meriden, Conn.). Scaled-up alachlor-GST assays were conducted in 25-ml Corex centrifuge tubes (final volume of 6.6 ml, 50 μM alachlor, 0.1 μCi ml^{-1}) under the above conditions. After incubating 3 to 5 h, the tubes were centrifuged (10 min, 7,000 × *g*), and the pellet was extracted with 10 ml of buffer and recentrifuged. Both supernatants were combined and passed through a 3-ml C_{18} solid-phase extraction (SPE) (Baker, Phillipsburg, N.J.) column. The column was eluted with CH_3CN, and the eluate was spotted on TLC plates and developed with solvent system A or B described above. Recovery of ^{14}C-material in the protein pellet, aqueous filtrate from the SPE column, and the CH_3CN eluate was quantified by liquid scintillation counting.

Induction of alachlor-GST activity was assessed for several *Pseudomonas* strains. In the first experiment, cells growing in TSB and exposed to 50 μM alachlor for 6 h during late logarithmic growth were compared with untreated cells. In the second experiment, cells were grown for 24 h in TSB or TSB containing 50 μM alachlor. CFE preparations and hexane partitioning assays was conducted as described above.

Glutathione-conjugate metabolism assays. The ability of selected isolates to metabolize CDNB- and alachlor-glutathione conjugates was evaluated by assaying L-γ-glutamyltranspeptidase activity. This activity was evaluated in CFEs from 10 representative rhizosphere bacterial strains with DNP-SG and GPNA (26) as substrates. In a third test, metabolism of alachlor-SG was determined in *P. fluorescens* BD4-13 and UA5-40 and *Pseudomonas putida* M-17. In the DNP-SG assay, 900 μl of the DNP-SG conjugate stock (0.25 mM, prepared as described above) was added to a 1-cm cuvette. Reactions were initiated by addition of 100 μl of CFE (1.5 to 2.5 mg of protein), and A_{340} and A_{410} were monitored for 2 min. Activity of DNP-γ-glutamyltranspeptidase was calculated on the basis of a decrease in A_{340} and the DNP-SG extinction coefficient. Controls with buffer instead of CFE were included.

In the GPNA assay, 900 μl of GPNA stock (0.40 mM, 0.1 M K-phosphate [pH 8.0]) was added to a 1.0-cm cuvette and reactions were initiated with 100 μl of CFE (0.6 to 1.6 mg of protein). The mixture was vortexed, and the formation of product (*p*-nitroaniline) was monitored at A_{410} in the kinetic mode for 3 min (nine scans every 20 s) (26).

Alachlor-SG (AL-SG) γ-glutamyltranspeptidase activity was determined for three *Pseudomonas* strains. The assay mixture consisted of 200 μl of CFE (3.0 mg of protein) and 200 μl of AL-SG (200 μM, 1.0 μCi ml^{-1}). Aliquots (100 μl) were removed after 1 and 3 h of incubation at 24°C. Equal volumes of CH_3CN were added to these subsamples to terminate the reaction, and the mixture was vortexed and centrifuged (5 min, 14,000 × *g*). The CH_3CN extract was spotted on TLC plates, developed in solvents B and C, and analyzed by radioimaging.

Alachlor whole-cell metabolism studies. Studies to assess alachlor metabolism by intact cells were conducted on 48-h TSB cultures. Cells were harvested by centrifugation and washed twice in buffer K. The pellet was suspended in 6 to 8 volumes of buffer K per volume of cells. Alachlor, 200 μl of a 500 μM stock (0.55 μCi ml^{-1}, in 20% methanol–80% buffer K), was added to 1.0 ml of cell suspension. Each strain was assayed in duplicate. The cells were incubated at 25°C for 24 h on a rotary shaker, and then a 200-μl subsample was removed and the reaction was terminated with 200 μl of CH_3CN, vortexed for 1 min, and centrifuged (5 min, 14,000 × *g*). The CH_3CN extract was spotted on TLC plates and developed with solvent B or C, and the distribution of radioactivity was analyzed as described above.

RESULTS

Validation of CDNB-GST assay. The linearity of CDNB-GST activity was verified in four strains under standard assay conditions. A linear increase in A_{340} was observed for all strains and the nonenzymatic conjugation in controls for 6 to 10 min (Fig. 1A). A greater increase in A_{410} was observed in the two pseudomonads compared with *Citrobacter diversus* JM92 or *E. cloacae* ECH1 (Fig. 1B). The rate of A_{410} increase was low during the first 2 min of incubation. Heat-treated CFEs of all strains exhibited levels of CDNB-GSH conjugation similar to those in controls with no increase at 410 nm. In the absence of exogenous GSH, there was little or no change in A_{340} or A_{410} (data not shown). DNP-SG formation following a 10-min assay was verified for several strains (*P. fluorescens* strains [UA5-40, PRA25, and BD4-13], *P. cepacia* [AMMD], *P. putida* [M-17], and *E. cloacae* strains [EC39979 and ECH1]) by TLC with solvent system B. These strains accumulated a compound with an R_f of 0.23, identical to that of DNP-SG. All *Pseudomonas* strains also accumulated the CysGly and Cys conjugates (R_fs, 0.32 and 0.43, respectively) of DNP in addition to intense yellow compounds with R_fs of 0.80 to 0.97 in solvent system B. The identity of these latter two compounds is unknown, but they are hypothesized to be products of DNP-SG metabolism.

CDNB-GST activity in rhizobacteria. Eight sets of assays (8 to 17 strains per set) with at least two batches of bacteria for most strains were evaluated (Table 1). An overall average least significant difference of 15.6 to 48.6, mean 25.2, was observed in these tests. The highest CDNB-GST specific activity (60 to 222) was measured in gram-negative isolates, especially *Pseudomonas* and *E. cloacae* strains. There was a wide range of specific activities observed among strains of the same species. CDNB-GST activity was low or undetectable (specific activity of 36) in certain genera of gram-negative bacteria (i.e., *Alcali-*

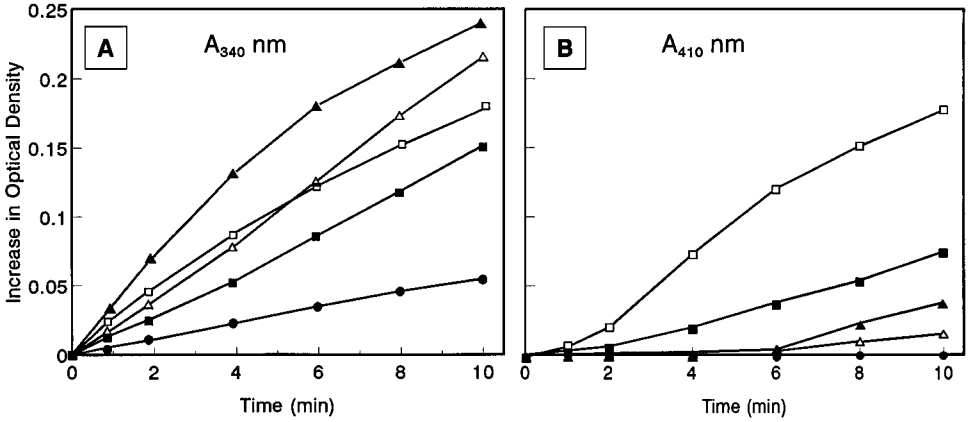

FIG. 1. CDNB-GST assay. DNB-GS formation rates were determined by monitoring increases in optical density (A_{340} [A] and A_{410} [B]) for 10 min in strains ECH1 (△), JM92 (▲), BD4-13 (□), M-17 (■), and control (●).

genes and *Bradyrhizobium* spp.) and in all gram-positive strains of the genera *Bacillus* and *Arthrobacter*. Although measurable, increases in A_{340} equal to or less than specific activity of 36 are within the standard deviation of control values.

Kinetic parameters for GSH-mediated CDNB-GST activity were determined for the four most active strains: *E. cloacae* EC39979 and ECH1, *P. fluorescens* PRA25, and *P. cepacia* AMMD. K_ms for GSH in *E. cloacae* ECH1 and EC39979 were 50 ± 10 and 106 ± 25 μM, respectively, compared with 336 ± 72 and 1,613 ± 270 μM for *P. cepacia* AMMD and *P. fluorescens* PRA25, respectively. V_{max}s were 192 ± 6,240 ± 12, 174 ± 12, and 438 ± 30 nmol · h^{-1} · mg^{-1} for strains ECH1, EC39979, AMMD, and PRA25, respectively. The higher K_ms for GSH found in the pseudomonads reflect a more rapid turnover of GS conjugates. These data indicated that the GSH concentration used was sufficient for enzyme saturation for most bacterial strains.

Validation of alachlor and alachlor conjugate separation in the hexane phase-partitioning assay. This assay was verified with 50 μM U-ring-^{14}C-labelled alachlor, AL-SG, AL-CysGly, and AL-Cys, in buffer K without GSH or enzyme, in addition to complete assay mixtures without CFEs or with CFEs of UA5-40 and BD4-13. After phase partitioning, 12.6% of the alachlor remained in the aqueous phase, while 98.7 to 99.2% of the alachlor conjugates were recovered in the aqueous phase as determined by liquid scintillation counting (data not shown). Conversely, 87.4% of the alachlor and approximately 1.0% of the conjugates were present in hexane fractions. In control assay mixtures (i.e., with GSH but no CFE), 17.9% of the radioactivity remained in the aqueous fraction compared with 41.2 and 28.8% in reaction mixtures containing CFEs of UA5-40 and BD4-13, respectively.

Alachlor-GST activity in CFE. Data are presented for two of four assays assessing alachlor-GST activity in CFEs of selected bacterial strains (total number tested = 18), by the hexane partioning assay (Table 2). Activity (specific activity = 0.6 to 2.3 nmol of AL-SG formed · h^{-1} · mg^{-1}) was found in all pseudomonads. However, there was little or no activity (specific activity <0.15 nmol · h^{-1} · mg^{-1}) in *Alcaligenes*, *Arthrobacter*, *Bacillus*, or *Enterobacter* strains and low activities in *Xanthomonas* and *Serratia* strains (some data not shown).

Scaled-up, longer-term assays were conducted for several pseudomonads to confirm alachor-GST activity in CFEs and to identify metabolites (Table 3). A low amount (10%) of ^{14}C-material was bound to precipitated protein, and about 5% was metabolized to polar compounds not retained by a reverse-phase SPE column. By using TLC with solvent system A, three regions of radioactivity were identified corresponding to R_fs of synthesized conjugates or alachlor. AL-SG was found only in the enzyme-free control, heat-treated CFE, and UA5-40 preparations. One major metabolite (R_f of 0.42, solvent system A) occurred in all CFE preparations but not in enzyme-free controls or heat-treated CFEs. In solvent system C, this radioactive component was resolved into AL-CysGly (dominant component) and AL-Cys (minor component). Strain UA5-40 was most active: 48% of the radioactivity was recovered in spots corresponding to AL-SG and AL-CysGly. Strains BD4-13 and M-17 produced only a trace of AL-SG with 10 to 13% recovered as AL-CysGly and AL-Cys after 5 h. Heat-treated CFEs of all strains had AL-SG levels similar to the buffer control, with no detection of metabolite production or radioactivity accumulation in the SPE column aqueous eluate. Denatured proteins may have had more exposed binding sites for alachlor.

The role of alachlor as a GST inducer was assessed by exposing cells to alachlor for the duration of growth (24 h) or during the late log phase (6 h). A significant increase in AL-GST activity occurred in *P. fluorescens* BD4-13 (48 to 78%) in both experiments (Table 4), while a significant increase in *P. putida* M-17 AL-GST activity (45%) was observed only after a

TABLE 2. Alachlor-GST activity of bacterial strains with a hexane phase-partitioning assay in two separate experiments

Strain	Sp.	Sp act[a] (nmol · h^{-1} · mg^{-1})	
		Expt. 1	Expt. 2
BD4-13	*P. fluorescens*	1.54 a	1.31 b
M-17	*P. putida*	1.36 b	0.71 c
PRA25	*P. fluorescens*	0.81 c	0.51 c
AMMD	*P. cepacia*	0.63 c	0.65 c
UA5-40	*P. fluorescens*	NT[b]	2.33 a
SP	*S. plymuthica*	0.15 de	0.25 de
XC	*X. campestris*	0.40 d	0.15 e
EC39979	*E. cloacae*	NT	0.14 e
UW85	*B. cereus*	0.04 e	0.12 e
UZ404	*B. thuringiensis*	NT	0.15 e
GB07	*B. subtilis*	NT	0.08 e
ECMS	*E. cloacae*	NT	0.07 e

[a] Mean of four replicate assays. Means within a column followed by the same letter do not differ significantly at the 95% confidence level.
[b] NT, not tested.

TABLE 3. Recovery of [U-ring-^{14}C]alachlor and metabolites from alachlor-GST assays of CFEs of three *Pseudomonas* strains as determined by TLC and radioimaging

Incubation period and strain	% ^{14}C detected in various components				
	Protein pellet	Aqueous filtrate	AL-SG	AL-CysGly and AL-Cys	Alachlor
3 h[a]					
UA5-40	13.3	1.6	9.6	4.2	71.1
UA5-40 (HT)[b]	39.8	ND[c]	3.2	ND	56.8
BD4-13	12.0	1.4	ND	4.7	81.7
BD4-13 (HT)	48.9	ND	2.9	ND	48.1
M-17	8.2	2.4	ND	5.3	84.3
M-17 (HT)	27.7	ND	3.1	ND	69.2
Control		ND	2.7	ND	97.3
5 h					
UA5-40	11.7	5.2	31.8	16.0	35.2
BD4-13	9.0	4.6	<0.2	13.3	73.2
M-17	9.5	3.3	<0.2	10.6	78.7
Control		ND	5.8	ND	94.2

[a] 50 μM alachlor, 2 mM GSH, and 4 to 5 mg of protein ml^{-1}.
[b] HT, heat-treated CFE (30 s, 100°C).
[c] ND, not detected.

24-h exposure. There was no significant change in AL-GST activity of *P. fluorescens* PRA25 in either case.

Glutathione conjugate metabolism. Metabolism of the DNP-SG conjugate was quantified on the basis of a decrease in A_{340} (Table 5). We observed rapid metabolism by all pseudomonads and by *Serratia plymuthica* SP. Little metabolism of the DNP-SG conjugate occurred with strains of *E. cloacae*, *Klebsiella planticola*, and *Bacillus cereus*. Specific activities for conjugate degradation by pseudomonads were one- to twofold higher than those for CDNB-GST activity measured by DNP-SG accumulation. The pseudomonads and *S. plymuthica* SP exhibited A_{410} increases of 0.01 to 0.09 min^{-1} · mg of protein^{-1} while all other strains showed little change (e.g., <0.002 min^{-1} · mg of protein^{-1} [data not shown]). This is further evidence that the yellow products formed during CDNB-GST assays result from metabolism of the DNP-SG conjugate. The initial concentration of DNP-SG used in this assay (0.23 mM) is greater than DNP-SG formed during a typical 2-min CDNB-GST assay. No metabolism of the DNP-SG conjugate was observed for heat-treated CFEs (data not shown). γ-Glutamyltranspeptidase activity was also detected with GPNA as substrate (Table 5). Higher activities were found with GPNA compared with DNP-SG for certain strains. Generally, GPNA activity was greater in pseudomonads than in enteric bacteria.

TABLE 4. Effect of alachlor exposure on alachlor-GST activity of CFEs of *Pseudomonas* strains in a hexane partitioning assay

Expt, exposure, and strain	Sp act[a] (nmol · h^{-1} · mg^{-1})	
	Alachlor treated	Control
Expt 1, 6 h		
BD4-13	2.66 a	1.54 b
M-17	1.24 a	1.36 a
PRA25	0.81 a	0.67 a
Expt 2, 24 h		
BD4-13	1.76 a	1.19 b
M-17	1.96 a	1.65 b
PRA25	0.65 a	0.60 a

[a] Mean of four replicates. Means within a row followed by the same letter do not differ significantly at the 99% confidence level.

TABLE 5. L-γ-Glutamyltranspeptidase activity of representative rhizosphere bacteria with DNP-SG and GPNA as substrates

Strain	Sp.	Sp act[a] (nmol · h^{-1} · mg^{-1})	
		DNP-SG	GPNA
AMMD	*P. cepacia*	396 a	180 c
UA5-40	*P. fluorescens*	72 d	48 f
BD4-13	*P. fluorescens*	144 c	294 a
PRA25	*P. fluorescens*	144 c	144 d
M-17	*P. putida*	288 b	240 b
EC39979	*E. cloacae*	6 e	18 g
ECH1	*E. cloacae*	12 e	42 f
JM676	*K. planticola*	6 e	138 d
SP	*S. plymuthica*	90 d	84 e
JM92	*C. diversus*	6 e	84 e

[a] Mean of three replicates. Means within a column followed by the same letter do not differ significantly at the 95% confidence level.

Alachlor-SG γ-glutamyltranspeptidase activity was assayed in CFEs from three *Pseudomonas* strains. UA5-40 exhibited less than 25% of the activity of either M-17 or BD4-13 (data not shown). This lower activity in UA5-40 corresponds with DNP- and GPNA-transpeptidase assays and the above CFE study in which the AL-SG accumulation was observed.

Whole-cell alachlor metabolism. Evidence for whole-cell GST-mediated alachlor metabolism was obtained from incubations with seven rhizobacterial cultures (Table 6). Two to seventy-five percent of the alachlor was recovered as AL-GlyCys and AL-Cys in the seven strains showing activity after 24 h. No AL-SG was detected, but the AL-CysGly conjugate was observed in all active strains. *P. fluorescens* UA5-40 accumulated a greater amount of AL-GlyCys than AL-Cys compared with other strains. Except for *E. cloacae* ECH1, the pseudomonads exhibited higher specific activities than the enteric bacteria. Whole-cell alachlor metabolism was not detectable in *B. cereus* UW85, *Bacillus thuringiensis* UZ404, *P. cepacia* AMMD, and *S. plymuthica* SP. These results are generally consistent with those from the phase-partitioning study in which the two strains of bacilli and *S. plymuthica* had low AL-GST activity whereas that of AMMD was moderate.

TABLE 6. Metabolism of [U-ring-^{14}C]alachlor and alachlor-GST activity by whole-cell suspensions of 11 rhizosphere bacterial strains after 24 h of incubation

Strain	% Total ^{14}C added[a]			Alachlor-GST activity[b]
	Alachlor-CysGly	Alachlor-Cys	Alachlor	
UA5-40	61.0	13.7	25.3	7.06
PRA25	5.4	16.4	78.2	1.61
BD4-13	1.9	18.5	79.6	1.64
M-17	4.4	20.4	75.2	2.03
AMMD	ND[c]	ND	100.0	ND
JM676	4.8	ND	95.2	0.46
ECH1	2.2	9.2	88.6	1.31
JM92	2.2	ND	97.2	0.25
SP	ND	ND	100.0	ND
UZ404	ND	ND	100.0	ND
UW85	ND	ND	100.0	ND
Control	ND	ND	100.0	ND

[a] Average of duplicate assays.
[b] Alachlor-GST activity, nanomoles 24 h^{-1} milligram of total protein^{-1}.
[c] ND, not detected.

DISCUSSION

CDNB-GST activities in bacterial CFEs that we examined are similar to those reported by others (22, 30) and severalfold lower than for many higher organisms (3, 9, 22). CDNB-GST activity of 30 nmol · h^{-1} · mg or less is difficult to ascertain because of limited sensitivity of the assay. Thus, we are limited in detecting GST in gram-positive bacteria such as *Bacillus* spp. It is evident, however, that many genera of gram-negative bacteria inhabiting the rhizosphere can use GST as a potential detoxification mechanism.

There is potential for rapid metabolism of glutathione conjugates, particularly in pseudomonads, which was confirmed with three substrates for γ-glutamyltranspeptidase activity, i.e., DNP-SG, GPNA, and AL-SG. *Pseudomonas* strains exhibited specific activities of 72 to 396 and 48 to 294 nmol · h^{-1} · mg^{-1} with DNP-SG and GPNA, respectively. Strains of the *Enterobacteriaceae* possessed lower specific activities of 6 to 90 and 18 to 138 nmol · h^{-1} · mg^{-1} for DNP-SG and GPNA, respectively. Detection of DNP-Cys indicates the presence of a second carboxypeptidase. Accumulation of CDNB metabolites (increased A_{410}) with either DNP-SG as substrate or following DNP-SG accumulation (Fig. 1) implicates possible cysteine-β-lyase activity. The downstream metabolism of DNP-SG by bacteria warrants further elucidation.

Feng (7) showed that the GST pathway is a major route for microbial degradation of chloroacetamide herbicides in soil. Although we assessed only a small subsample of bacteria for AL-GST activity, we have demonstrated GST-mediated detoxification of alachlor by three assays. AL-GST activity was quantified in CFEs by a hexane fractionation technique, and AL-SG, AL-CysGly, and/or AL-Cys conjugates were identified in select *Pseudomonas* strains. Whole-cell studies confirmed AL-GST activity in strains of the *Pseudomonas* and *Enterobacteriaceae*. The phase-partitioning assay described here is a useful method to screen microorganisms for chloroacetamide-GST activity.

Whole-cell studies demonstrated that metabolites of GSH conjugation accumulate in the absence of added exogenous GSH. Thus, these bacteria contain sufficient GSH pools to allow some degree of endogenous AL-GST activity. GSHs, both oxidized and reduced forms, are the dominant active thiols in gram-negative bacteria but are minor or lacking in gram-positive bacteria (5). The AL-GST activity in whole-cell preparations in some of the pseudomonads was lower than that observed in CFEs. However, whole-cell specific activities were calculated on the basis of total, not soluble, protein, and they were not supplemented with exogenous GSH.

In our assays, AL-GST activity was 50- to 100-fold lower than that observed for CDNB. Solubility limits and the potential for sorption of alachlor to protein or other cellular components reduce the alachlor concentration that can be used in CFE assays. Alachlor assays were conducted at 50 to 100 μM while CDNB assays were conducted at 1.0 mM. In our CFE studies, a significant amount of alachlor remained in the pellet after washing, analogous to high sorption of another chloroacetamide herbicide, metolachlor, by bacterial communities (23). Specific activities of other herbicide-GST assays are typically lower than those for CDNB-GST activity in plants (1, 17, 29).

In plant and animal systems, some GSTs are inducible (17). In the facultative anaerobe *Methylobacterium* sp. strain DM4, the GST-like dichloromethane dehalogenase activity is enhanced over 100-fold following exposure to dichloromethane (20). In our induction studies, a small but significant increase in AL-GST activity (42 to 78%) was observed in two *Pseudomonas* strains after alachlor exposure. This indicates that AL-GST activity in these strains is predominantly constitutive. The effects of induction on other enzymes in the glutathione-conjugate metabolic sequence are unknown.

Cysteine-β-lyase has been implicated in the downstream metabolism of propachlor conjugates by gastrointestinal bacteria (21), as well as 2,4-dichloro-1-nitrobenzene in *Mucor javanicus* (10). Evidence for further metabolism of the AL-Cys conjugate by several of these bacterial strains was observed in whole-cell studies in incubations greater than 24 h and in CFEs with various substrates (34). Our studies (34) indicate that GST-mediated dechlorination of alachlor by rhizosphere bacteria is a mechanism for the formation of polar metabolites of chloroacetamide herbicides (7, 19).

High γ-glutamyltranspeptidase activity observed in most strains tested here indicates that the glutathione conjugates are short-lived transient catabolic intermediates in rhizosphere bacteria. A 30-min half-life of acetochlor-SG compared with a 7-day half-life for acetochlor in the same soil has been reported (7). A strain of *Pseudomonas alcaligenes* that metabolized propachlor to the cysteine conjugate has been reported (19); however, neither GST activity nor GSH intermediates could be demonstrated. Thiol-containing metolachlor metabolites have been observed in mixed bacterial culture (23) and are likely GSH conjugation products. Our studies show that metabolism of GSH conjugates is rapid, and thus, products of these reactions can be significant environmental contaminants and therefore might be considered for inclusion in groundwater monitoring programs.

Various studies have demonstrated the potential for bacterial metabolism of alachlor (6, 24, 25), but metabolic products have not always been identified. Two alachlor metabolites characterized by R_f values with solvent system A (25) are most likely the AL-CysGly and AL-Cys conjugates observed here. Recently, a unique amide cleavage of propachlor, yielding a catechol and the corresponding acetamide, was reported (31). This mechanism and aryl acylamidase cleavage of propachlor (24) may be minor pathways for degradation of chloroacetamide herbicides compared with GST-mediated dechlorination. The enrichment and diversity of gram-negative bacteria in the rhizosphere (14, 28) suggest that these bacteria (especially pseudomonads) could potentially be involved in xenobiotic detoxification in rhizosphere soil. GST activity of rhizosphere bacteria thus may play an important role in the aerobic dechlorination of xenobiotics such as the chloroacetamide herbicides.

Detoxification of compounds in the spermosphere and rhizosphere could potentially be mediated by plant growth-promoting rhizobacteria (15). Certain chemicals used as herbicide safeners induce biochemical changes in susceptible plants, increasing their tolerance to herbicides such as the chloroacetamides and thiocarbamates (11). Karns (13) suggested that bacterial inoculants developed for rapid pesticide detoxification might be useful as protectants. This technology has been used to protect susceptible plant species from the herbicide dicamba (16). Many *Pseudomonas* strains are excellent colonizers of seeds and plant roots (14, 15). Our data show that these organisms have the highest GST activities, and thus, they may be vehicles for delivering plant protection activities to plant roots. Recent studies have shown that inoculation of soil containing high alachlor concentrations (100 μg · g^{-1}) with *P. fluorescens* UA5-40 or *Mucor* strain SSF-1 (a fungus also possessing AL-GST activity) enhanced alachlor degradation (33). Addition of cornmeal enhanced the survival of UA5-40 and alachlor biodegradation.

These studies show that a diverse collection of rhizobacterial

strains possess GST activity. Furthermore, there appears to be some diversity in these enzymes as indicated by the differential specificity for CDNB and alachlor. Thus, GST-mediated reactions may be important metabolic transformations of rhizobacteria exposed to xenobiotics. In addition to the diversity in GST activity, the fluorescent pseudomonads also showed a high potential to metabolize GSH conjugates. Determination of the distribution of conjugate metabolism in rhizospheres might improve our overall understanding of the fate of alachlor and other potential bacterial GST substrates in the environment.

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