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Transformation of Low Concentrations of 3-Chlorobenzoate by *Pseudomonas* sp. Strain B13: Kinetics and Residual Concentrations

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The transformation of 3-chlorobenzoate (3CB) and acetate at initial concentrations in the wide range of 10 nM to 16 mM was studied in batch experiments with *Pseudomonas* sp. strain B13. Transformation rates of 3CB at millimolar concentrations could be described by Michaelis-Menten kinetics (K_m, 0.13 mM; V_{max}, 24 nmol · mg of protein^{-1} · min^{-1}). Experiments with nanomolar and low micromolar concentrations of 3CB indicated the possible existence of two different transformation systems for 3CB. The first transformation system operated above 1 μM 3CB, with an apparent threshold concentration of 0.50 ± 0.11 μM. A second transformation system operated below 1 μM 3CB and showed first-order kinetics (rate constant, 0.076 liter · g of protein^{-1} · min^{-1}), with no threshold concentration in the nanomolar range. A residual substrate concentration, as has been reported for some other *Pseudomonas* strains, could not be detected for 3CB (detection limit, 1.0 nM) in batch incubations with *Pseudomonas* sp. strain B13. The addition of various concentrations of acetate as a second, easily degradable substrate neither affected the transformation kinetics of 3CB nor induced a detectable residual substrate concentration. Acetate alone also showed no residual concentration (detection limit, 0.5 nM). The results presented indicate that the concentration limits for substrate conversion obtained by extrapolation from kinetic data at higher substrate concentrations may underestimate the true conversion capacity of a microbial culture.

The production and use of chlorinated organic chemicals have increased dramatically in the last few decades, and concomitantly, large amounts of these chemicals have been discharged into the environment. Numerous chlorinated organic contaminants are almost ubiquitously present at low concentrations in soil, groundwater, and wastewater (nanomoles to micromoles per liter), though some are intrinsically biodegradable. Obviously, there are factors which prevent a complete degradation of these pollutants. These factors also set limits to the applicability of bioremediation techniques for the cleanup of contaminated sites.

The biodegradation kinetics of organic chemicals at low, environmentally relevant concentrations can differ significantly from the kinetics of such chemicals at higher concentrations. Some individual bacterial strains seem to cause multiple degradation kinetics for different concentration ranges. Transformation of methyl parathion by a *Flavobacterium* sp. involved at least two transformation systems, one operating below a concentration of 20 μg · liter^{-1} and another one operating below a concentration of 4 mg · liter^{-1} (10). According to several studies, the presence of a second, easily degradable organic substrate can have both inhibiting and enhancing effects on the transformation rate. In slurries of aquifer solids, the mineralization of toluene, *p*-nitrophenol, or ethylene dibromide at a 100-μg · kg^{-1} concentration was inhibited when glucose or amino acids (at 0.1 and 2 mg · kg^{-1}, respectively) were added (17). Batch studies with a *Pseudomonas* sp. strain showed an increase in the utilization rate of methylene chloride (0.01 to 1 mg · liter^{-1}) when 1 mg of acetate liter^{-1} was amended (8).

Residual concentrations in the aqueous phase (i.e., concentrations that remain after biodegradation has stopped) have been observed for some xenobiotic compounds under various experimental conditions (8, 12, 15, 19). But for more common substrates, such as acetate and benzoate, residual concentrations have also been found (5, 7, 13). After the initial degradation of 40 μg of 1,3- and 1,4-dichlorobenzene and 1,2,4-trichlorobenzene liter^{-1} by a *Pseudomonas* strain in batch incubations, residual concentrations of 10 to 20 μg · liter^{-1} could still be observed (19). In batch studies with a *Pseudomonas* strain degrading 10 μg of methylene chloride liter^{-1}, a residual concentration of ca. 2.5 μg · liter^{-1} could be reduced significantly in the presence of 1 mg of acetate liter^{-1}. Initial concentrations below the measured residuals were not tested in these studies. Up to now, little about the processes causing a residual concentration in aerobic batch systems has been outlined.

This paper presents data on residual substrate concentrations and transformation kinetics in a batch system using *Pseudomonas* sp. strain B13 to degrade 3-chlorobenzoate (3CB). It is shown that below a concentration of about 1 μM 3CB, kinetic parameters which are different from the parameters that govern the degradation of 3CB at higher concentrations are valid. Residual concentrations of 3CB in the batches could not be observed within the range of detection. The presence of acetate as an additional, easily degradable substrate neither affected the transformation kinetics nor induced a detectable residual 3CB concentration.

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MATERIALS AND METHODS

Medium and culture conditions. *Pseudomonas* sp. strain B13, which can use 3CB as a sole energy and carbon source, was previously isolated from a wastewater treatment plant (3). The cells were pregrown aerobically at 20°C on a rotary shaker in 2.3-liter Erlenmeyer flasks (culture volume, 500 ml). The mineral salts medium consisted of (per liter of demineralized water) 2.86 g of $Na_2HPO_4 \cdot 2H_2O$, 1.46 g of KH_2PO_4, 1 g of NH_4NO_3, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $Ca(NO_3)_2$, and 1 ml of a trace-element solution (as modified from reference 23). The solution contained (per liter of demineralized water) 2.0 g of $FeCl_3 \cdot 6H_2O$, 0.05 g of H_3BO_3, 0.05 g of $ZnCl_2$, 0.05 g of $MnCl_2 \cdot 4H_2O$, 0.05 g of $CuSO_4 \cdot 5H_2O$, 0.05 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 g of $AlCl_3$, 0.05 g of $CoCl_2 \cdot 6H_2O$, 0.05 g of $NiCl_2$, 0.5 g of sodium EDTA, 0.05 g of $Na_2SeO_3 \cdot 5H_2O$, 0.05 g of Na_2WO_4, 0.05 g of $Na_2MoO_4 \cdot 2H_2O$, and 1 ml of concentrated HCl. For growth, 3CB was added from a 0.5 M stock solution of both 3CB and NaOH (final concentration, 5 mM). The pH in the growth medium was about 6.9. For the experiments with acetate alone, cells were pregrown on 6 mM acetate. In the late exponential growth phase, cells were harvested aseptically by centrifugation (10,200 × *g*), washed twice in mineral medium, and resuspended in the same medium. Purity checks were done by plating bacteria on nutrient agar.

Before use, all glassware was cleaned with a 5% solution of $K_2Cr_2O_7$ in 50% H_2SO_4. The mineral salts medium was prepared with highly purified water (Milli-Q Systems; Millipore Co., Bedford, Mass).

Transformation kinetics experiments. Transformation rates of 3CB at three different initial concentration ranges—1.5 μM to 16 mM 3CB, 1 to 5 μM 3CB, and 100 nM to 3 μM 3CB—were measured. In this last study, 100 nM 3CB of the total 3CB applied was added as ^{14}C-labeled 3CB, giving a total activity of 2,300 dpm/ml in each bottle. To the middle range, acetate at 0, 50, 500, and 5,000 μM was added, whereas the lowest range received 0 or 500 μM acetate.

A series of 100-ml serum bottles with 20 ml of sterile mineral medium was inoculated with washed cell suspensions, giving a final cell concentration of up to 10^9 cells · ml^{-1}. The bottles were sealed with a viton septum and incubated stationary for 20 h at 20°C. This incubation was done to allow the residual intracellular substrate as well as residual carbon in the medium to be degraded. Starvation experiments had demonstrated that cells remained fully active for at least 24 h after harvesting. After starvation, each bottle was sampled to measure protein (initial biomass concentration). A few bottles were checked for purity. The substrate was provided as a solution of 3CB-NaOH or a mixture of acetate and 3CB-NaOH in mineral medium. Bottles with medium and substrate but without cells were used as controls. At the start of the experiment and after all the manipulations, the total volume of each culture was 24 ml. Cultures were incubated on a rotary shaker at 20°C. Each bottle was regularly sampled (1-ml aliquots) during periods varying from 20 min to 12 h, depending on the initial substrate concentration. The data in the linear part of the depletion curve (four to eight datum points) were used for calculating the initial transformation rates (Fig. 1). Incubations in NaCl-phosphate buffer were compared with incubations in mineral medium, and this comparison showed that in this linear part of the depletion curve, transformation rates were not yet affected by growth of the bacteria (Fig. 1). Only in the mineral medium at the high concentration range was an effect of growth seen after 300 min of incubation. Conversion of the 3CB in the samples was stopped by acidification with HCl (final pH, 1.5).

Adsorption of 3CB to the cell material did not affect the disappearance of the compound, even at the lowest concentration range. At the start of the experiments, cell suspensions and controls without cells contained equal 3CB concentrations. Adsorption of 3CB to the cell material could not be observed in a test in which cells were inactivated with sodium azide.

Residual concentration experiments. The occurrence of a residual substrate concentration was investigated in three different batch experiments. Each experiment consisted of about 60 independent batch incubations in which 3CB, acetate, or a mixture of 3CB and acetate was tested. In the first experiment, 3CB was added at an initial concentration of 1.2 μM or 11 nM. The second set of batches was incubated with 8.6 μM or 85 nM acetate. In the third experiment, 1.5 μM 3CB was added in combination with 23 μM acetate. To be able to detect a low residual concentration, ^{14}C-labeled substrates were used. The third experiment consisted of two series: one with [^{14}C]3CB combined with cold acetate and a second with cold 3CB combined with [^{14}C]acetate. To test whether any residual concentration was associated with the radiolabel alone (e.g., nondegradable impurities), a set of batches was spiked for a second time with the same amount of labeled substrate after 4 h of incubation. Sterile controls with chemical concentrations in both the micromolar and nanomolar ranges as well as unlabeled controls were included in all experiments to check for abiotic disappearance of the compounds and for contamination with the radiolabel during the experimental procedure.

A series of Hungate tubes (Bellco, Vineland, N.J.) containing 4 ml of sterile mineral medium and sealed with butyl rubber stoppers was inoculated with washed cell suspensions to give a final cell concentration of about 10^9 cells · ml^{-1} in the experiments with 3CB. In the experiment with acetate alone, the concentration of bacteria was 10^7 cells · ml^{-1}. After a 20-h starvation period, four of the batches were sacrificed to verify the purity and the number of cells. The experiment was started by adding the substrate to the incubations. The total final volume was 5 ml. All tubes were incubated stationary at 20°C. Substrate degradation was followed for 3 days. Samples were taken in duplicate by sacrificing two tubes at a time. Microbial activity was stopped by injecting sodium azide solution through the septum (final concentration, 25 mM).

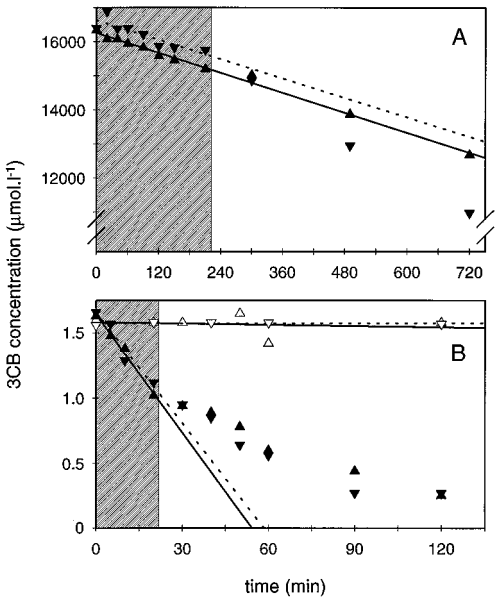

FIG. 1. 3CB depletion curves in the millimolar (A) and micromolar (B) ranges. Regression through the linear part of the curves (shaded) gives the initial transformation rates. Cells were incubated in NaCl-phosphate buffer (▲, solid regression line) and mineral medium (▼, dashed regression line). Open triangles represent the values obtained with sterile controls.

Analytical procedures. (i) Transformation kinetics. Samples from the experiments done with 3CB concentrations in the millimolar and micromolar ranges were centrifuged at 10,000 × *g* with a table centrifuge (Beun de Ronde, Amsterdam, The Netherlands). The supernatant was analyzed for 3CB by high-performance liquid chromatography (HPLC) (LKB 2150 pump and LKB 2152 controller; LKB, Woerden, The Netherlands). A total of 20 μl was injected into a ChromSphere C_8 reversed-phase column (Chrompack, Middelburg, The Netherlands). 3CB was detected by UV A_{206} (LKB 2158 Uvicord SD) and quantified with an SP 4290 computing integrator (Spectra Physics Inc., San Jose, Calif.). The mobile phase was a mixture of acetonitrile and 5 mM H_2SO_4 in water with a volume ratio of 40:60 and a flow rate of 0.6 ml · min^{-1}. Acetate concentrations in these experiments were determined with the same HPLC equipment and under the same conditions. The column, an RT300-6,5 PolySphere OAHY organic acids column, was run at 60°C (Merck, Darmstadt, Germany) with 5 mM H_2SO_4 in water as the mobile phase at 0.6 ml · min^{-1}.

Experiments with concentrations in the nanomolar range were done with ^{14}C-labeled 3CB. Acidified samples (final pH, 1.5) were left open for 4.5 h in a safety hood to release all labeled CO_2. Thereafter, no $^{14}CO_2$ was detectable in the medium. 3CB concentrations were not influenced by this $^{14}CO_2$ removal. Cells were removed by centrifugation with a table centrifuge (10,000 × *g*). Unlabeled 3CB (100 μM) was added to the supernatant. The cold 3CB allowed UV detection and prevented loss of labeled 3CB. The supernatant (200 μl) was injected into a ChromSphere C_8 reversed-phase column. The mobile phase was a mixture of acetonitrile and 5 mM H_2SO_4 (30:70) at a flow rate of 0.5 ml · min^{-1}. Fractions (1 ml each) were captured and counted in 4.5 ml of Aqualuma scintillation cocktail (Lumac, Olden, Belgium) in an LKB Wallac scintillation counter (LKB). Quench corrections were made by the external standard-channel ratio method.

Control experiments showed that the different analytical methods used for the separate concentration ranges gave the same results. The data could therefore be directly compared.

(ii) Residual concentrations. Before analysis, a known amount of unlabeled 3CB or acetate was added to each batch to minimize losses of the radiolabel during the analytical procedure. After they were mixed, the batches were brought to pH 1.5 with HCl and $^{14}CO_2$ was stripped from the solution by purging it for 12 min with 30 ml of air min^{-1}. Longer purging did not result in higher $^{14}CO_2$ recovery. Controls showed no loss of either acetic acid or 3-chlorobenzoic acid. $^{14}CO_2$ was trapped in a 1 M NaOH solution. After the suspensions were purged, the pHs of the suspensions with 3CB were increased to 6.9 to dissolve the 3-chlorobenzoic acid precipitate that had been formed at pH 1.5.

The amount of the radiolabel incorporated in the biomass was determined by filtering 0.5-ml suspensions through 0.45-μm-pore-size filters. Filters were washed with 5 ml of a 10 mM solution of unlabeled substrate in mineral medium.

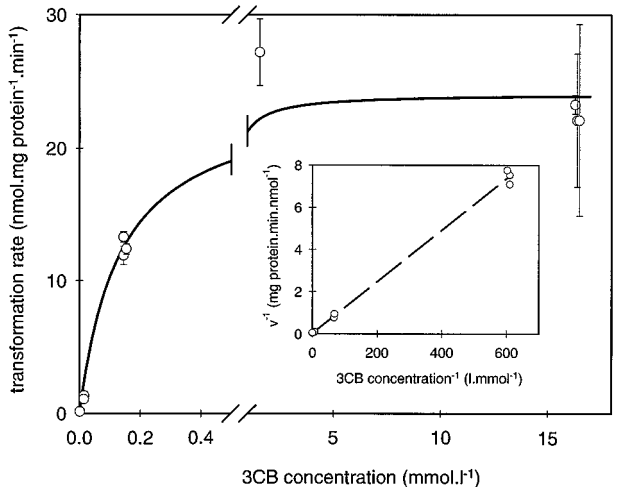

FIG. 2. Kinetics of 3CB transformation in the 3CB concentration range from 1.5 μM to 15 mM. The data are presented as a direct plot and as a Lineweaver-Burk linearization (inset). Error bars show standard deviations. v^{-1} is the inverse 3CB transformation rate.

The filters were dissolved in 4.5 ml of scintillation fluid, and the radioactivity was counted.

For the analysis of 3CB and acetate, each of the remaining suspensions was filtered (filter pore size, 0.45 μm), brought to pH 11.5 with NaOH, freeze-dried, and dissolved again in 100 μl of 0.8 M H_2SO_4 solution. This procedure concentrated the 3CB or acetate by a factor of about 50. Acetate and 3CB were separated by HPLC, and the fractions containing them were analyzed for radioactivity.

Biomass measurements. Protein measurements in the transformation kinetics experiments were done according to the method of Lowry et al. (11), with bovine serum albumin being used as the standard protein (Boehringer, Mannheim, Germany). In the residual concentration experiments, biomass was determined by direct microscopic counting with a Bürker Türk counting chamber.

Chemicals and radiochemical purity. [1-^{14}C]sodium acetate (53 mCi · mmol^{-1}) was obtained from Amersham International plc (Buckinghamshire, England). The radiochemical purity reported by the manufacturer was 99.0%. 3-Chlorobenzoic acid as well as 3-chlorobenzoic acid-ring-UL-^{14}C (10.8 mCi · mmol^{-1}) was purchased from Sigma Chemical Company (St. Louis, Mo.). The radiochemical purity reported by the manufacturer was >98%.

3-Chlorobenzoate was contaminated with about 1% 4-chlorobenzoate, which cannot be utilized by strain B13. This contamination was positively identified with a 4-chlorobenzoate standard after 3CB biodegradation. The radiolabeled 3CB was contaminated with 0.9 ± 0.4% of a nondegradable (by strain B13) compound which comigrated with 3CB in the HPLC analysis. The compound is presumably also 4-chlorobenzoate. The contamination was quantified by repeatedly adding [^{14}C]3CB to a B13 culture. Each addition increased the radioactive, nondegradable residual by about 0.9% of the [^{14}C]3CB supplied. [^{14}C]acetate was tested in the same way for purity. It contained 0.6 ± 0.3% of a compound which can be very slowly degraded by strain B13 and which comigrated with acetate.

All other chemicals were of analytical grade and were used without further purification.

RESULTS

Transformation kinetics. 3CB is converted by *Pseudomonas* sp. strain B13 following Michaelis-Menten kinetics over a wide concentration range from 1.5 μM to 16 mM (Fig. 2). Each of the initial transformation rates presented in Fig. 2 was based on at least four datum points in the linear part of the substrate depletion curve. Nonlinear regression analysis according to the Michaelis-Menten model yielded an apparent half-saturation constant K_m of 0.13 mM and a maximum specific transformation velocity V_{max} of 24 nmol · mg of protein^{-1} · min^{-1}.

At 3CB concentrations far below the half-saturation constant, the Michaelis-Menten model approaches a first-order relationship, with V_{max}/K_m as the first-order rate constant. Experiments in this lower concentration range were done to investigate whether this pattern of the Michaelis-Menten model was valid for the transformation rates at initial concentrations of a few micromoles per liter. A linear relationship in the concentration range of 1 to 5 μM was indeed observed, with a rate constant of 0.113 liter · g of protein^{-1} · min^{-1} (Fig. 3). Each of the initial transformation rates presented in Fig. 3 was based on four or five datum points in the linear part of the substrate depletion curve. Extrapolation of these data gives an intercept on the *x* axis, with a threshold concentration for transformation of about 0.5 μM. This value differs significantly from zero, as was calculated with a 95% confidence interval. In the third, independent experiment, which used a 3CB concentration range from 100 nM to 3 μM, a threshold of ca. 0.5 μM can also be calculated by linear regression through the transformation data for the range from 1 to 3 μM 3CB (Fig. 4). This regression results in the same first-order rate constant as that for Fig. 3. Below 1 μM, however, conversion followed a linear relationship, with a first-order rate constant of only 0.076 liter · g of protein^{-1} · min^{-1}.

The influence of an easily degradable additional substrate was studied by incubating the cells with different concentrations of acetate in addition to micromolar concentrations of 3CB. Acetate and 3CB analyses showed that simultaneous uptake of acetate and 3CB occurred in these incubations. The transformation rate of 3CB was not affected by the presence and simultaneous uptake of acetate, as is demonstrated in Fig. 3. At the lowest 3CB concentration range from 100 to 500 nM, transformation rates were also not affected by the presence of acetate (Fig. 4).

Residual concentrations. As part of the search for a lower limit for the degradation of 3CB, residual concentration experiments were carried out down to the lower nanomoles-per-liter range. Figure 5 shows the disappearance of radiolabeled 3CB over time, expressed as 3CB equivalents of the radiolabel. Each datum point represents the measurement of one individual tube sacrificed at a given incubation time.

In the batches with an initial concentration of 1.2 μM 3CB, the residual detected was about 1.2% of the 3CB added; thus, the residual represented the contamination. After 48 h, the cells were still active. They degraded a second 3CB spike at a rate comparable to that for the first addition (12 nmol · liter^{-1} · min^{-1} after the first spike and 13.3 nmol · liter^{-1} · min^{-1} after the second spike) (Fig. 5).

When the incubations were started with 11 nM 3CB, 3CB disappeared immediately. After a few hours, the label was below the detection limit of about 1.0 nmol · liter^{-1}. Table 1 shows that the total amount of radiolabel that had been added in this low-concentration series was recovered as $^{14}CO_2$, as biomass, or as dissolved compounds. In the batches in which all 3CB had been degraded (after 27 to 72 h of incubation), 10% of the ^{14}C was recovered as biomass and 8% of the ^{14}C was collected in HPLC fractions other than the 3CB fraction. The amount of $^{14}CO_2$ produced was 82%.

Similar results were obtained in batch experiments with acetate as the only substrate (Fig. 6). An initial concentration of 8.6 μM acetate was rapidly transformed to about 0.3% of the radiolabel added, which was followed by a very slow disappearance of this residual label. This residual corresponded to the contamination. Sterile controls at the nanomolar level did not show any loss of label. A 100-times-lower initial acetate concentration showed an analogous pattern but with a 50- to 100-times-lower residual concentration, which was around the detection limit (about 0.5 nmol of acetate equivalents per liter [Fig. 6]).

In the third experiment, in which both 3CB and acetate were added, both substrates were transformed simultaneously (Fig.

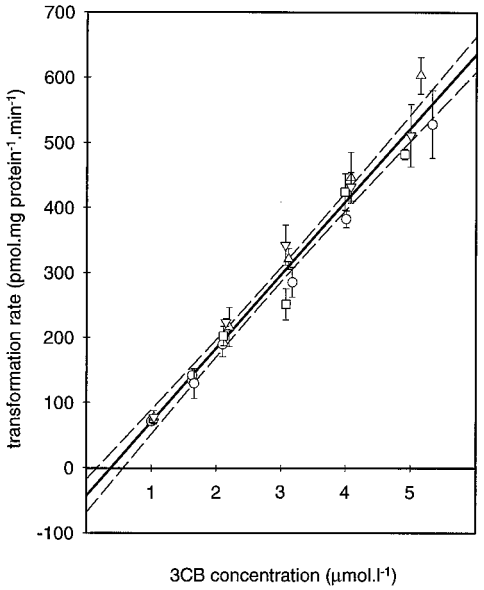

FIG. 3. Kinetics of 3CB transformation in the 3CB concentration range from 1 to 5 μM in the presence of 0 (○), 50 (▽), 500 (△) and 5,000 (□) μM acetate. Regression through all the data is presented with a 95% confidence interval. Error bars show standard deviations.

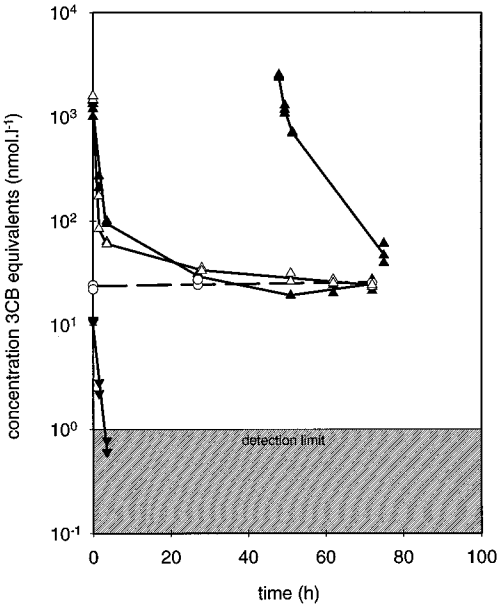

FIG. 5. Transformation of 3CB over time, expressed as 3CB equivalents of radiolabel that were measured in the HPLC fraction with 3CB. Cells were incubated with [^{14}C]3CB alone at two different substrate concentration levels (solid triangles). The activity of the cells was tested with a second spike after 48 h. Open triangles correspond to the radiolabel in the incubations with [^{14}C]3CB and 23 μM cold acetate. Open circles represent the values obtained with sterile controls.

5 and 6). The observed residual radiolabel was about 1.3% of the initial label in the incubations with [^{14}C]3CB and cold acetate. In the suspensions with [^{14}C]acetate and cold 3CB, the acetate was transformed to about 0.9% of the initial radiolabel. This residual label subsequently disappeared at a very slow rate. Both residuals represented the contaminations. It can therefore be concluded that the presence of an additional substrate did not markedly influence the residual radioactivity.

DISCUSSION

The data presented here suggest that *Pseudomonas* sp. strain B13 possesses two different uptake or transformation systems for 3CB. Our experimental setup did not allow us to distinguish the transport and transformation kinetics. Only experiments with membrane vesicles could give the necessary insights. Therefore, in the following text, transformation system for strain B13 should be read as combined uptake and transformation systems. Multiphasic kinetics of uptake or conversion of substrate have been reported for a number of organisms (4, 6, 10, 21, 22). In most of these studies, the individual kinetic systems can be described by separate sets of Michaelis-Menten parameters. The systems working for the high-concentration range are often found to have high capacities with regard to their maximal uptake and transformation rates but low affinities for the substrate. Systems active at low concentrations can be categorized as high-affinity, low-capacity systems.

At 3CB concentrations in the millimolar range, the transformation kinetics of strain B13 can be described by a Michaelis-Menten-type model. However, for predicting transformation rates at concentrations below 0.1 mM 3CB, an extended Michaelis-Menten model should be used: $V = V_{max} \times S/(K_m + S) - a$, where V is the transformation rate, S is the substrate concentration, and a is the virtual negative conversion rate. This equation is analogous to the growth model of Monod,

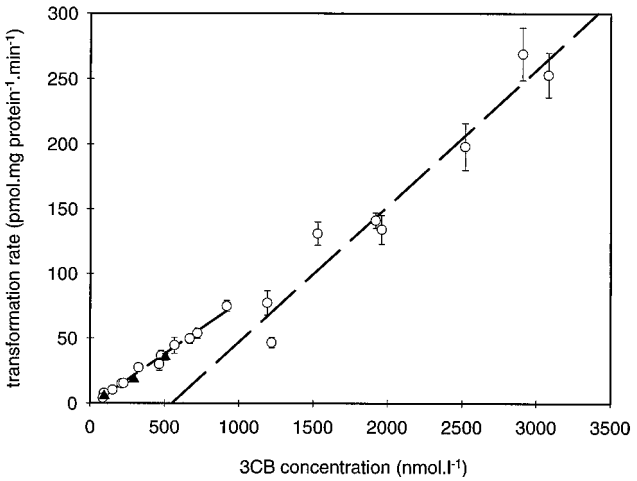

FIG. 4. Kinetics of 3CB transformation in the 3CB concentration range from 0.1 to 3 μM in the presence of 0 (○) and 500 (▲) μM acetate. Symbols without error bars indicate datum points with standard deviations that are so small that they fall within the size of the symbols used.

TABLE 1. Mass balance of radiolabel in the incubation series at an initial concentration of 11 nM

Label category	Amt of label ± SD (dpm)	Percentage
Total label added in 14 incubations	40,700 ± 2,100	100 ± 5
Total label recovered in solution or as biomass	10,700 ± 3,200	26 ± 8
Label cumulatively trapped as $^{14}CO_2$[a]	32,900 ± 6,900	81 ± 17
Total label recovered	43,600 ± 10,100	107 ± 25

[a] The trapped $^{14}CO_2$ was measured after all 14 incubations of this series, which were sacrificed at different time intervals, were purged.

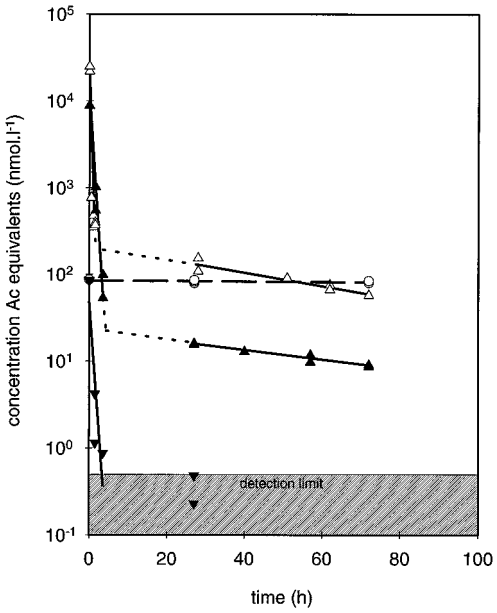

FIG. 6. Transformation of acetate over time, expressed as acetate (Ac) equivalents of radiolabel that were measured in the HPLC fraction with acetate. Cells were incubated with [^{14}C]acetate alone at two different concentration levels (solid triangles). Open triangles correspond to the radiolabel in the incubations with [^{14}C]acetate and 1.5 μM cold 3CB. Open circles represent the values obtained with sterile controls.

extended with a term for maintenance (20). The virtual negative conversion rate a at a zero substrate concentration does not have a practical meaning, but the essence of this equation is to describe transformation kinetics at low concentrations. Far below the K_m value, this model approaches the linear relationship $V = S \times V_{max}/K_m - a$ and predicts a substrate threshold concentration of $a \times K_m/V_{max}$. Combination of all the data for the 3CB concentration range of 1 to 5 μM (Fig. 3 and 4) gives an apparent threshold concentration of 0.50 ± 0.11 μM for *Pseudomonas* sp. strain B13 and 3CB.

Interestingly, *Pseudomonas* sp. strain B13 could convert 3CB in the concentration range from 0.1 to 0.9 μmol · liter^{-1} apparently by using another transformation system. The kinetics of this system are first order, which is not in agreement with the high-affinity, low-capacity systems described for other organisms (6, 10). Possibly, only the first part of a Michaelis-Menten relationship was observed, and at concentrations higher than 1 μM 3CB, the second transformation system was taking over. The data suggest that only one of these systems was operating at a time. The kinetic data around 1 μM 3CB do not show the curvature that would indicate the summation of two transformation systems operating simultaneously. A similar conclusion was drawn for conversion of methyl parathion by a *Flavobacterium* species (10).

No effects of the presence and simultaneous uptake of acetate on the transformation kinetics of 3CB could be demonstrated. This result is in disagreement with those of other studies in which both enhancing and adverse effects on the transformation rate of specific xenobiotic compounds was seen (1, 8, 14, 17, 18).

Residual substrate concentrations above the detection limits for 3CB and acetate (1.0 and 0.5 nmol · liter^{-1}, respectively) could not be detected for *Pseudomonas* sp. strain B13 in batch studies. This observation contradicts observations from batch studies with other organisms (7, 15, 19). In aerobic liquid batch cultures, a residual concentration in the lower nanomoles-per-liter or picomoles-per-liter range is not likely when adsorption or diffusion processes are not limiting the availability of the substrate. At 3CB and acetate concentrations around our detection limits, $\Delta G'$ values of −3,134 kJ/mol of 3CB and −815 kJ/mol of acetate can be calculated for their total oxidation (at around 10 mM 3CB and acetate, concentrations at which aerobic cultures are often grown, the $\Delta G'$ values are −3,175 kJ · mol^{-1} for 3CB and −858 kJ · mol^{-1} for acetate). This outcome is in contrast with those of acetate conversions in methanogenic systems, in which the limits for the change in free energy are clearly the cause for the observed residual concentration (7).

A bacterial cell has to meet a substrate molecule to enable biodegradation. The number of collisions between particles A and B (Z_{AB}) is given by the formula $Z_{AB} = N_A N_B d_{AB}{}^2 [8\pi kT (m_A + m_B)/m_A m_B]^{1/2}$, in which N is the concentration, d_{AB} is the average diameter, k is the Boltzmann constant, T is the absolute temperature, and m is the mass of the particles. At a substrate concentration of around 0.1 nmol · l^{-1} (0.1 pmol · ml^{-1}) at 20°C and a cell concentration of 10^9 cells · ml^{-1}, each cell collides around 10^7 times per s with substrate molecules. This frequency is more than sufficient for an active metabolism if we consider the 24-h starvation period, after which cells of strain B13 are still fully active. As long as we assume the Michaelis-Menten concept to be correct, namely, that for a reaction to occur one substrate molecule has to meet one enzyme, two factors will determine a possible residual concentration for a given time period. First, there is the frequency with which substrate molecules make successful contacts with the enzymes converting them. Obviously, the time period between two successful contacts should be shorter than the average lifetimes of the enzymes. Unfortunately, data on enzyme decay rates are extremely scarce, and no relevant data on the enzymes of the 3CB degradation system exist. Second, the change in free energy must be sufficiently negative for the overall conversion to allow the cell to maintain a necessary proton motive force. In our case, the second prerequisite is certainly fulfilled, and on the basis of the fact that we could not detect a residual concentration in this study, it can be assumed that the first one is fulfilled too.

A residual concentration in a resting cell suspension is something entirely different from the residual concentration or minimum substrate concentration for growth (S_{min}) in a continuous system or a system with a growing cell population. A residual substrate concentration may be related to the substrate concentration required for the induction of the responsible enzymes. In chemostats, the residual concentrations at steady state are determined by the growth kinetic parameters of the organism. For mixtures of glucose, fructose, and galactose, Lendenmann (9) could show that for *Escherichia coli* growing in a chemostat at 0.3 h^{-1}, the residual concentrations of each sugar added up to the residual substrate concentration, which was obtained with only one of the sugars as the single substrate. S_{min} has been defined as the substrate concentration at which the substrate flux into the cells equals the maintenance requirement of the entire bacterial population, i.e., the substrate concentration at which the cell population stays constant. For chemostats, S_{min} values can be obtained by extrapolation of the data at low specific growth rates (2). In a continuous biofilm reactor, Rittmann and McCarty could measure no significant biofilm activity once the steady-state substrate concentration equaled the predicted value for S_{min} (13). Continuously fed soil columns, repeatedly inoculated with a *Pseudomonas* strain, showed residual effluent concentrations

of about 70 nM 1,2-dichlorobenzene, independent of the feeding concentration. Subsequent batch incubation of the effluent, however, could reduce this residual to the detection limit of 0.7 nM (19). This result illustrates that residual substrate concentrations obtained in batches under nongrowth conditions are generally lower than residual concentrations obtained in continuous systems.

For in situ bioremediation of polluted aquifers, the treatment has to be, at least partly, continuous. A regular supply of limiting nutrients or primary substrates is necessary to maintain a microbial population which (co)metabolically or after primary substrate depletion degrades a pollutant. A feasible method to bring the residual contaminant concentration below the S_{min} of the bacterial population is a pulsing substrate addition. Systems with pulses of the limiting substrates can be regarded as a series of batch incubations between the successive pulses. A well-studied example of a pulsing system is the one involving the degradation of chlorinated ethenes in an aquifer to which methane (electron donor) or oxygen (electron acceptor) was added as a growth stimulator at specific time intervals (16). On the basis of the data available up to now, fed batch or pulsing systems are superior to continuously fed systems for the attainment of low pollutant concentrations, providing that adsorption and diffusion do not limit the availability of the substrate.

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