APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 427–434 Vol. 63, No. 2
0099-2240/97/$04.00+0

Evidence that Formation of Protoanemonin from Metabolites of 4-Chlorobiphenyl Degradation Negatively Affects the Survival of 4-Chlorobiphenyl-Cometabolizing Microorganisms

RAFAEL BLASCO,† MEGHARAJ MALLAVARAPU,‡ ROLF-MICHAEL WITTICH, KENNETH N. TIMMIS, AND DIETMAR H. PIEPER*

Department of Microbiology, GBF-National Research Centre for Biotechnology, 38124 Braunschweig, Germany

Received 7 May 1996/Accepted 23 October 1996

A rapid decline in cell viability of different PCB-metabolizing organisms was observed in soil microcosms amended with 4-chlorobiphenyl. The toxic effect could not be attributed to 4-chlorobiphenyl but was due to a compound formed from the transformation of 4-chlorobiphenyl by the natural microflora. Potential metabolites of 4-chlorobiphenyl, 4-chlorobenzoate and 4-chlorocatechol, caused similar toxic effects. We tested the hypothesis that the toxic effects are due to the formation of protoanemonin, a plant-derived antibiotic, which is toxic to microorganisms and which has been shown to be formed from 4-chlorocatechol by enzymes of the 3-oxoadipate pathway. Consistent with our hypothesis, addition to soil microcosms of strains able to reroute intermediary 4-chlorocatechol from the 3-oxoadipate pathway and into the *meta*-cleavage pathway or able to mineralize 4-chlorocatechol by a modified *ortho*-cleavage pathway resulted in reversal of this toxic effect. Surprisingly, while direct addition of protoanemonin influenced both the viability of fungi and the microbial activity of the soil microcosm, there was little effect on bacterial viability due to its rapid degradation. This rapid degradation accounts for our inability to detect this compound in soils amended with 4-chlorocatechol. However, significant accumulation of protoanemonin was observed by a mixed bacterial community enriched with benzoate or a mixture of benzoate and 4-methylbenzoate, providing the metabolic potential of the soil to form protoanemonin. The effects of soil heterogeneity and microcosm interactions are discussed in relation to the different effects of protoanemonin when applied as a shock load and when it is produced in small amounts from precursors over long periods.

The majority of microorganisms isolated on the basis of their capability to mineralize chloroaromatic compounds initiate metabolism by transformation to chlorocatechols by nonspecific peripheral enzymes (20, 33, 44). Thus, a wide variety of chloroaromatics are converted to a group of related metabolic products. So far, three pathways which transform chlorocatechols have been described. Mineralization is usually achieved by enzymes of the chlorocatechol pathway (13, 14, 25, 36, 40). Unfortunately, only a small fraction of bacteria able to transform chloroaromatics into chlorocatechols simultaneously contain the complete metabolic potential for mineralization (30, 32, 34). Therefore, in natural bacterial populations, where chlorocatechol genes are usually not very abundant, cometabolism will lead to the formation of chlorocatechols. Thus, routes other than the chlorocatechol pathway (e.g., *meta*-cleavage and the 3-oxoadipate pathway), which have the potential to transform these compounds, are of considerable interest.

It has recently been shown that transformation of 4-chlorocatechol by enzymes of the classical 3-oxoadipate pathway, which, in comparison to enzymes of the chlorocatechol pathway, were formerly believed to catalyze the same reactions at a low rate (36), results in the quantitative production of the toxic product (antibiotic) protoanemonin (6) and thus may account for the toxicity of a variety of chloroaromatic pollutants which can be transformed into 4-chlorocatechol. Protoanemonin is an active constituent of plants of the *Ranunculaceae* family (38) and is antibiotically active against a wide spectrum of microorganisms (6, 11). The formation of protoanemonin could also account for the poor effectiveness of polychlorinated biphenyl (PCB) cometabolizing organisms in natural environments (24), where metabolites (such as chlorobenzoates) excreted by those specialized organisms most probably will be further metabolized by the natural microflora. In this study, we investigated the potential for the soil microflora to transform potential precursors of protoanenomin and investigated the ecotoxicity of such transformed halogenated aromatic compounds.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Most of the bacterial strains used in this study have been described previously: *Pseudomonas putida* KT 2442 (17), *Pseudomonas* sp. strain LB400 (7), *Sphingomonas paucimobilis* Q1 (43), *Alcaligenes eutrophus* H850 (4), and *Rhodococcus globerulus* P6 (2). *Pseudomonas* sp. strain PS121, which is able to degrade 4-chlorobenzoate via 4-chlorocatechol and a chlorocatechol pathway, was supplied by the Deutsche Sammlung von Mikroorganismen (DSM 8961). *Pseudomonas* sp. strain LB400 was tagged with *lacZ* Km^r, using a mini-Tn5 transposon system (10).

Soil microcosms consisted of 1 g of standard soil collected from Rübenberge, Niedersachsen, Germany (carbon, 1.2%; sand, 75%; clay, 3.9%; silt, 21%; pH 6.1; maximum water-holding capacity, 35%) in 15-ml sterile culture tubes fitted with Teflon-covered screw caps. This soil had no history of contamination with chlorinated aromatics. Soils were sterilized by autoclaving for 45 min on three alternate days; on intervening days, the soils were moistened with sterile water and incubated at 25°C. Prior to inoculation into soils, cells were grown in Luria-Bertani (LB) medium, harvested by centrifugation, and washed twice with M9 medium. Biphenyls and chlorobiphenyls (in hexane) were added to soils up

* Corresponding author. Mailing address: Department of Microbiology, GBF-National Research Centre for Biotechnology, Mascheroder Weg 1, D-38124-Braunschweig, Germany. Phone: 49 531 6181 467. Fax: 49 531 6181 411. E-mail:pieper@gbf-braunschweig.de.

† Present address: Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, 14071 Córdoba, Spain.

‡ Present address: Department of Microbiology, University of Otago, Dunedin, New Zealand.

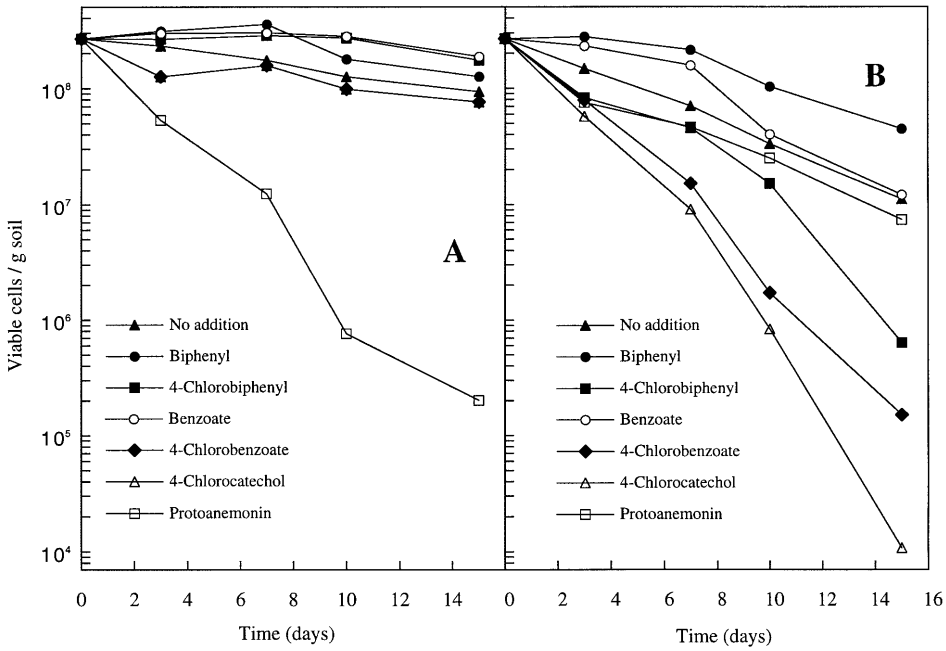

FIG. 1. Survival of *Pseudomonas* sp. strain LB400 in sterilized (A) and nonsterilized (B) soil contaminated with the indicated chemicals at concentrations of 0.3 mg/g of soil.

to a final concentration of 1 mg/g of soil; other compounds were dissolved in M9 medium without a carbon source and applied to a final concentration of 0.3 mg/g of soil. Following addition of chemicals and/or bacteria, the soils were brought to 80% of water-holding capacity with M9 buffer and incubated at 30°C.

Extraction and analysis of soil samples. Microorganisms and water-soluble chemicals were extracted from soil samples by shaking with 5 ml of phosphate-buffered saline on a rotatory shaker for 3 h and then decanting off the soil particles. PCBs were extracted similarly with cyclohexane instead of phosphate-buffered saline. One soil sample served for a single determination.

The viability of soil-supplemented bacteria was monitored by plating soil extracts on LB plates containing antibiotics and using the antibiotic resistance of the bacteria as a marker. In this way, LB plates containing streptomycin were used to monitor the fate of *S. paucimobilis* Q1 and *R. globerulus* P6; LB plates containing rifampin were used for a spontaneous rifampin-resistant mutant of *A. eutrophus* H850; and LB–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates with or without kanamycin were used for *Pseudomonas* sp. strain LB400 (mini-Tn*5* *lacZ* Km^r). Indigenous bacteria were estimated as CFU on LB plates containing cycloheximide (100 μg/ml) to avoid the growth of fungi. Viable fungi were estimated as previously described (49). The difference in viable counts between triplicate determinations was always lower than 30%.

Substrates and products (the sample size was usually 20 μl) were analyzed with a Beckman System Gold high-pressure liquid chromatograph equipped with SC columns (125 by 4.6 mm) filled with 5-μm-diameter particles of Lichrospher 100 RP8 (Bischoff, Leonberg, Germany), with 60% (vol/vol) methanol–0.1% (vol/vol) H_3PO_4 as the mobile phase (flow rate, 1 ml/min). The column effluent was monitored simultaneously at 210 and 270 nm with a diode array detector, and the scans of the significant peaks between 200 and 300 nm were stored by the system. Protoanemonin was analyzed similarly with 15% methanol as the mobile phase.

Transformation of 4-chlorocatechol by bacteria enriched from soil by (methyl)benzoate. Soil samples (1 g), in baffled Erlenmeyer flasks, were supplemented with 50 ml of M9 medium containing either 2 mM benzoate or 1 mM benzoate–1 mM 4-methylbenzoate or 50 ml of LB medium and incubated at 30°C on a rotary shaker at 140 rpm for 2 days. Additional benzoate (1 mM), benzoate–4-methylbenzoate (0.5 mM each), or LB was then added, and the flasks were incubated for a further 16 h. Soil particles were then removed by centrifugation at 2,000 × *g* for 10 min, and the supernatant was centrifuged at 9,000 × *g* for 10 min. The pellet containing bacteria was then resuspended in 2 ml of M9 medium. Resuspended cells were incubated at 30°C for 4 h with 4-chlorocatechol (1 mM), and samples were analyzed by high-pressure liquid chromatography, as described above.

Preparation of protoanemonin. *Pseudomonas* sp. strain B13 (12) was grown in M9 medium supplemented with 5 mM benzoate as the sole carbon source and incubated at 30°C on a rotary shaker at 140 rpm in baffled Erlenmeyer flasks. Cells were harvested by centrifugation at the end of the logarithmic growth phase, resuspended in 50 mM Tris-HCl buffer (pH 7.5) supplemented with 2 mM $MnSO_4$ and passed once through a French pressure cell (Aminco) operating at 18,000 lb/in^2. Cell debris were removed by centrifugation at 20,000 × *g* for 20 min.

The reaction mixture contained 50 mM morpholineethanesulfonic acid (MES)–NaOH (pH 6.5), 2 mM $MnSO_4$, 200 μM 4-chlorocatechol, and an appropriate amount of cell extract of *Pseudomonas* sp. strain B13 (final concentration, ca. 100 μg/ml). The reaction was performed at 25°C, and the progress of the reaction was monitored by high-pressure liquid chromatography. Following complete conversion (usually 2 h), the reaction mixture was extracted twice with diethyl ether and concentrated carefully under vacuum. Pure protoanemonin was obtained in yields greater than 90%.

Chemicals. 4-Chlorocatechol was purchased from Aldrich (catalog for rare chemicals). All other chemicals were of analytical grade and were obtained from Fluka AG, Merck AG, and Aldrich Chemie GmbH.

RESULTS

Effect of 4-chlorobiphenyl and its potential precursors on the survival of PCB-metabolizing organisms. To explore the hypothesis that 4-chlorobiphenyl or its potential metabolites could be converted by soil microflora to toxic products, in particular protoanemonin; 4-chlorobiphenyl, 4-chlorobenzoate, and 4-chlorocatechol, as well as their corresponding unsubstituted analogs, were added to sterilized and nonsterilized soil inoculated with *Pseudomonas* sp. LB 400.

In sterile soil, no effect of these chemicals on the survival of LB 400 was observed (Fig. 1A). Catechol, 4-chlorocatechol, and 4-chlorobenzoate were not transformed in sterile soils by LB400, excluding any major toxic effects due to these compounds. Benzoate and biphenyl were quantitatively utilized without accumulation of intermediates (thus indicating mineralization). 4-Chlorobiphenyl was quantitatively transformed into 4-chlorobenzoate as a dead-end product (Fig. 2).

In dramatic contrast to the results observed in sterile soils, severe decreases in the cell viability of LB400 (approximately 3 to 4 orders of magnitude [Fig. 1B]) occurred upon addition of 4-chlorocatechol, 4-chlorobenzoate, and 4-chlorobiphenyl to nonsterilized soils. The decreases in viability are much more pronounced than in the control experiment without any addition (1 order of magnitude), where the decline in viability is

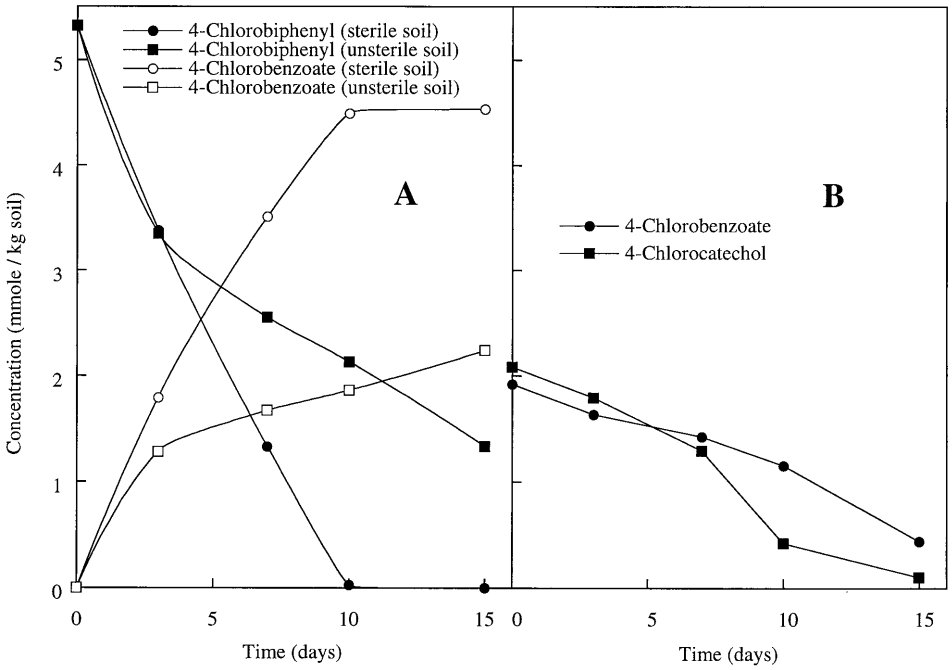

FIG. 2. Metabolism of 4-chlorobiphenyl, 4-chlorobenzoate, and 4-chlorocatechol in soil microcosms. (A) The disappearance of 4-chlorobiphenyl and the accumulation of 4-chlorobenzoate (1 mg/g of soil, corresponding to 5.3 mmol/kg of soil) in the presence of *Pseudomonas* sp. strain LB400 was quantified in nonsterilized and sterilized soil. (B) Transformation of 4-chlorobenzoate and 4-chlorocatechol (0.3 mg/g of soil corresponding to 1.9 and 2.1 mmol/kg of soil, respectively) was monitored in nonsterilized soil. Data points represent the mean of three independent experiments.

most probably due to restricted competitiveness (21) and/or predation (19, 48). The dramatic decreases in viability in the case of addition of chlorinated chemicals are clearly not due to the compounds themselves (see above), strongly suggesting that the effect is due to the conversion of these compounds by the indigenous microflora to a toxic product. This is further supported by the observation that the viability of LB400 still significantly decreased as the concentration of the applied compounds diminished, i.e., were converted (Fig. 1 and 2) and that the conversion of these compounds was completely different from that observed in sterile soil. In sterilized soil, degradation of 4-chlorobiphenyl by LB400 resulted in the almost stoichiometric accumulation of 4-chlorobenzoate (as in liquid culture), whereas in nonsterilized soil, 4-chlorobenzoate was further transformed by the natural microflora and therefore accumulated to a much smaller extent. In addition, the degradation of 4-chlorobiphenyl was slower in nonsterilized than in sterilized soil (Fig. 2A), correlating with the decline in the number of viable LB 400 cells in nonsterilized soil. However, despite the considerable evidence for the formation of a toxic product from 4-chlorobenzoate and 4-chlorocatechol, both compounds were transformed without accumulation of detectable amounts of metabolites.

Consistent with the effect on LB400, the addition of 4-chlorobenzoate to nonsterilized soil microcosms containing the PCB-metabolizing organisms *S. paucimobilis* Q1 (43), *A. eutrophus* H850 (4), and *R. globerulus* P6 (2) separately, resulted in a rapid decrease in the viability of these organisms while only small decreases were observed in nonsterilized soil. No such decrease was observed in sterilized soil.

Effect of coinoculation with a 4-chlorobenzoate degrader and a cometabolizer on the survival of *Pseudomonas* sp. strain LB400 in soil containing 4-chlorobenzoate. The transformation of 4-chlorocatechol is known to occur by three pathways: the 3-oxoadipate pathway, leading to the formation of protoanemonin (6); the chlorocatechol pathway, operating via a nontoxic *cis*-dienelactone (14, 36); and the *meta*-cleavage pathway, operating via 5-chloro-2-hydroxymuconic semialdehyde (46), most probably with chloroacetate as an intermediate (1). If the toxic effects of 4-chlorobenzoate are due to its transformation via 4-chlorocatechol to protoanemonin, then an increase in the metabolic flux through pathways other than the protoanemonin-producing 3-oxoadipate pathway may result in a better survival rate of soil microbes. We investigated this hypothesis by inoculating nonsterilized soil, supplemented with 4-chlorobenzoate, with LB400 and *Pseudomonas* strain PS121, a strain able to degrade 4-chlorobenzoate via 4-chlorocatechol and a modified *ortho*-cleavage (chlorocatechol) pathway. In contrast to the severe decrease in the viability of LB400 in nonsterilized soil containing 4-chlorobenzoate no such decrease in viability was observed in soil also containing PS121 (Fig. 3). A similar protective effect from 4-chlorobenzoate was also observed when *P. putida* KT2442 harboring the TOL plasmid was added to soil (Fig. 3). In this case, KT2442 is capable of transforming 4-chlorobenzoate into 5-chloro-2-hydroxymuconic semialdehyde (31), reducing the potential of 4-chlorocatechol to be converted to protanemonin. No 4-chlorocatechol or protoanemonin accumulation was observed in any of the experiments.

Effect of protoanemonin on the indigenous soil microflora. To investigate the effect of protoanemonin on the soil microflora, this compound was added to nonsterilized soils up to concentrations of 300 ppm. Surprisingly, although protoanemonin is toxic to bacterial isolates grown in artificial growth media (5, 6), the addition of protoanemonin to nonsterilized soils had no observable effect on the viability of indigenous bacteria (Fig. 4). Moreover, a rapid depletion of protoanemonin was observed (Fig. 4), indicating a rapid microbial transformation. However, although there were no apparent effects on the soil microflora viability, within 2 days after addition of

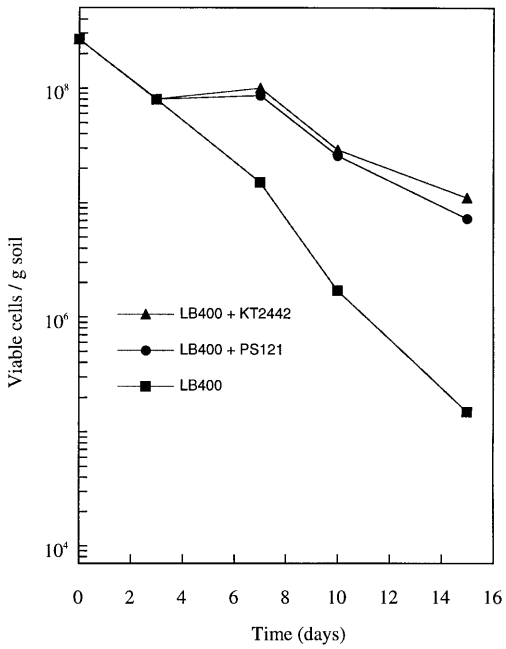

FIG. 3. Survival of *Pseudomonas* sp. strain LB400 in soil contaminated with 4-chlorobenzoate (0.3 mg/g of soil), without a helper strain (as shown in Fig. 1), in the presence of the 4-chlorobenzoate degrader *Pseudomonas* sp. strain PS121 or the 4-chlorobenzoate cometabolizer *P. putida* KT2442 (pTOL). The two strains were inoculated at the same order of magnitude as *Pseudomonas* sp. strain LB400 ($\sim 10^8$ CFU/g of soil). This indicates survival of LB 400 without a helper strain (as shown in Fig. 3). Data points represent the mean of three independent experiments.

75 and 150 ppm of protoanemonin, a significant decrease in dehydrogenase activity (40 and 60%, respectively) occurred, showing a severe effect on the physiology of the microflora.

To investigate whether other antibiotic compounds may behave like protoanemonin, streptomycin was added to nonsterilized soil (200 ppm, final concentration). As was the case for protoanemonin, streptomycin had no observable effect on the viability of the indigenous bacteria, although only 10^{-6} of the culturable indigenous bacteria were resistant to streptomycin (data not shown).

Toxicity of protoanemonin for *Pseudomonas* sp. LB400 in sterilized and nonsterilized soil. A large difference in the viability of strain LB400 was observed between protoanemonin-containing sterilized and nonsterilized soil. Consistent with the toxic effect of protoanemonin on LB400 observed in liquid culture (6), a severe decrease in the viability of LB400 was observed in sterilized soil containing protoanemonin (Fig. 1). In contrast, in nonsterilized soil, protoanemonin had little effect on the viability of strain LB400. A statistically significant decrease in the number of viable cells more pronounced than that in the unamended control was observed only after 2 days. Overall, the viability decreased only by 1 order of magnitude in 1 week, a result similar to that of controls which did not contain protoanemonin. The nontoxic effect of protoanemonin in nonsterilized soil is attributed to the rapid breakdown of this compound by the indigenous microflora, as observed in the experiments discussed above (Fig. 4).

Formation of protoanemonin from 4-chlorocatechol by mixed bacterial cultures enriched from soil. As protoanemonin appeared to accumulate at barely detectable levels in soils amended with 4-chlorobiphenyl, 4-chlorobenzoate, or 4-chlorocatechol, we decided to investigate whether indigenous bacteria are actually capable of producing this compound. In contrast to controls enriched on complex medium, bacterial samples prepared from soil cultures grown on benzoate accumulated up to 2.5% of the amended 4-chlorocatechol as protoanemonin, and those grown on benzoate–4-chlorobenzoate accumulated up to 4%. The accumulation was followed by a depletion of protoanemonin due to the conversion by the bacteria. This depletion suggests that the conversion of 4-chlorocatechol to protoanemonin is even greater than the net accumulation observed.

DISCUSSION

Bioremediation is potentially a powerful technique for the cleanup of contaminated sites. In the case of chloroaromatics, usually the absence, or the presence in small numbers, of an acclimated microbial population capable of degrading the pollutant limits biodegradation (9, 28). These deficiencies can be overcome by the introduction of specific organisms into contaminated sites (16, 45). These introduced organisms frequently perform poorly; this has been attributed to a number of factors, such as poor survival (21), predation (19, 48), and inappropriate regulation of the degradative capability (8).

It is generally accepted that metabolites of the transformation of aromatic compounds can be more toxic than the original substrate (23, 26, 35, 41). Consequently mineralization rather than biotransformation has to be the goal of bioremediation strategies. In the case of bicyclic aromatics such as biphenyls, naphthalenes, dibenzofurans, and dioxins, most organisms available until now were isolated on the basis of degradation of the unchlorinated compound and only cometabolized but did not mineralize some chlorinated congeners (4, 15,

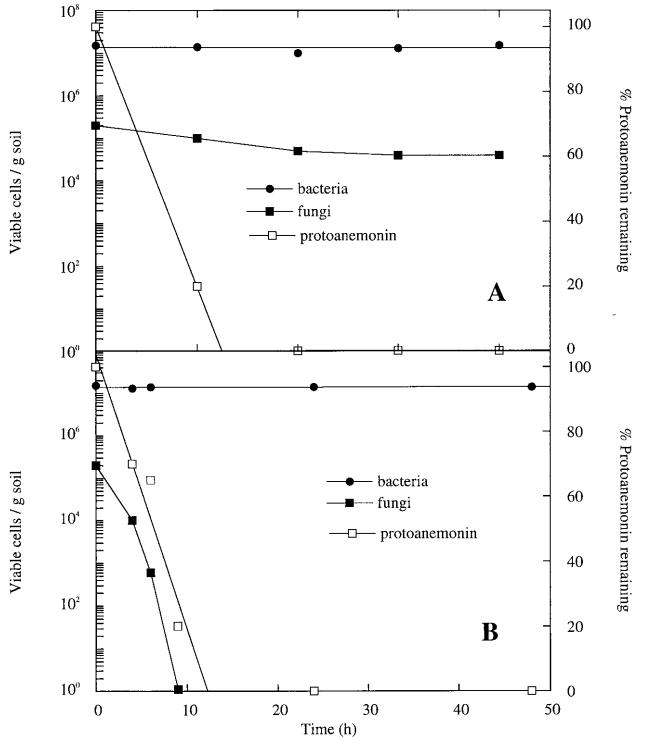

FIG. 4. Effect of protoanemonin on soil microcosms. Soil samples were treated with 40 ppm (A) or 300 ppm (B) of protoanemonin. At the indicated times, viable bacterial and fungal cells as well as the residual concentration of protoanemonin were analyzed as described in Materials and Methods.

18, 22, 29, 47). Depending on the microorganism and the congener of interest, different pathway bottlenecks were described, but, most commonly, only one aromatic ring was degraded. For example, chlorobenzoates accumulate as dead-end products of chlorobiphenyls (39). Furthermore, while dead-end products accumulate in pure culture and transformation of chlorobiphenyls into chlorobenzoates can be regarded as a detoxifying process, in natural environments those compounds may be subsequently transformed by other members of the microbial community.

Negative effects of chlorobenzoate metabolism on chlorobiphenyl degradation have already been reported. 3-chlorobenzoate can be transformed by benzoate 1,2-dioxygenase followed by a dehydrogenase into 3-chlorocatechol, which, upon *meta* cleavage, give rise to a reactive acylchloride (3), inactivating the ring cleavage enzyme. Actual accumulation of hydroxylated metabolites was observed when biphenyl-grown cells of *Pseudomonas testosteroni* were incubated with 3-chlorobenzoate (42). Havel and Reineke (24) assumed the formation of a toxic compound from 4-chlorobenzoate by the natural microflora, leading to a rapid die-off of the biphenyl-metabolizing strain used in their study, an effect similar to that observed in this study.

In this paper, we present strong evidence that 4-chlorobenzoate is converted to a toxic product, most probably protoanemonin (see below), by the indigenous soil microflora. This toxic product has severe effects on the cell viability of both the indigenous organisms and organisms that were supplemented to the soil.

Because 4-chlorocatechol is produced from 4-chlorobenzoate (Fig. 5) and because addition of both compounds to nonsterilized soil had similar effects on the viability of LB400, we deduce that a metabolite downstream of 4-chlorocatechol is responsible for toxicity. 4-Chlorocatechol can be metabolized via either *meta* or *ortho* cleavage. We discount the production of a toxic product via the *meta*-cleavage pathway, since the addition of an organism containing a *meta*-cleavage pathway. (*P. putida* KT2442 harboring the TOL plasmid) actually protected against the toxicity of 4-chlorobenzoate in nonsterilized soil (Fig. 3). Thus, by default, the production of a toxic compound from 4-chlorobenzoate is assumed to proceed via 4-chlorocatechol and subsequent *ortho* cleavage.

The *ortho* cleavage of 4-chlorocatechol produces 3-chloro-*cis,cis*-muconate, which can then be catabolized via three distinct routes (Fig. 5). In the chlorocatechol pathway, chloromuconate cycloisomerase transforms 3-chloro-*cis,cis*-muconate into *cis*-dienelactone (36), which is then further metabolized to Krebs cycle intermediates (25, 36). Alternatively, muconate cycloisomerase from *Trichosporon cutaneum* can catalyze the 1,4-cycloisomerization of 3-chloro-*cis,cis*-muconate to form 3-chloromuconolactone (27). Most importantly, enzymes of the 3-oxoadipate pathway, usually involved in the bacterial degradation of benzoate, can form protoanemonin (6).

Even though the actual production of protoanemonin from potential precursors (such as 4-chlorocatechol) was observed only in enriched mixed cultures, on the basis of known metabolic pathways this compound is the most likely candidate to account for the observed toxic effects. *Pseudomonas* sp. strain LB400 is very sensitive to protoanemonin in pure culture (6) and in sterilized soil (Fig. 1A). 4-Chlorobenzoate itself is not toxic to LB400, and a metabolic product of this compound is clearly responsible for toxicity. Metabolic pathways exist that convert 4-chlorobenzoate to protoanemonin via *ortho* cleavage of 4-chlorocatechol. Addition of *meta*-cleavage pathway-containing organisms that can reroute the flux of 4-chlorocatechol away from *ortho* cleavage actually inhibited the toxic effects of 4-chlorobenzoate on LB400 in nonsterilized soil.

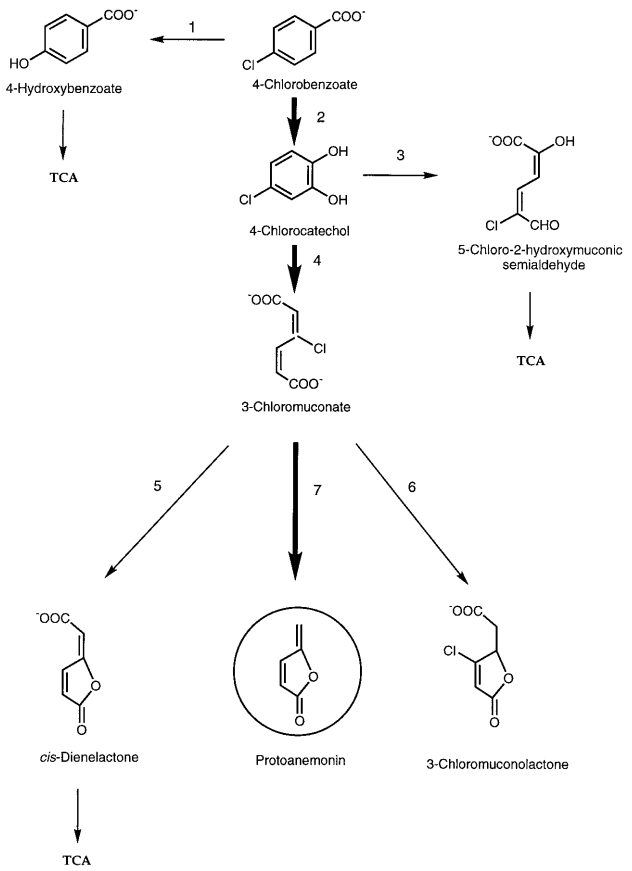

FIG. 5. Pathways for the aerobic degradation of 4-chlorobenzoate. 1, Hydrolytic dehalogenation of 4-chlorobenzoate occurs to form 4-hydroxybenzoate (37). 2, Dioxygenation of 4-chlorobenzoate is catalyzed by benzoate dioxygenases of relaxed substrate specificity such as toluate dioxygenase (32). After dehydrogenation, 4-chlorocatechol is formed. 3, *meta* cleavage of 4-chlorocatechol is caused by catechol 2,3-dioxygenases (46). 4, *ortho* cleavage of 4-chlorocatechol is catalyzed by catechol and chlorocatechol 1,2-dioxygenases (14). 5, The formation of *cis*-dienelactone from 3-chloro-*cis,cis*-muconate is catalyzed by chloromuconate cycloisomerases (36) of specialized bacteria. 6, 1,4-Cycloisomerization of 3-chloro-*cis,cis*-muconate is catalyzed by muconate cycloisomerase of *Trichosporan cutaneum* (27). 7, Formation of protoanemonin is catalyzed by bacterial muconate cycloisomerases (6). TCA, trichloroacetic acid.

While the conversion of 4-chlorocatechol and 4-chlorobenzoate seems to result in the production of protoanemonin, which produces the toxic effects, it was surprising that the addition of this compound to nonsterilized soil had no severe effect on the viability of LB400 or soil organisms. We attribute this observation to the transient presence of the compound: direct addition of protoanemonin to nonsterilized soil resulted in a rapid decrease in its concentration, thereby allowing toxic effects to occur only during the first 1 to 2 days of incubation.

If the toxic effects of 4-chlorobenzoate are due to its conversion to protoanemonin by indigenous soil microbes, how can the resulting protoanemonin exert its toxic effects, given the rapid degradation of this compound and its failure to produce a severe toxic effect when applied as a shock load to nonsterilized soil? Two reasons may account for the difference. First, 4-chlorobenzoate is most probably continually converted to protoanemonin, such that microorganisms are exposed to this compound for as long as the precursor is present and not only for a few hours. Second, soil is heterogeneous, and so

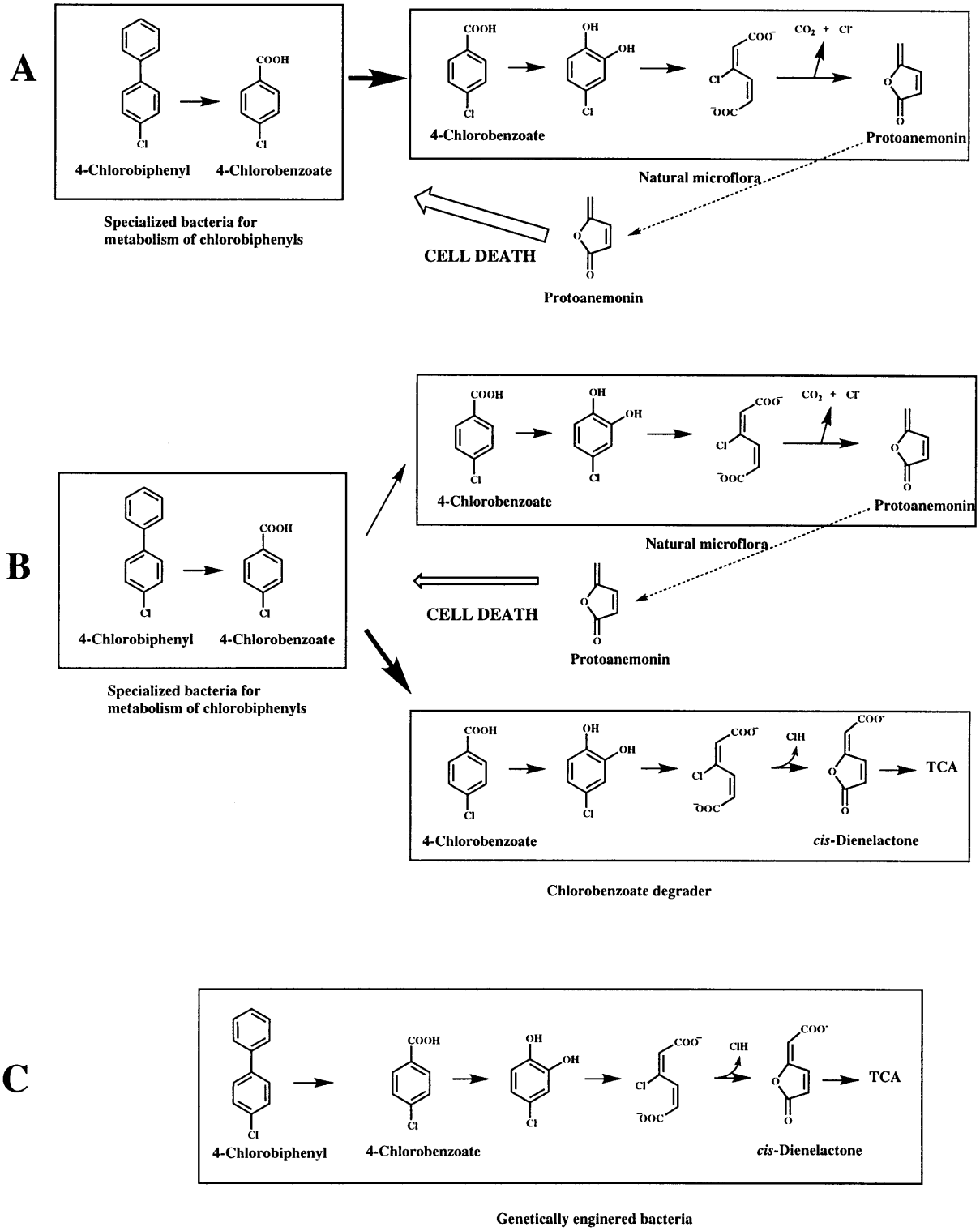

FIG. 6. Schematic representation of the formation of protoanemonin in soil contaminated with 4-chlorobiphenyl in the presence of a PCB degrader (A) and its potential circumvention by coinoculation of a chlorobenzoate degrader (B) or by the use of a suitable genetically engineered bacterium (C).

protoanemonin could be produced in a particular area, giving rise to locally relatively high concentrations, and could exert its toxic effect before diffusing to another area and being degraded. Thus, only very small amounts of protoanemonin need to be produced for a toxic effect to occur prior to its degradation. This would explain the pronounced toxic effects, as exposure, which is a combination of concentration (high local concentrations) and time (much longer when applied as a shock

load) can be regarded as high. On the macroscopic level, when soil is assayed for this compound, no protoanemonin is observed.

If a general problem in haloaromatic-contaminated environments is the production of protoanemonin from 4-chlorobenzoate (Fig. 6A), a possible solution will be to coinoculate the soil with a chlorobenzoate degrader (Fig. 6B) or to equip those organisms able to degrade the pollutants as far as halobenzoates with the additional catabolic enzymes needed to prevent the formation and accumulation of protoanemonin (Fig. 6C). However, coinoculation of a chlorobenzoate degrader involves the problem of the relative affinity for the chemical of the natural microflora, which may misroute intermediates to protoanemonin. The problem of the accumulation of intermediates can be avoided if all the information for the mineralization of the xenobiotic compound is in a single bacterium. This should not only permit survival of the degraders in such environments but also result in protection of the entire microbial community from protoanemonin poisoning. Since such catabolic enzymes are known, the engineering of the respective genes into appropriate degraders should provide an effective solution to the protoanemonin problem.

ACKNOWLEDGMENTS

We thank David McKay for help in preparation of the manuscript. This work was supported by the Federal Ministry of Research and Technology (award 0319-433A). R.B. was supported by the Spanish Ministerio de Educación y Ciencia and the European Environmental Research Organization. K.N.T. gratefully acknowledges generous support by the Fonds der Chemischen Industrie.

REFERENCES

1. **Arensdorf, J. J., and D. D. Focht.** 1995. A *meta* cleavage pathway for 4-chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepacia* P166. Appl. Environ. Microbiol. **61:**443–447.
2. **Asturias, J. A., and K. N. Timmis.** 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. J. Bacteriol. **175:**4631–4640.
3. **Bartels, I., H.-J. Knackmuss, and W. Reineke.** 1984. Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl. Environ. Microbiol. **47:**500–505.
4. **Bedard, D. L., M. L. Haberl, R. J. May, and M. J. Brennan.** 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1103–1112.
5. **Bérdy, J., A. Aszalos, M. Bostian, and K. L. McNitt.** 1982. CRC handbook of antibiotic compounds. CRC Press, Inc., Boca Raton, Fla.
6. **Blasco, R., R.-M. Wittich, M. Mallavarapu, K. N. Timmis, and D. H. Pieper.** 1995. From xenobiotic to antibiotic. Formation of protoanemonin from 4-chlorocatechol by enzymes of the 3-oxoadipate pathway. J. Biol. Chem. **270:**29229–29235.
7. **Bopp, L.** 1986. Degradation of highly chlorinated PCBs by Pseudomonas strain LB400. J. Ind. Microbiol. **1:**23–29.
8. **Brunner, W., F. H. Sutherland, and D. D. Focht.** 1985. Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. J. Environ. Qual. **14:**324–328.
9. **Chen, S., and M. Alexander.** 1989. Reasons for the acclimation for 2,4-D biodegradation in lake water. J. Environ. Qual. **18:**153–156.
10. **de Lorenzo, V., M. Herrero, U. Jakubzik and K. N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
11. **Didry, N., L. Dubreuil, and M. Pinkas.** 1991. Activité antibacterienne des vapeurs de protoanémonine. Pharmazie **46:**546–547.
12. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. **99:**61–70.
13. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. Biochem. J. **174:**85–94.
14. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. Biochem. J. **174:**73–84.
15. **Durham, D. R. and D. B. Stewart.** 1987. Recruitment of naphthalene dissimilatory enzymes for the oxidation of 1,4-dichloronaphthalene to 3,6-dichlorosalicylate, a precursor for the herbicide dicamba. J. Bacteriol. **169:** 2889–2892.
16. **Edgehill, R. U., and R. K. Finn.** 1983. Microbial treatment of soil to remove pentachlorophenol. Appl. Environ. Microbiol. **45:**1122–1125.
17. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of the genes for the entire regulated aromatic ring *meta*-cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
18. **Furukawa, K., N. Tomizuka, and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. **38:**301–310.
19. **Goldstein, R. M., L. M. Mallory, and M. Alexander.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. **50:**977–983.
20. **Häggblöm, M. M.** 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. **103:**29–72.
21. **Harkness, M. R., J. B. McDermott, D. A. Abramowicz, J. J. Salvo, W. P. Flanagan, M. L. Stephens, F. J. Mondello, R. J. May, J. H. Lobos, K. M. Carroll, M. J. Brennan, A. A. Bracco, K. M. Fish, G. L. Warner, P. R. Wilson, D. K. Dietrich, D. T. Lin, C. B. Morgan, and W. L. Gately.** 1993. In situ stimulation of aerobic PCB biodegradation in Hudson River sediments. Science **259:**503–507.
22. **Harms, H., H. Wilkes, V. Sinnwell, R.-M. Wittich, K. Figge, W. Francke, and P. Fortnagel.** 1991. Transformation of 3-chlorodibenzofuran by *Pseudomonas* sp. HH69. FEMS Microbiol. Lett. **81:**25–30.
23. **Haugland, R. A., D. J. Schlemm, R. P. L. III, P. R. Sferra, and A. M. Chakrabarty.** 1990. Degradation of the chlorinated phenoxyacetate herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid by pure and mixed bacterial cultures. Appl. Environ. Microbiol. **56:**1357–1362.
24. **Havel, J., and W. Reineke.** 1992. Degradation of Aroclor 1221 and survival of strains in soil microcosms. Appl. Microbiol. Biotechnol. **38:**129–134.
25. **Kaschabek, S. R., and W. Reineke.** 1993. Degradation of chloroaromatics: purification and characterization of maleylacetate reductase from *Pseudomonas* sp. strain B13. J. Bacteriol. **175:**6075–6081.
26. **Liu, D., R. J. Maguire, B. J. Dutka, and G. J. Pacepavicius.** 1990. Rationale for including metabolites in chemical toxicity bioassay. Toxicol. Assess. **5:** 179–188.
27. **Mazur, P., W. A. Pieken, S. R. Budihas, S. E. Williams, S. Wong, and J. W. Kozarich.** 1994. Cis,cis-muconate lactonizing enzyme from *Trichosporon cutaneum*: evidence for a novel class of cycloisomerases in eucaryotes. Biochemistry **33:**1961–1970.
28. **Morgan, P., and R. J. Watkinson.** 1989. Microbiological methods for the cleanup of soil and ground water contaminated with halogenated organic compounds. FEMS Microbiol. Rev. **63:**277–300.
29. **Morris, C. M., and E. A. Barnsley.** 1982. The cometabolism of 1- and 2-chloronaphthalene by *Pseudomonas*. Mikrobiologiya **28:**73–79.
30. **Oltmanns, R. H., H. G. Rast, and W. Reineke.** 1988. Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl. Microbiol. Biotechnol. **28:**609–616.
31. **Reineke, W., D. J. Jeenes, P. A. Williams, and H.-J. Knackmuss.** 1982. TOL plasmid pWW0 in constructed halobenzoate-degrading *Pseudomonas* strains: prevention of *meta* pathway. J. Bacteriol. **150:**195–201.
32. **Reineke, W., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. Biochim. Biophys. Acta **542:**412–423.
33. **Reineke, W., and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. **42:**263–287.
34. **Rubio, M. A., K.-H. Engesser, and H.-J. Knackmuss.** 1986. Microbial metabolism of chlorosalicylates. Accelerated evolution by natural genetic exchange. Arch. Microbiol. **145:**16–122.
35. **Ruckdeschel, G., G. Renner, and K. Schwarz.** 1987. Effects of pentachlorophenol and some of its known and possible metabolites on different species of bacteria. Appl. Environ. Microbiol. **53:**2689–2692.
36. **Schmidt, E., and H.-J. Knackmuss.** 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleoylacetic acid. Biochem. J. **192:**339–347.
37. **Scholten, J. D., K.-H. Chang, P. C. Babbitt, H. Charest, M. Sylvestre, and D. Dunaway-Mariano.** 1991. Novel enzymic hydrolytic dehalogenation of a chlorinated aromatic. Science **253:**182–185.
38. **Seegal, B. C., and M. Holden.** 1945. The antibiotic activity of extracts of *Ranunculaceae*. Science **101:**413–414.
39. **Seeger, M., K. N. Timmis, and B. Hofer.** 1995. Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. Appl. Environ. Microbiol. **61:**2654–2658.
40. **Seibert, V., K. Stadler-Fritzsche, and M. Schlömann.** 1993. Purification and characterization of maleylacetate reductase from *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **175:**6745–6754.
41. **Snyder, R., G. Witz, and B. D. Goldstein.** 1993. The toxicology of benzene. Environ. Health Perspect. **100:**293–306.
42. **Sondossi, M., M. Sylvestre, and D. Ahmad.** 1992. Effects of chlorobenzoate

transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl. Environ. Microbiol. **58:**485–495.
43. **Taira, K., N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa.** 1988. Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1. Biochemistry **27:**3990–3996.
44. **van der Meer, J. R., W. M. de Vos, S. Harayama, and A. J. B. Zehnder.** 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. **56:**677–694.
45. **van der Meer, J. R., W. Roelofsen, G. Schra, and A. J. B. Zehnder.** 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. FEMS Microbiol. Ecol. **45:**333–341.
46. **Wieser, M., J. Eberspächer, B. Vogler, and F. Lingens.** 1994. Metabolism of 4-chlorophenol by *Azotobacter* sp. GP1: structure of the *meta*-cleavage product of 4-chlorocatechol. FEMS Microbiol. Lett. **116:**73–78.
47. **Wilkes, H., R.-M. Wittich, K. N. Timmis, P. Fortnagel, and W. Francke.** 1996. Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. Appl. Environ. Microbiol. **62:**367–372.
48. **Zaidi, B. R., Y. Murakami, and A. M.** 1989. Predation and inhibitors in lake water affect the success of inoculation to enhance biodegradation of organic chemicals. Environ. Sci. Technol. **23:**859–863.
49. **Zapek, D., and J. P. Martin.** 1950. Use of the acid Rose Bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci. **69:**215–233.